In vitro modulation of epileptiform activity in the hippocampal formation of immature rodents by GABAergic antagonists, dopamine and methylxanthines

Dissertation

Zur Erlangung des Grades
Doktor der Naturwissenschaften

Am Fachbereich Biologie
Der Johannes Gutenberg-Universität Mainz

vorgelegt von

Salim Sharopov

geb. am 10.07.1975 in Tadschikistan

Mainz, 2013

Tag der mündlichen Prüfung: 09.09.2013

Table of contents

List of symbols and abbreviations

α	Alpha frequency range (8-13 Hz)
β	Beta frequency range (13-30 Hz)
γ	Gamma frequency range (30-80 Hz)
δ	Delta frequency range (0.5-4 Hz)
θ	Theta frequency range (4-7 Hz)
ACSF	Artificial cerebrospinal fluid
ADC	Analogue-to-digital conversion
aEEG	Amplitude-integrated EEG
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
APV	(2*R*)-amino-5-phosphonovaleric acid
A_{1A}Rs	Adenosine A1 receptors
A_{2A}Rs	Adenosine A2 receptors
CA1	Cornu Ammonis area 1
CA2	Cornu Ammonis area 2
CA3	Cornu Ammonis area 3
CA4	Cornu Ammonis area 4
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate

CHF	Corticohippocampal formation
CICR	Calcium–induced calcium release
CLC-2	Voltage - gated chloride channel 2
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CT	Computer tomography
DA	Dopamine
D1	Dopamine receptor 1
D2	Dopamine receptor 2
D3	Dopamine receptor 3
D4	Dopamine receptor 4
D5	Dopamine receptor 5
D4	Dopamine receptor 4
D5	Dopamine receptor 5
DG	Dentate gyrus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E_{Cl}	Chloride reversal potential
EC	Entorhinal cortex
EEG	Electroencephalography
ENOs	Early network oscillations

EPSP	Excitatory postsynaptic potential
Fgf8	Fibroblast growth factor 8
Fig.	Figure
FP	Field potential
GABA	γ-aminobutyric acid
GBZ	Gabazine
GTCS	Generalized Tonic-Clonic Seizures
Hz	Hertz
IIE	Inter ictal event
KCC2	Potassium chloride cotransporter 2
ILAE	International League against Epilepsy
L-DOPA	L-dihydroxy-phenylalanine
MRI	Magnetic resonance imaging
MZ	Marginal zone
MΩ	Megaohm
NH_2	Amine group
NICU	Neonatal intensive care unit
NKCC1	Na-K-Cl cotransporter
NMDA	N-methyl-D-aspartic acid
Non-SLE	Non-Seizure like events
NO	Nitric oxide

P	Postnatal day
PDE	Phosphodiesterase
preBötC	pre-Bötzinger complex
PZ	Proliferative zone
sPFPs	Spontaneous paroxysmal field potentials
PTX	Picrotoxin
RMP	Resting membrane potential
RrF	Retrorubal field
SEM	Standard error of the mean
Shh	Sonic hedgehog
SLE	Seizure like event
SNc	Substantia nigra pars compacta
SVZ	Subventricular zone
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol
TEA	Tetraethylammonium
Th	Tyrosine hydroxylase
VTA	Ventral tegmental area
VDCC	Voltage-dependent calcium channel
$[Cl^-]i$	Intracellular chloride concentration

List of figures

1 Introduction

1.1 Anatomy and development of the hippocampal formation

The hippocampal formation belongs to the limbic system and is positioned in the medial temporal lobe. It is considered to play an important role in the pathogenesis of various neurological disorders such as epilepsy, Alzheimer disease and depression (Aronica et al., 2007; Benedetti et al., 2006; Blume, 2006; Schwartzkroin, 1994). There are many studies which investigated developmental stages of this brain structure in detail (Nowakowski and Rakic, 1981; Rickmann et al., 1987).

Anatomically, the hippocampal formation is referred to the hippocampus proper, dentate gyrus (DG), subiculum and entorhinal cortex (EC). The hippocampus proper itself consists of the four Cornu Ammonis (CA) subdivisions: CA4, a comparatively small part underlying the DG, CA3, which is followed by very small region CA2 and then CA1. All CA areas consist of tightly packed pyramidal neurons. The DG is separately located and closely packed layer of relatively small neurons enveloped at the end of the hippocampus proper, forming a semicircle shape. Subiculum, presubiculum and parasubiculum then mostly transit to the EC (Andersen, 2007). Simplified anatomy of the hippocampus with most important inputs from EC is shown in Fig.1.

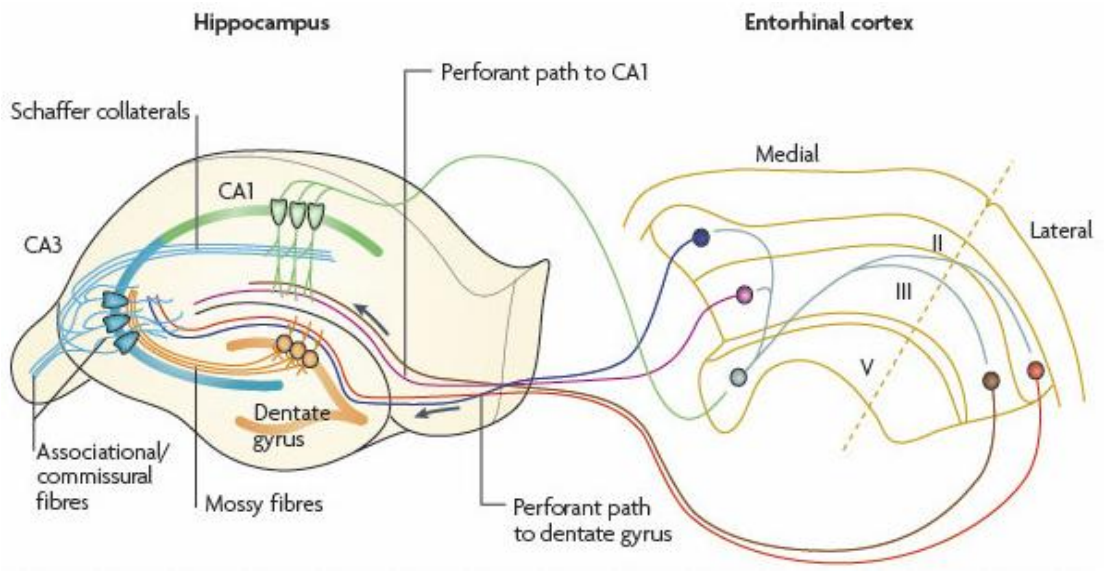

Figure 1. Basic anatomical representation of the hippocampus. The perforant path axons carry sensory information from cells of layer II of the EC to the DG. The axons from the lateral EC make excitatory connections to the different part of granule cell dendrites. Granule cells send information then through mossy fibres to the dendrites of CA3 pyramidal cells which, one after the other, make connections to the ipsilateral part of CA1 pyramidal neurons via Schaffer collaterals and to contra-lateral CA3 and CA1 neurons by commissural connections (Picture from Neves et al., 2008).

In the development of the hippocampal formation like for other structures of the central nervous system (CNS), very complex procedures are involved, which belong to three categories: proliferation of the cells, their differentiation and migration. The process of neurogenesis in the hippocampal formation takes place during distinctive intervals of prenatal development with an exception for the neurons of the DG (Miller, 1995; Nowakowski, 1988). In rodents, the generation of pyramidal cells continues until the second part of embryogenesis, and granule cell generation begins some days before birth. Then they are generated during the first month after birth and continue being generated throughout adulthood (Angevine, 1965; Eriksson et al., 1998; Kuhn et al., 1996). Thus, 85% of the DG neurons are formed after the birth (Andersen, 2007). In humans, the exact time of formation of pyramidal neurons is not identified, whereas the DG cells layer appears approximately at E90 (Humphrey, 1967). At around E170, all parts of the hippocampal formation become architectonically similar to what we observe in the adult state (Arnold and Trojanowski, 1996; Humphrey, 1967). Generation of the cells of the hippocampal formation is restricted only to one proliferative zone (PZ). The majority of the cells, including all of the hippocampal pyramidal neurons, initiate from the ventricular zone (VZ) coating the lateral ventricle (Nowakowski, 1988), while the dentate gyrus neurons are produced from the intrahilar PZ. However, some dentate gyrus neurons can originate also from the VZ and cells of the intrahilar PZ also originate from the VZ (Nowakowski and Rakic, 1981).

The newborn neurons have to travel to the length of radial glial fibers across an obscured terrain to reach the intrahilar PZ or their specific destination (Nowakowski, 1988; Rickmann et al., 1987). In contrast, the entire pathway for newborn cells to the targeted area, which are predestined for the subiculum or areas CA1, CA2 of the hippocampus, is relatively fair and smooth (Nowakowski, 1988). However, the migration process of neurons destined for the CA3 is even longer and they pass it also along a circuitous and tortuous pathway (Rickmann et al., 1987). The migration

procedure of cells intended for the DG and intrahilar PZ is along a path close to the way followed by the CA3-destined neurons, but located closer to the surface of the transverse fissure (Nowakowski, 1988). As a result, the CA3 and DG newborn neurons migrate radially, however the increasing hippocampus and DG change the moving track of the cells that is parallel to the pial surface (Nowakowski, 1988). Overall this leads to the consequence that the radial and apposed migrating cells reproduce the topographic image of the regions of the hippocampal formation (Nowakowski, 1988).

1.2 Seizures and epilepsy

A predisposition to have recurrent seizures is defined as epilepsy. Some descriptions also require seizures to be unprovoked (Berg et al., 2010; Chang and Lowenstein, 2003). Seizure as a principal constituent of epilepsy is a rapid surge of abnormal excessive and hypersynchronous neuronal activity in the brain which can have sufficient magnitude to change motor or sensory function, behaviour and consciousness (Berg et al., 2010; Chang and Lowenstein, 2003; Fisher et al., 2005; Freeman, 2009). The term epilepsy include numerous syndromes, in which the main aspect remains a tendency for persistent seizures (Chang and Lowenstein, 2003). An epileptic syndrome is an assemblage of seizures, their localization, frequency, EEG patterns, family histories, and age-specific features sufficient to generate a reproducible seizure pattern. Many studies suggested that the incidence of seizures and epilepsy is higher during early childhood (Briggs and Galanopoulou, 2011; Hauser et al., 1993; Kotsopoulos et al., 2002).

1.2.1 Seizures and epilepsy in the developing brain

Seizures can be categorized into two groups: partial and generalized (Commission of ILAE, 1981; Commission of ILAE, 1989; Shah et al., 1992). Partial seizures (may also be called local or focal seizures) happen when excessive electrical brain activity cover one or many areas of one hemisphere of the brain. When during the seizure consciousness is kept such onsets are categorized as partial, in contrast, when consciousness is lost, than they are classified as complex partial seizures. Partial seizures can spread to neighbouring areas or throughout both hemispheres and

this kind of onset is termed secondary generalization. Either form of seizure may develop into the secondary generalization (Freeman, 1995).

Seizures that involve the entire surface of the hemispheres and impair consciousness are called generalized. There are many types of generalized seizures, where the main three forms are tonic-clonic seizures (grand mal), myoclonic and absence (petit mal seizures, staring spells) (Freeman, 1995). Seizures can be also categorized by their cause. Seizures with identified cause are symptomatic, for instance, fits that develop after head injury, infection (meningitis, encephalitis etc.), cerebrovascular abnormality, infarction, tumor and developmental abnormalities. An undiscovered cause for the seizures associated with obvious abnormal neurologic problems or psychological retardation (which is not detectable on computer tomography (CT) or magnetic resonance imaging (MRI) is called cryptogenic. The most common form of seizures is idiopathic. In this kind of seizures there are no apparent underlying causes and no evidence for neurologic disorders. Possibly, idiopathic seizures are caused by tiny structural lesions, but how these abnormalities lead to the epilepsy is unknown (Freeman, 1995; Guerrini et al., 1992; Guerrini et al., 2003; Prayson et al., 1993).

Table 1: Seizures and epilepsy syndromes occurring in childhood (adapted from Schwartzkroin, 1995)

1. Neonatal seizures
1.1 Benign neonatal convulsions
2. Infantile spasms (West's syndrome)
3. Lennox-Gastaut syndrome (childhood epileptic encephalopathy)
4. Benign focal epilepsies
4.1 Benign rolandic seizures
5. Juvenile absence epilepsy (petit mal)
6. Juvenile myoclonic epilepsy
7. Progressive unilateral encephalopathy of childhood (Rasmussen's syndrome)

Neonatal seizures are epileptic onsets taking place in the first month of life or until the end of the neonatal period (Freeman, 1995; Holmes et al., 2002; Lombroso, 1996; Panayiotopoulos, 2005). Seizures occur due to developmental malfunctions, hypoxia, trauma, vitamin deficiency and chemical imbalances. Some benign and genetic forms have also been reported (Quattlebaum, 1979; Ryan et al., 1991). Seizures are unremarkable and very difficult to distinguish from the normal baby behaviors. Mostly neonatal seizures are described as subtle since the important clinical symptoms are normally overlooked. These include deviation of the eyes, jerking, blinking or fluttering, sucking, smacking movements, swimming, pedaling or rowing movements. However, these imperceptible seizures may have long-term neurological consequences (Holmes, 2009; Holmes and Ben-Ari, 2001). But, not all children that have such abnormalities develop seizures. It is currently not understood why some structural malfunctions recruit sufficient neighbouring neurons to become highly excitable or manifest as seizures while others do not?

The higher seizure vulnerability and incidence in the immature CNS, among others, has been also associated to immature neuronal properties of the GABAergic system (Ben Ari et al., 2007; Fritschy et al., 1994; Sanchez and Jensen, 2001; Taketo and Yoshioka, 2000).

1.3 The development and variety of functions of the GABAergic system

In the mammalian CNS GABA has been shown to be the most important inhibitory neurotransmitter which acts primarily by connecting to ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (Farrant and Kaila, 2007; Kaila, 1994). The $GABA_A$ receptors open a Cl^- channel in response to neurotransmitter binding, and in the mature nervous system (channel is permeable also for HCO_3^-), GABA activation causes Cl^- influx and membrane hyperpolarization due to the low intracellular Cl^- concentration established by the K^+ -Cl^- cotransporter KCC2 (Li et al., 2002; Rivera et al., 1999; Wang et al., 2002) (Fig.2b). In contrast, in the developing nervous system, $GABA_A$ receptor activation mediates depolarizing membrane responses (Ben-Ari et al., 1989; Luhmann and Prince, 1991; Mienville, 1998; Mueller et al., 1984; Owens et al., 1996) (Fig.2a). This is due to elevated intracellular Cl^- concentration, which in most neurons is caused by the abundance of NKCC1 (Na-K-

Cl cotransporter) mediating Cl^-- uptake (Farrant and Kaila, 2007; Rivera et al., 1999; Yamada et al., 2004). NKCC1 imports Cl^- into the immature cells, thereby causing Cl^- efflux and membrane depolarization upon $GABA_A$ receptor activation (Luhmann and Prince, 1991; Plotkin et al., 1997; Wang et al., 2002).

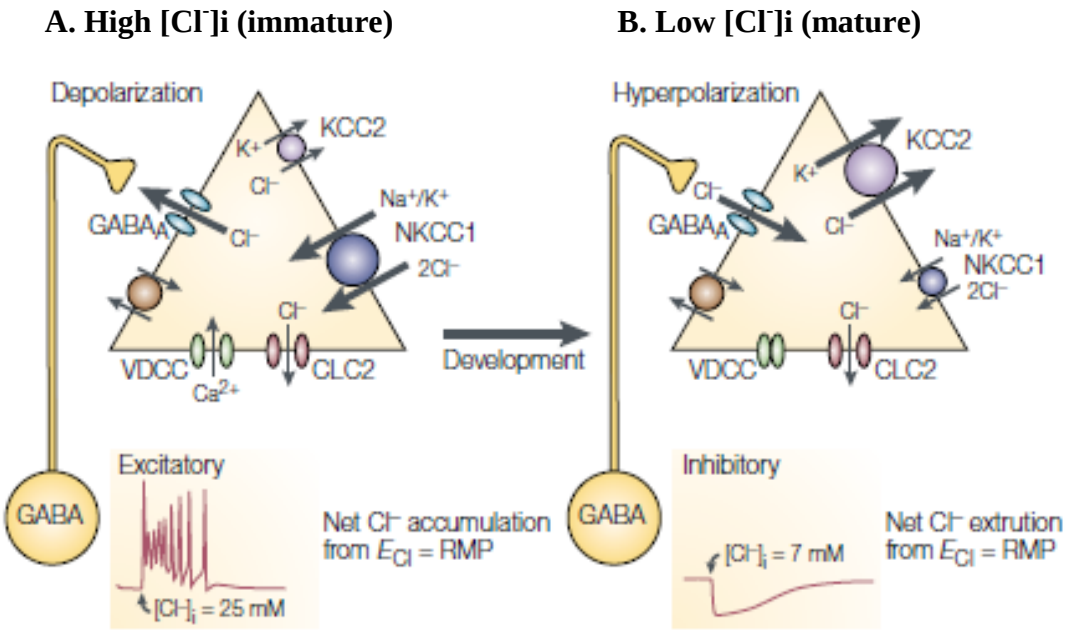

Figure 2. Expression of NKCC1 and KCC2 establishes developmental changes in chloride homeostasis. ***A.*** *NKCC1 domination in immature neurons leads to relatively high chloride concentration in the cytoplasm - [Cl⁻]i.* ***B.*** *KCC2 expression prevails in mature neurons. Note that $GABA_A$ receptor activation induces an extrusion of chloride ions, which leads to excitation of immature neurons, and an influx of chloride that inhibit the adult neurons. CLC-2, chloride channel 2 (voltage gated); E_{Cl}, chloride reversal potential; RMP, resting membrane potential; VDCC, voltage-dependent calcium channel (picture from Ben-Ari, 2002).*

The GABAergic activation induce depolarization in the human CNS in the initial first postnatal weeks of in-term babies (Dzhala et al., 2006). Depolarizing GABA neurotransmission in the immature brain mediates important functions. For example, GABAergic activation influences the proliferation of cortical and hippocampal neurons. It has been shown that GABA activation in the VZ promotes proliferation and in contrast, it attenuates neuronal proliferation in the subventricular zone (SVZ) (Haydar et al., 2000; LoTurco et al., 1995). The hippocampal (Manent et al., 2005) and cortical (Behar et al., 2000) migration processes are also modulated by $GABA_A$ receptors. For the proper integration into a functional network and morphological differentiation an activation of $GABA_A$ receptors and resulting depolarization of neurons is necessary (Cancedda et al., 2007). Moreover, it has been shown that

GABAergic depolarization can play a crucial role in inducing oscillatory activity patterns (Sipila et al., 2005) and removing the Mg^{2+} block from NMDA receptors since functional expression of AMPA receptors occurs later (Ben-Ari et al., 1997) (Fig. 3). Depolarizing GABAergic responses not always exert excitatory action; their activation can also lead to inhibition (Eccles et al., 1962; Edwards, 1990a; for review Kilb, 2012).

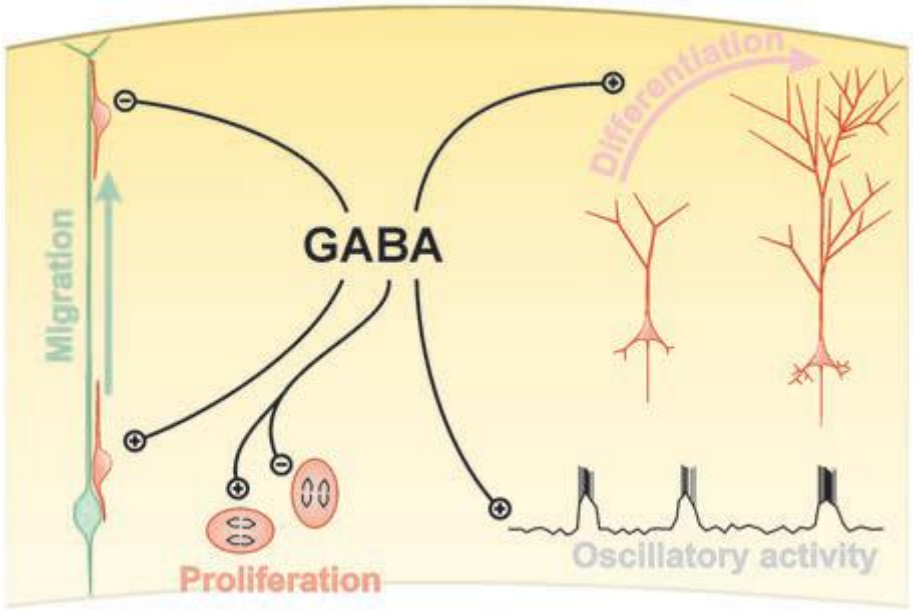

Figure 3. Activation of GABAergic receptors has many effects during brain development. $GABA_A$ receptor activation is essential for cell precursor proliferation in the VZ and attenuation of proliferation in the SVZ (Haydar et al., 2000). For radial migration of the lower cortical layers $GABA_A$ activation is needed, where under the cortical surface it terminates the migration process (Behar et al., 2000; Denter et al., 2010). Excitatory synaptogenesis, differentiation of dendrite branches and facilitation of the generation of oscillatory activity are also depending on GABAergic depolarization (Cancedda et al., 2007; Sipila et al., 2005; Wang and Kriegstein, 2008). (Picture from Kilb, 2012).

It has been suggested that GABAergic depolarization makes the immature hippocampus prone to the generation of epileptiform activity (Dzhala and Staley, 2003; Dzhala et al., 2005). However, GABAergic inhibition in the immature hippocampus has also been shown to exert epileptiform activity (Baram and Snead, III, 1990; Khazipov et al., 2004; Kilb et al., 2007; Lamsa et al., 2000; Wells et al., 2000), which demonstrates an inhibitory effect of GABA already at this developmental period. The study by Baram and Snead indicated that the GABA antagonist bicuculine is able to induce seizures in rats younger than 6 days, which

was not shown before (Baram and Snead, III, 1990). Blocking of $GABA_A$ receptors elicits spontaneous paroxysmal field potentials (sPFPs) in the immature CNS, which directly indicate that GABA can be an inhibitory neurotransmitter already at early postnatal ages (Wells et al., 2000). The $GABA_A$ antagonists gabazine and picrotoxine have also been shown to induce epileptiform discharges in immature neocortical neurons (Richter et al., 2010). Moreover, drugs that positively modulate $GABA_A$ receptors such as midazolam or phenobarbital are recommended as efficient medication for the treatment of neonatal seizures (Wheless et al., 2007), although some clinical studies do not support their use (Booth and Evans, 2004).

GABAergic activation elicits heterogenic neurotransmission that can be the reason for these incompatible observations (Farrant and Nusser, 2005). For the period of early hippocampal development the role of synaptic and extrasynaptic GABA receptors is very important. In the immature hippocampus extrasynaptic $GABA_A$ receptors are responsible to induce tonic inhibition and these currents strongly modulate the neuronal activity (Marchionni et al., 2007; Sipila et al., 2005). Since synaptic and extrasynaptic GABAergic activation elicit different functions in the neuronal activity modulation (Farrant and Nusser, 2005; Walker and Semyanov, 2008), induction of phasic versus tonic GABAergic currents in the immature hippocampus crucially influences the effect of GABA on the excitability processes.

This contradictory information suggests that the functional consequences of depolarizing GABAergic responses on the excitability of immature neurons are not fully understood and further investigations are necessary to clarify the issue.

1.4 The development and function of the dopaminergic system

Dopamine (DA) is one of the catecholaminergic neurotransmitters of the vertebrate central nervous system, where it is synthesized in a common biosynthetic pathway as a precursor to noradrenaline and adrenaline. The name of dopamine originates from its chemical composition, which combines an amine group (NH_2) connected to a catechol structure named dihydroxyphenethylamine (Fig.4). Tyrosine hydroxylase (Th) is an enzyme in this pathway that converts the essential amino acid tyrosine to L-dihydroxy-phenylalanine (L-DOPA). For production of DA L-DOPA should be then decarboxylated using enzyme L-aromatic amino acid decarboxylase.

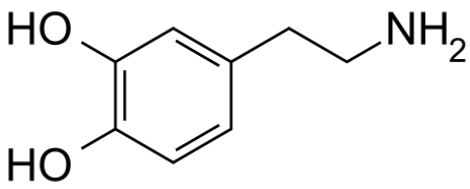

Figure 4. The chemical structure of DA. DA consists of two main parts: amine group (NH_2) and catechol structure dihydroxyphenethylamine. (Picture from http://en.wikipedia.org/wiki/Dopamine)

Th expression therefore has been commonly used as a molecular marker for DA-synthesizing cells (Prakash and Wurst, 2006). Axons of DAergic neurons, which transmit the neurotransmitter DA from one to another region of the brain, constitute DAergic pathways. Axons of such DAergic neurons are projected along the whole length of the pathways. DAergic pathways in the mammalian brain are divided into four groups: the nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular pathways that derive from substantia nigra pars compacta (SNc), ventral tegmental area (VTA) and retrorubal field (RrF) groups of DA-containing cells (Anden et al., 1964; Beaulieu and Gainetdinov, 2011; Dahlstroem and Fuxe, 1964). The projection of the main DAergic neurons and pathways are illustrated in Fig.5.

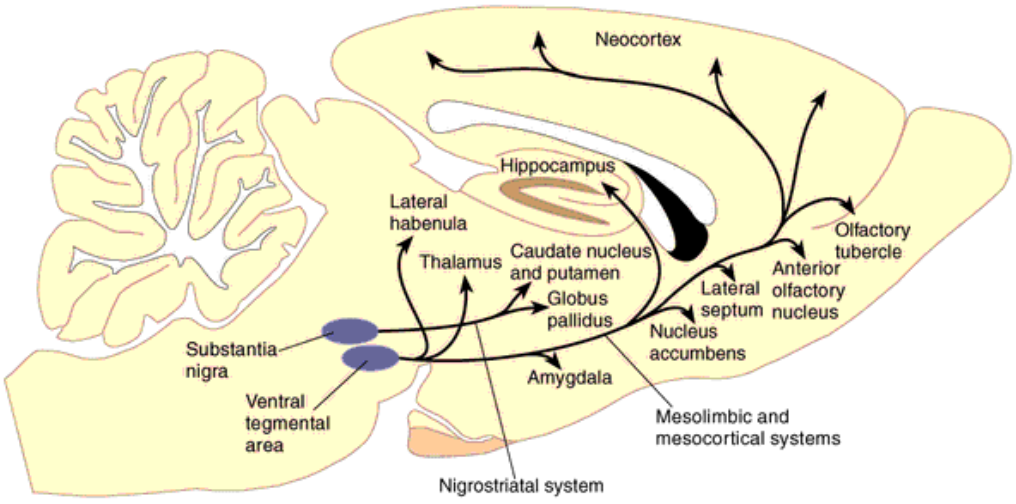

Figure 5. Schematic saggital view of a rat brain showing the positions of DA cell groups and their main axonal projections.The mesolimbic and mesocortical systems start in the VTA of the midbrain and send axons to the limbic system via the amygdala, the hippocampus and the nucleus accumbens as well as to the frontal lobe of the medial prefrontal cortex. The nigrostriatal pathway connects the substantia nigra to the neostriatum. DAergic neurons from the substantia nigra project their axons into the nucleus caudate, putamen and globus pallidus (Picture from http:// homepage.psy.utexas.edu/homepage/class/Psy308/Salinas/Schizophrenia/Schizophrenia.html

DA neurons and pathways in the brain have crucial regulatory functions and several severe neurological diseases like Parkinson's disease, drug addiction,

depression and schizophrenia etc. have been related to improper functioning of DA neurons and pathways. The modulating role of DA in epilepsy was described many years ago (Starr, 1996). In this modulation DAergic neurons from VTA projecting their axons to the hippocampus are involved (Luo et al., 2011).

The development of most of DAergic neurons depends on a single cell group during embryogenesis that initiates from the diencephalon-mesencephalon junction, which targets then to different forebrain structures. Investigations of DA pathways during development have identified a number of factors that affect the final formation of DAergic neurons in adulthood. The two signalling proteins sonic hedgehog (Shh) and fibroblast growth factor 8 (Fgf8) determine the identity of proliferating DA progenitor cells (Chinta and Andersen, 2005). Transcription factors important for post-mitotic DA neuron development are Nurr1, Lmx1b, Pitx3 and En1/En2 (Wallen and Perlmann, 2003), while transcription factors exclusively expressed in the DA proliferating neurons are not well studied yet. Nurr1 seems to be responsible for neurotransmitter synthesis (Zetterstrom et al., 1997), while Lmx1b is important for the Pitx3 expression (Smidt et al., 2000). Pitx3 is expressed specifically in mesencephalic DA neurons, where it takes part in the maintenance and development of DAergic cells (Nunes et al., 2003).

Investigation of DAergic pathways and neurotransmitter release in the targeting region will not only increase the understanding of how DAergic neurons are generated and function in vivo, but also allow us to understand the mechanisms and develop new therapeutic strategies for many neurological diseases.

1.5 Role of methylxanthines in the prevention and treatment of apnoea of prematurity

Xanthine is a product of the purine metabolism, which was found in most human body tissues and fluids (Alexiou and Leese, 1992). As a methylated form xanthines (methyxanthines) are ingested as a component of tea, coffee and cola drinks (Ashihara and Suzuki, 2004; Eteng, 1997). These alkaloids commonly used for their effects as soft stimulants and as bronchodilators, particularly in the treatment of asthma symptoms and also stimulate the respiratory centre (Eldridge et al., 1983) They act mainly as both: competitive nonselective phosphodiesterase (PDE)

inhibitors (Essayan, 2001) and nonselective adenosine receptor blockers (Daly et al., 1987). Methylxanthines caffeine and theophylline (Fig.6) share very similar structural and pharmacological identites. Methylxanthines are used as a standard medication for prevention of frequently occurring apnoea of prematurity in preterm human infants

A. Caffeine **B. Theophylline**

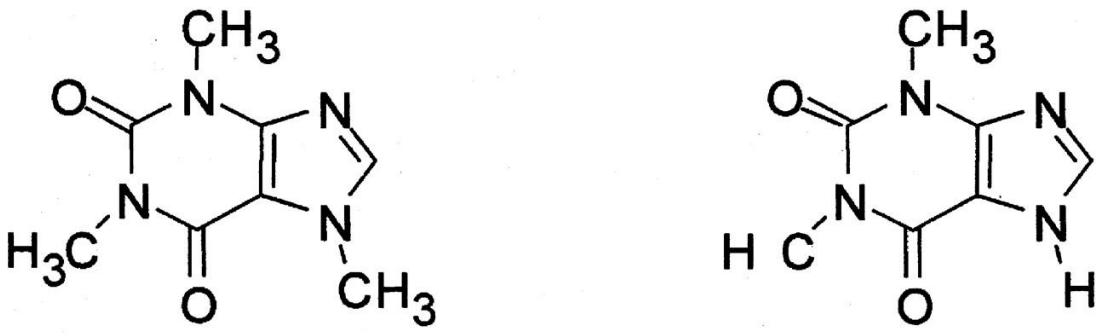

Figure 6. Chemical structure of methyxanthines caffeine and theophylline. Caffeine (1,3,7-Trimethyl-1H-purine-2,6(3H,7H)-dione) and theophylline (1,3-dimethyl-7H-purine-2,6-dione) accepted as a standard medication for the treatment of apnoea in preterm human babies. Picture from http://www.jacn.org/content/25/2/79/F2.expansion.html

(Bhatia, 2000; Daly, 2007; Schmidt et al., 2006). Some early studies (Ballanyi et al., 1999; Ruangkittisakul and Ballanyi, 2010) demonstrate that the excitatory respiratory methylxanthine action targets inspiratory active neuron-glia networks in the pre-Bötzinger complex (preBötC) which is involved in the initiation and control of normal breathing (Feldman and Del Negro, 2006). In rodents, during early postnatal period other neural networks including the neocortex and hippocampus also show spontaneous rhythmic network bursts, which have been termed 'early network oscillations' (ENOs) (Allene and Cossart, 2010; Ben-Ari et al., 1989; Garaschuk et al., 2000; Khazipov and Luhmann, 2006; Sipila and Kaila, 2008). While the preBötC must function already at birth, cortical networks are more immature and ENOs are thought to be important for stabilizing synaptic connectivity particularly via depolarization-related rises of cytosolic Ca^{2+} (Bonifazi et al., 2009; Garaschuk et al., 2000; Goodman and Shatz, 1993; Leinekugel et al., 1997; Spitzer, 2006).

There is a long clinical and experimental history of use of methylxanthines for the treatment of premature apnea (Aranda et al., 1981; Gannon, 2000; Schmidt et al., 2006), despite little information about the underlying molecular mechanisms.

Methylxanthine doses above therapeutic plasma levels of 100-400 µM, but sometimes also notably lower doses, can evoke seizures among other adverse effects such as nausea, hyperglycemia, tachycardia or tachypnea (Comer et al., 2001; Delanty et al., 1998; Fredholm et al., 1999; Lee et al., 1996) . Though theophylline and caffeine have been reported both to be effective (Aranda and Turmen, 1979; Bairam et al., 1987; Brouard et al., 1985; Gannon, 2000; Henderson-Smart and Steer, 2010; Scanlon et al., 1992) a comparative study should be carried out to identify the methylxanthine with the least adverse effects for the treatment of apnea of prematurity.

ENOs have been attributed to a variety of neurodevelopmental events (Ackman et al., 2009; Ben-Ari et al., 2007; Hanganu-Opatz, 2010; Kilb et al., 2007). Therefore any disturbance of these ENOs may interfere with normal brain development and can lead to persistent structural or functional alterations of the CNS (Wang and Kriegstein, 2011).

1.6 Aims of the present study

In my PhD thesis I concentrated on three questions that could improve our understanding of the role of distinct receptors which might be involved in the manifestation of epilepsy in the immature CNS. It has been shown that early child seizures are an additional risk factor for the later progression into epilepsy (Holmes, 1997; Khalilov et al., 2003). Neonatal seizures have a different etiology (Silverstein and Jensen, 2007) and respond poorly to the existing AEDs, which points out the importance of an alternative therapeutic strategy to adult forms (Booth and Evans, 2004; Sankar et al., 2005). I addressed particularly, the following questions: i) What is the role of phasic and tonic GABAergic activity on the excitability of the developing hippocampus, ii) What is the influence of DAergic receptors on epileptiform activity in the immature hippocampus and iii) What are the effects of the methylxanthines theophylline and caffeine on a hippocampal preparation corresponding to preterm conditions.

GABAergic transmission plays an essential role during early hippocampal development (Ben-Ari et al., 1989). But the influence of synaptic and/or extrasynaptic GABAergic currents on the excitability of the immature hippocampus was not fully investigated. Regarding the first question I investigated the role of $GABA_A$ receptor antagonists and modulators with different affinity to synaptic and extrasynaptic GABAergic receptors of hippocampal slices from immature rats using field potential recordings. These experiments revealed that in the immature hippocampus synaptic GABAergic activity is required to limit excitability, whereas extrsynaptic GABAergic currents can promote excitability.

Although many studies addressed the role of DAergic receptors in the epilepsy (Starr, 1996), there is very little information about DAergic modulation of epilepsy in immature hippocampus available. Therefore, concerning the second question, I investigated the effect of DAergic agonists and antagonists on epileptiform activity in the corticohippocampal formation of immature mice, using field potential recordings. These experiments revealed that DA already in immature CNS can modulate the epileptiform activity.

In human preterm infants, the methylxanthines caffeine and theophylline are routinely administered to stabilize respiration. Whether methylxanthines promote epileptic seizures *in-toto* hippocampal preparations remains unanswered. To answer the third question, I investigated in the third part of my work the effect caffeine and theophylline on the spontaneous and evoked field potential responses of the hippocampus. It will be shown that both theophylline and caffeine at higher concentrations modify basal synaptic transmission in the hippocampus CA1 region. The effects on postsynaptic responses and spontaneous epileptiform discharges were less pronounced for caffeine, suggesting that this substance may be potentially advantageous for therapeutic applications in preterm infants.

2 Materials

2.1 Chemicals

APV	Competitive antagonist for NMDA receptors
Bumetanide	Antagonist of the NKCC1 cation-chloride cotransporter
Caffeine	Antagonist of adenosine receptors
CNQX	Competitive AMPA/kainate receptor antagonist
Dopamine hydrochloride	Dopamine receptor agonist
Furosemide	Inhibitor of the KCC2 and NKCC1 Antagonist of α6-subunit of $GABA_A$ receptors
GABA	Agonist of $GABA_{A/B}$ receptors
Gabazine	Selective, competitive $GABA_A$ receptor antagonist
GSK 789472 hydrochloride	Selective dopamine D_3 receptor antagonist and D_2 partial agonist
GYKI 52466	Selective non-competitive AMPA receptor antagonist
L-741,626	D_2 receptor selective antagonist
L-655,708	Selective inverse agonist for the benzodiazepine site of $GABA_A$ receptors containing the α5 subunit
Muscimol	g-aminobutyric acid (GABA) agonist
Nomifensine	Selective dopamine uptake inhibitor
Phentolamine mesylate	A reversible nonselective alpha-adrenergic antagonist

Picrotoxin	$GABA_A$ receptor antagonist
Propranolol hydrochloride	Non-selective beta-adrenergic antagonists
Quinpirole hydrochloride	Selective dopamine D_2 receptor agonist
SB 277011A	Selective dopamine D_3 receptor antagonist
SCH-39166	High affinity dopamine D_1/D_5 receptor antagonist
SKF-38393	D_1-like dopamine receptor selective partial agonist
Sodium metabisulfide	Antioxidant
Sulpiride	Selective D_2-like dopamine antagonist
Tetrodotoxin	Selective blocker of the voltage-gated sodium channels
Theophylline	Nonselective adenosine receptor antagonist, competitive nonselective PDE inhibitor
THIP	Selective agonist of delta-subunit containing $GABA_A$ receptors
4-aminopyridine (4-AP)	Non-selective voltage-dependent K^+-channel blocker

2.2 Solutions

Normal ACSF

	MW	Concentration (mMol)
NaCl	58,44	126,0
NaH_2PO_4 x H_2O	137,99	1,25
$MgCl_2$ x $6H_2O$	203,3	1,0
$CaCl_2$ x $2H_2O$	147,02	2,0
KCl	74,56	2,5

Glucose	180,16	10,0
$NaHCO_3$	84,01	26,0

Mg^{2+} -free ACSF

	MW	Concentration (mMol)
NaCl	58,44	126,0
NaH_2PO_4 x H_2O	137,99	1,25
$MgCl_2$ x $6H_2O$	-	-
$CaCl_2$ x $2H_2O$	147,02	2,0
KCl	74,56	2,5
Glucose	180,16	10,0
$NaHCO_3$	84,01	26,0

Furosemide, bumetanide, γ-aminobutyric acid (GABA), 4-aminopyridine (4-AP), 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazine butanoic acid hydrobromide (gabazine, SR-95531), picrotoxin, 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP) and ethyl-(S)-11,12,13,13a-tetrahydro -7- methoxy -9- oxo-9H imidazo[1,5-a]pyrrolo[2,1-c] [1,4]benzodiazepine-1-carboxylate (L-655,708) were purchased from Sigma (Taufkirchen, Germany), and tetrodotoxin (TTX), DL-2-amino-5-phosphonopentanoic acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Biotrend (Cologne, Germany). GABA was dissolved in H_2O, furosemide in 0.2M NaOH solution and 4-AP, picrotoxin, gabazine, L-655,708, bumetanide, CNQX, and APV in dimethylsulfoxide (DMSO) (Kolbaev et al., 2012). DA hydrochloride was used at concentrations 0.1, 0.3, 1, 3, 10, and 30 µM in the presence of 5µM nomifensine (1,2,3,4-tetrahydro-2-methyl-4-phenyl-8-isoquinolinamin maleate) and 100 µM sodium metabisulfide (Sigma, Steinheim, Germany) to avoid internal dopamine reuptake and oxidation of dopamine. (6)-SKF-38393 (Sigma), GSK 789472 hydrochloride (Tocris, Ellisville, MO), and (2)-quinpirole hydrochloride (Sigma) were used as a subtype-specific agonists of DA receptors. To block DA receptors we used following antagonists: (R)-(1)-SCH-39166 hydrochloride (Sigma), L-741626 (3-[[4-(4-chlorophenyl)-4-hydroxypiperidin-l-yl]

methyl-1H-indole; Tocris), (2)-sulpiride (Sigma), and SB 277011A (Sigma). In order to antagonize adrenergic receptors the combined application of (RS)-propranolol hydrochloride (Biotrend, Zurich, Switzerland) and phentolamine mesylate (Biotrend) were used. Gabazine (SR- 95531) and DL-2-amino-5-phosphonopentanoic acid (6-APV) were used as antagonists of NMDA and $GABA_A$ receptors. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or GYKI 52466 (4-(8-methyl-9H-1,3-dioxolo[4,5-][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride were used to antagonize AMPA receptors; Tocris). A fresh solution containing DA and sodium metabisulfite was prepared from ACSF every day. For preparation of (6)-APV, GSK 789472, propranolol, and phentolamine we used an aqueous stock solution and all other substances from a stock solution in dimethylsulfoxide (DMSO) (Sharopov et al., 2012). Coffeine and Theophylline were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Concentration of the DMSO in the final solution never exceeded 0.2%.

2.3 Software

Adobe Reader 9	Adobe Systems Inc., CA, USA
Corel Draw X4	Corel GmbH, Unterschleiβheim, Germany
MS Office 2007	Microsoft, Redmond, USA
Matlab 7	The MathWorks Inc., Natick, USA
Reference Manager 12	THOMSON REUTERS,CA, USA
TIDA 5.17	HEKA Elektronik, Dr. Schulze GmBH, Germany

2.4 Hardware

AD/DA Border ITC - 16	Instrutech Corp., Greatneck, N.Y. USA
Tungsten microelectrodes	FHC, Bowdoinham, ME
WPI stimulus isolator A360	World Precision Instruments Germany GmbH, Berlin

3 Methods

3.1 Preparation of hippocampal formation and slices

3.1.1 Hippocampal slice preparation

All experiments were performed according to EU directive 86/609/EEC for the animal use in research and the National Institutes of Health (NIH) Guidelines for the care and use of laboratory animals and by agreement of the local ethical committee (Landesuntersuchungsanstalt RLP, Koblenz, Germany). Maximal efforts were made to decrease the number of experimental animals and their suffering. Wistar rat pups of P 4–7 were taken from the institute's animal facility and were deeply anesthetized with enflurane (Ethrane; Abbot Laboratories, Wiesbaden, Germany). After decapitation, the brains were quickly removed and immersed for 2–3 min in ice-cold standard artificial cerebrospinal fluid (ACSF). All solutions were equilibrated with 95% O_2 / 5% CO_2 at least 1 h before use. For field potential recordings 600-µm–thick slices were used, then slices were transferred to an interface-type recording chamber and constantly were supplied with ACSF at a rate of 1–2 ml/min at 31± 1°C (Kolbaev et al., 2012).

3.1.2 Preparation of corticohippocampal formation (CHF)

For the CHF preparation neonatal mice (C57Bl/62) of P 3–4 were used. Pups were decapitated and the brain was quickly transferred to oxygenated, ice-cold (2–5°C) ACSF. After the brains had equilibrated for 2–3 min, basal brain structures were removed using two spatulas then the hemispheres were dissected, and the CHF was isolated by a series of perpendicular cuts with a scalpel blade. The intact preparation of the CHF includes the hippocampus and large parts of the posterior cortex, including entorhinal and temporal cortex (Quilichini et al., 2002; Quilichini et al., 2003). Each CHF was transferred to a fully submerged chamber and continuously superfused with ACSF at 31°C ± 1°C at a flow rate of ~5 ml/min. After an incubation period of >45 min, the CHF was superfused with low-Mg^{2+} solution to induce epileptiform discharges (Sharopov et al., 2012).

3.1.3 Preparation of in toto whole hippocampus preparation

The preparation of in toto whole hippocampus was performed as described in details before (Kilb et al., 2007). Neonatal rats were decapitated and brain was quickly transferred to oxygenated, ice-cold ACSF. After the brains equilibrated for 2-3 min in the ice-cold ACSF, hemispheres were dissected and the whole hippocampus separated with a spatulum from hippocampal formation and overlying neocortex. Each hippocampi was transported to a fully submerged chamber and superfused with ACSF at 31°C ± 1°C at a flow rate of ~5 ml/min. After an incubation period of >45 min, the hippocampi was superfused with low-Mg^{2+} solution to induce epileptiform discharges or stimulated in CA3 and recorded from CA1 region for evoked responses.

3.2 Data acquisition and analysis

3.2.1 Extracellular field potential recordings from hippocampal slices

Before starting measurements hippocampal slices were allowed to recover in an interface chamber minimum for 1 hour without electrodes and then another 1 hour with electrodes. Field potential recordings were performed from the stratum radiatum of the hippocampal CA3 region as described earlier (Kilb et al., 2006). Signals were amplified by extracellular amplifier, low-pass filtered at 3 kHz and stored on a PC using an AD/DA board (ITC-16, HEKA, Lamprecht, Germany) and TIDA software (HEKA). Hipocampal slices that did not respond with epileptiform activity upon the application of 50-100 μM 4-AP in low-Mg^{2+} solution were removed from analysis. Epileptiform activity was identified according to their amplitude and shape by eye and quantified using the MiniAnalysis program (Synaptosoft, Leonia, NJ) (Kolbaev et al., 2012).

3.2.2 Field potential recordings from the intact CHF

Field potential extracellular activity was recorded from the CA3 region of the intact CHF using tungsten 4–5-MΩ microelectrodes (FHC, Bowdoinham, ME). Signals were AC recorded with a purpose - built amplifier, low-pass filtered at 1 kHz, stored, and analyzed with an eight-channel PC-base software program (Tida 4.11; Heka, Lambrecht, Germany). Extracellular field potentials recorded from up to

four separate CHFs were obtained simultaneously and were analyzed independently. Recordings were analyzed by using algorithms developed on the basis of a Matlab environment (Matlab R2006a; Mathworks, Natick, MA). This algorithm identified spikes by a threshold-crossing detector (with the threshold set manually above the noise level), and subsequently the epileptiform discharges were analyzed according to their occurrence (i.e., the frequency of discharges) and the average amplitude, frequency, and number of spikes per discharge. Epileptiform discharges were classified as seizure-like or interictal events according to the number of spike and the duration of discharges. Power spectrograms of epileptiform activity were calculated using TIDA 4.11. For further analysis, the power was averaged in five frequency bands (delta 0.5–4 Hz, theta 4–8 Hz, alpha 8–14 Hz, beta 14–30 Hz, and gamma 30–80 Hz) (Sharopov et al., 2012).

3.2.3 Extracellular recordings from in toto whole hippocampus

Field potentials were AC-recorded using tungsten micro-electrodes connected to custom-made extracellular amplifiers, low-pass filtered at 1 kHz, digitized (ITC-16, Heka, Lambrecht, Germany), stored and analyzed with PC-based software (Tida 4.11, Heka). Synaptic responses (FP-s) were evoked by electrical stimulation in the CA3 using bipolar stimulation electrode consisting of two tungsten wires (resistance 4 Mega-Ohm), fixed in a theta glass capillary (TGC 150-7.5, 1.5; Harvard Apparatus) for mechanical stability. For stimulation we applied 100µs constant voltage pulses at a frequency of 2/min. Pulse amplitude was set to obtain 50% of the maximal response amplitude. Spontaneous recordings were analyzed using algorithms developed program mentioned above (MATLAB R2006a, Mathworks, Natic, MA, USA). The epileptiform discharges were analyzed according to their occurrence (i.e. the frequency of discharges) and the average amplitude, frequency and number of spikes per discharge.

3.2.4 Statistical analyses

Values throughout this report are given as mean ± SEM. For statistical comparisons, the sign test and Mann-Whitney U test (Systat 11; SPSSInc., Chicago, IL) were used. Significance levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered.

4 Results

4.1 Effect of GABAergic agonists and antagonists on the excitability of the immature hippocampus

4.1.1 In vitro modulation of epileptiform activity by GABAergic antagonists

Activation of GABAergic receptors in the immature CNS has been shown to have opposing effects (inhibitory and excitatory) and accordingly alter seizure susceptibility of the developing hippocampus (Khazipov et al., 2004). Since different influences of synaptic and extrasynaptic GABAergic receptors may underlay this discrepancy (Farrant and Nusser, 2005; Walker and Semyanov, 2008), we performed experiments to check the properties of phasic and tonic $GABA_A$ receptor mediated currents on the excitability of the immature hippocampus. For this purpose we analyzed the effect of GABAergic antagonists and modulators with different affinities to synaptic and extrasynaptic GABAergic receptors on field potential activity of hippocampal slices from immature (P 4–7) rats using field potential recordings (Kolbaev et al., 2012).

4.1.2 $GABA_A$ antagonists induce epileptiform activity in the immature hippocampus

First, we investigated the effect of $GABA_A$ receptor antagonists on field potential recordings in the CA3 region of hippocampal slices of P4-7 rats to clarify the comprehensive effect of $GABA_A$ receptors activation. The first results demonstrated that the specific $GABA_A$ antagonist gabazine (3 μM) induces spontaneous epileptiform activity in 12 of 14 slices investigated (Fig. 7A). This epileptiform activity consisted mainly of interictal discharges composed of 7 ± 2.3 population spikes (Fig. 7B) at a frequency of 7 ± 0.9 Hz occurring at an average interval of 23 ± 6.3 s (n = 12). The non-competitive $GABA_A$ receptor antagonist picrotoxin (100 μM), which at this concentration is able to block tonic $GABA_A$-mediated currents, induced epileptiform activity in all out of nine hippocampal slices (Fig. 7C) with similar properties. Mainly interictal discharges composed of 8 ± 2.6 population spikes (Fig. 7D) at a frequency of 9 ± 0.7 Hz per burst occurred at an average interval of 36.7 ± 11.9 s (n = 9) (Kolbaev et al., 2012).

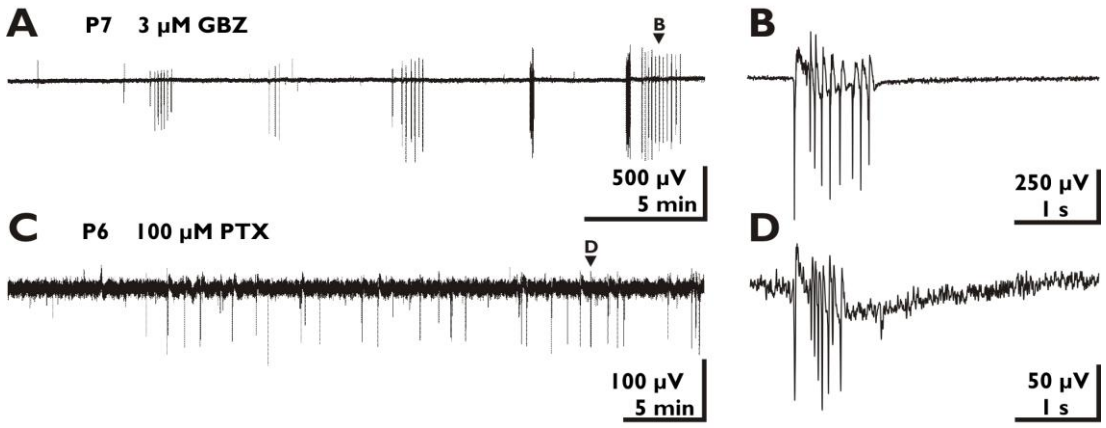

Figure 7. GABAergic antagonists evoke spontaneous epileptiform discharges in immature hippocampal slices. (A, B) Bath application of 3µM gabazine (GBZ)–induced spontaneous epileptiform discharges in a hippocampal slice from a P7 rat. (C, D) Bath application of 100 µM picrotoxin (PTX)–provoked spontaneous epileptiform discharges in a hippocampal slice from a P6 rat. The traces in B and D display typical epileptiform events at a higher temporal resolution (Kolbaev et al., 2012).

In summary, these experiments demonstrate that GABAergic antagonists promote hyperexcitability in immature hippocampal slices, suggesting that $GABA_A$ receptor activation contribute to inhibition in this preparation (Kolbaev et al., 2012).

4.1.3 GABAergic antagonists provoke epileptiform activity

To reveal at what concentration gabazine evoke spontaneous epileptiform activity we did the further experiments using field potential recordings. These experiments were performed in low-Mg^{2+} solution to avoid that the low sensitivity of hippocampal slices to epileptogenic stimuli during the first postnatal week (Psarropoulou and Descombes, 1999; Wong and Yamada, 2001) exaggerates the threshold concentrations for GABAergic antagonists. Lowering of Mg^{2+} in ACSF induced epileptiform activity only in 15 of 167 slices investigated and in none of 12 control slices incubated for 4 h in low-Mg^{2+} solution. At gabazine concentrations below 100 nM the incidence of epileptiform activity was not significantly different from control conditions in low-Mg^{2+} solution (Fig. 8B). 100 nM gabazine significantly ($p = 0.00001$) increased the incidence for epileptiform activity (63.2% of 19 slices; Fig. 8A, B). Our next experiments showed that 300 nM gabazine, evoked epileptiform activity already in 90% of 20 slices, whereas 1µM gabazine evoked spontaneous epileptiform activity in all investigated slices. In 68% of slices,

epileptiform activity consisted of both ictal- and interictal-like discharges, whereas in 16% only ictal- and in 16% only interictal-like activity was observed.

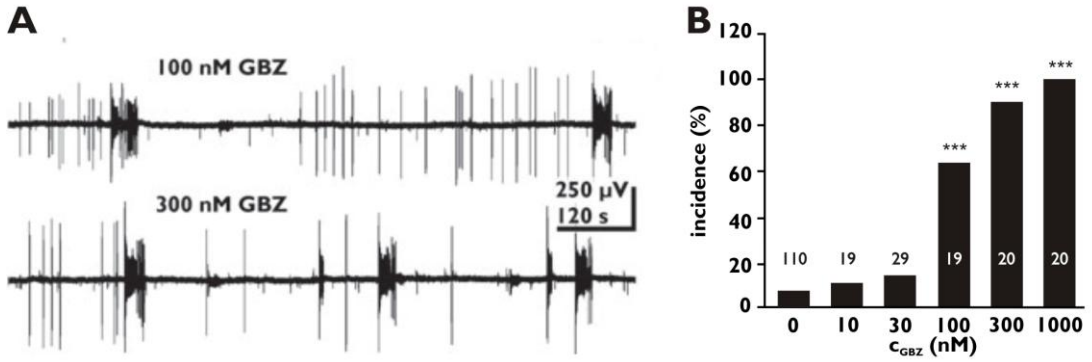

Figure 8. Gabazine provokes epileptiform activity at low concentrations. (A) Rather low GBZ concentrations induce spontaneous epileptiform activity. (B) Bar diagram illustrating the incidence of epileptiform activity at different GBZ concentrations. Note the steep increase in incidence at GBZ concentration >100 nM.

Next the effect of picrotoxin, which in the immature hippocampus affects both synaptic and extrasynaptic receptors, was investigated. The field potential recordings revealed that picrotoxin induced epileptiform activity already at low concentrations of 0.3 µM (Fig. 9A, B). Picrotoxin, at a concentration of 1 µM, provoked epileptiform activity in 53.8% (n = 26) of the slices, whereas higher picrotoxin concentrations induced epileptiform activity in virtually all slices investigated (Fig. 9A, B). Epileptiform activity consisted of ictal- (in 74.6% of active slices) and interictal-like discharges (in 73% of active slices) in most preparations.

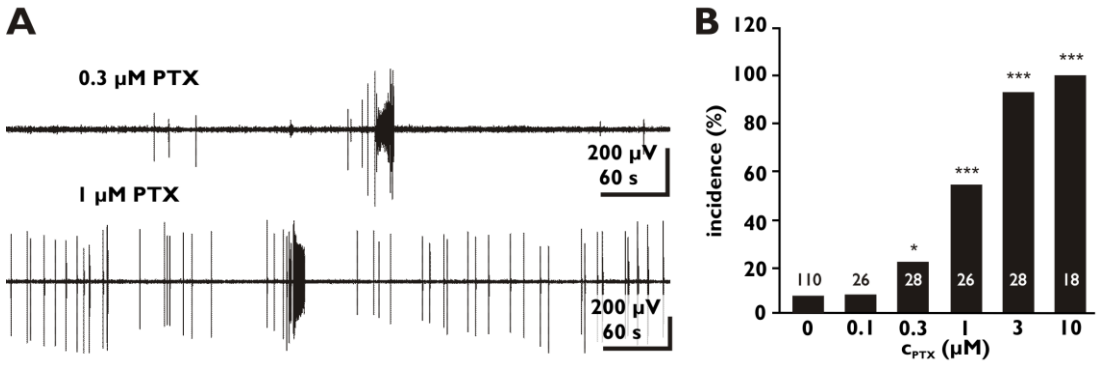

Figure 9. Picrotoxin provokes epileptiform activity at concentrations that suppress phasic synaptic currents. Picrotoxin provokes epileptiform activity at concentrations that suppress phasic synaptic currents. (A) Representative field potential recordings illustrating that 0.3- and 1 µM PTX induced epileptiform activity. (B) Bar diagram illustrating the incidence of epileptiform activity at different

PTX concentrations. Note that PTX at concentration >0.3 µM induced a significant increase in the incidence of epileptiform activity.

In summary, the results of these two sets of experiments demonstrate that GABAergic antagonists at concentrations that reduce phasic GABAergic transmission, but did not affect tonic currents, provoke epileptiform activity, suggesting that a phasic GABAergic inhibition is required to prevent hyperexcitability in the immature hippocampus (Kolbaev et al., 2012).

4.1.4 Agonists of tonic GABAergic receptors promote epileptiform activity

Because picrotoxin could not be used to selectively inhibit tonic GABAergic currents in the immature hippocampal slice preparation (Kolbaev et al., 2012), we next activated tonic GABAergic currents of CA3 pyramidal neurons. They were activated by 1µM THIP, a specific agonist of extrasynaptic α5, 6 - and δ-subunit containing $GABA_A$ receptors (Lindquist et al., 2003). These experiments revealed that this THIP concentration significantly ($p = 0.04$) increased the incidence of epileptiform activity in low-Mg^{2+} solution (Fig.10A). In 12 of 21 slices, epileptiform activity was observed, with both an ictal-like and interictal-like appearance.

To unravel which $GABA_A$-receptor subtype mediates this tonic effect, we used L-655,708, an antagonist of α5-subunit–containing $GABA_A$ receptors (Caraiscos et al., 2004). Field potential recordings showed that 1µM L-655,708 significantly ($p = 0.0003$) reduced the incidence of THIP-induced epileptiform discharges to only 7% ($n = 26$; Fig.10B). In addition, we investigated the effect of furosemide, which inhibits α6-subunit–containing $GABA_A$ receptors (Korpi et al., 1995). Although 500 µM furosemide did not significantly reduce THIP-induced tonic currents (117 ± 11.1%, $n = 10$), it significantly ($p = 0.015$) reduced the proconvulsive effect of 1µM THIP (Fig.10B). Only in 2 of 13 preparations could epileptiform discharges be induced under this condition. Because furosemide is a potent inhibitor of NKCC1-mediated Cl^- accumulation in immature neurons (Payne et al., 2003), we also performed control experiments with bumetanide, another potent NKCC1 inhibitor that did not interfere with α6-subunit containing $GABA_A$ receptors. In the presence of 10 µM bumetanide, the proconvulsive effect of 1µM THIP was completely blocked ($n = 13$), suggesting that the furosemide effect was most probably not due to inhibition of α6-subunit–containing $GABA_A$ receptors.

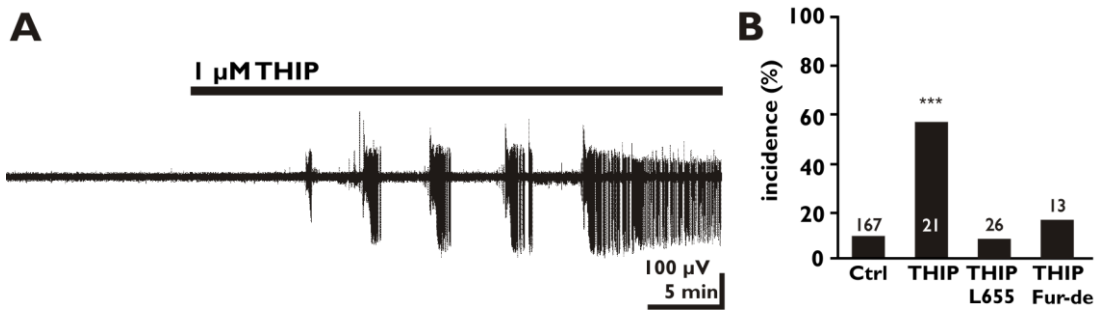

Figure 10. THIP promotes epileptiform activity. (A) Typical field potential recording illustrating that bath application of 1µM THIP in low-Mg^{2+} solution induced epileptiform discharges. (B) Statistical analysis revealed that 1µM THIP significantly increased the incidence of epileptiform discharges as compared to the control condition in low-Mg^{2+} solution (Ctrl). The proconvulsive THIP effect was completely blocked in the presence of 1µM L-655,708 and 500 µM furosemide.

In summary, these results suggest that activation of a tonic GABAergic component can contribute to ictogenesis in hippocampal slices. This proconvulsive effect of tonic GABAergic currents is at least partially mediated via α5-subunit–containing $GABA_A$ receptors and requires NKCC1-mediated Cl^- transport (Kolbaev et al., 2012).

4.2 DA receptors mediate epileptiform activity in the mouse CHF

4.2.1 The involvement of DA receptors in epileptic seizures

It has gradually become evident that the DAergic system also plays a profound role in epileptogenesis (Starr, 1996). Initial clinical observations suggested that various DAergic agonists have an anticonvulsant effect, whereas DA antagonists exacerbate seizures or lower seizure thresholds (Clemens, 1988; Gattereau et al., 1990; Lipka and Lathers, 1987). However, surprisingly little information is available on the influence of the DAergic system on seizure susceptibility in the immature nervous system. Therefore, using field potential recordings, we investigated the effect of DAergic agonists and antagonists on epileptiform activity in the corticohippocampal formation of immature mice.

4.2.2 DAergic modulation of low-Mg^{2+} - induced epileptiform activity

Exposure to 0.2 mM Mg^{2+}-containing ACSF (low-Mg^{2+} solution) induced spontaneous epileptiform activity in the CHF of immature mice, as previously shown (Moser et al., 2006). In all preparations, the epileptiform activity consisted of seizure-like and interictal-like events. To elucidate the effect of DA on epileptiform activity, it was bath applied in concentrations ranging from 0.1 to 30 µM to CHF preparations that showed stable epileptiform discharges in low-Mg^{2+} solution. DA was added in the continuous presence of 5 µM nomifensine to prevent endogenous DA reuptake. Bath application of 5 µM nomifensine significantly ($P < 0.01$) reduced the occurrence of seizure-like and interictal-like events by 14% ± 5.9% (n = 33) and 24% ± 3.6% (n = 33), respectively (data not shown). Application of 0.1 µM DA in the presence of nomifensine resulted in a slight but obvious attenuation of epileptiform activity (Fig. 11A, B). At intermediate concentrations (0.3–3 µM) no changes in epileptiform activity were obvious (Fig. 11C, D), but higher DA concentrations (>10 µM) induced an exaggeration of epileptiform activity (Fig. 11E, F). The averaged power spectrogram revealed that the DA effect led to alterations in a frequency band between ~3 and 50 Hz (Fig. 11G), so we analyzed DA's effect on the average power in the classical EEG-frequency bands. These analyses revealed that 0.1 µM DA induced a significant reduction in theta (93.3% ± 0.8%, P = 0.0386, n = 12), alpha (90.9% ± 0.8%, P = 0.0386), beta (90% ± 1.3%, P = 0.0386), and gamma (90.8% ± 1.5%, P = 0.0063) bands, whereas the power in the delta frequency band was not altered (Fig. 11H). Bath application of 0.3 µM (n = 11), 1 µM (n = 11), or 3 µM (n = 11) DA did not significantly change the power of epileptiform activity in any frequency band (Fig. 11H). In contrast, 10 µM DA significantly increased the power of epileptiform activity in theta (115.1% ± 1.9%, P = 0.0352, n = 15), alpha (147% ± 1.8%, $P < 0.0001$), beta (159.4% ± 2.7%, $P < 0.0001$), and gamma (157.3% ± 2.8%, $P < 0.0001$) frequency bands, whereas the delta frequency range was not altered (Fig. 11H). Application of 30 µM DA led to a significant increase of the power in all frequency bands (delta 132.5% ± 2.6%, P = 0.0117, n = 11; theta 134.4% ± 2.4%, P = 0.0117; alpha 166.5% ± 3.6%, P = 0.001; beta 176.8% ± 3.8%, P = 0.001; gamma 158.9% ± 4.7%, P = 0.001; Fig. 11H).

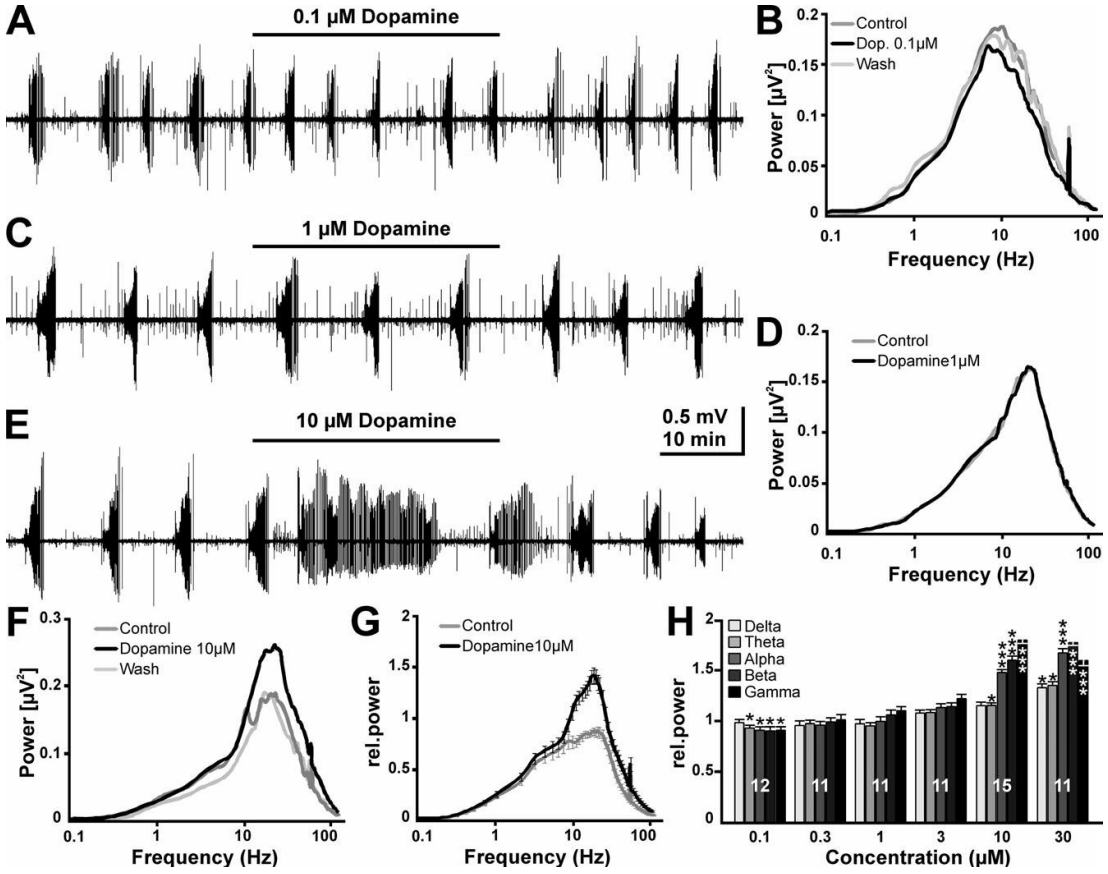

*Figure 11. Effect of DA on low-Mg^{2+} -induced epileptiform activity. A: Field potential recording in the CA3 region of the intact in vitro corticohippocampal formation (CHF) from a P4 mouse, illustrating the effect of 0.1 µM DA. B: Power spectrogram of EA shown in A before (control, dark gray line) and during (black line) the application of 0.1 µM DA and after wash out (light gray line). C: Field potential recordings of low-Mg^{2+}- induced EA illustrating the effect of 1 µM DA. D: Power spectrogram of EA shown in C before (control, gray line) and during (black line) the application of 1 µM DA. E: Field potential recordings of low-Mg^{2+} -induced EA illustrating the effect of 10 µM DA. F: Power spectrogram of EA induced by low-Mg^{2+} ACSF (control, dark gray line), during the application of 10 µM DA (black line) and after wash out (light gray line). G: Averaged power spectrogram of 15 experiments before (control, gray line) and during (black line) the application of 10 µM DA. H: Statistical analysis of different DA concentrations on the power of epileptiform discharges in different frequency ranges (delta 0.5–4 Hz, theta 4–8 Hz, alpha 8–14 Hz, beta 14–30 Hz, and gamma 30–80 Hz). Bars represent mean ± SEM. Number of experiments is given in the bars. Statistically significant differences are marked by *P < 0.05 and ***P < 0.001.*

To address the question of which alterations in the discharge pattern underlie the observed changes in the power of epileptiform activity, we next investigated the epileptiform activity patterns in more detail. Low-Mg^{2+} - induced epileptiform activity consisted of interictal events (IIE; Fig. 12A, B1) and seizure-like events (SLE; Fig. 12B2). IIE occurred at a frequency of 1.3 ± 0.09 min^{-1} (n = 55) and

consisted on average of 4 ± 0.2 spikes at a frequency of 13.6 ± 0.6 Hz. SLE occurred at a frequency of 0.14 ± 0.006 min^{-1} (n = 55). These events started with high-frequency spikes, followed by separated burst discharges at the end of the SLE reminiscent of the clonic phase in human epilepsy. These late bursts during SLE mostly had a spindle-like appearance (Fig. 12B3). In the presence of 10 µM DA, the structure of epileptiform patterns changed considerably. SLE were nearly unaffected (Fig. 12C1), but virtually no IIE could be detected under this condition. On the other hand, DA induced short patterns (0.5–2 sec) outside of SLE that had no corresponding patterns under control conditions (Fig. 12C2–4). Therefore, we classified all patterns outside of SLE as non-SLE. This non-SLE occurred at a frequency of 2.2 ± 0.16 min^{-1} and consisted on average of 13 ± 1.4 spikes at a frequency of 14. ± 0.4 Hz (n = 72).

By using semiautomatic classification of all epileptiform events, we analyzed the effect of different DA concentrations on SLE and non-SLE regarding their occurrence, amplitude, and frequency and the number of spikes per discharge. Application of 0.1 µM DA significantly (P = 0.0386) reduced the average occurrence of SLE by 25.1% ± 5.1% (n = 12; Fig.12D), whereas amplitude, frequency, and number of spikes were not significantly affected. At DA concentrations between 0.3 and 10 µM SLE were mostly unaffected (Fig. 12D), except for a slight reduction in the frequency of spikes by 2.2% ± 4.1% (n = 25, P = 0.0026) at 3 µM DA. Only in the presence of 30µM DA was the occurrence of SLE significantly (P = 0.0352) increased by 36.2% ± 13.5% (n = 15; Fig. 12D).

DA exerted stronger effects on the properties of non-SLE. Application of low DA concentrations reduced the occurrence of non-SLE, whereas amplitude, frequency, and number of spikes per non- SLE were not affected (Fig. 12E). Specifically, 0.1 µM DA reduced the occurrence by 24.6% ± 6.7% (P= 0.0386, n = 12), 0.3 µM DA by 23.2% ± 8.5 (P = 0.6291, n = 16), and 1 µM DA by 25.9% ± 12.5% (P = 0.0192, n = 19). In contrast, 10 µM and 30 µM DA significantly increased the occurrence of non-SLE by 64.4% ± 14.4% (P = 0.0004, n = 72) and 61.2% ± 22.6% (P = 0.0352, n = 15), respectively. In addition, the numbers of spikes per non-SLE were also significantly increased by 87.7% ± 21.4% (P = 0.0004, n = 72) and 89.7% ± 24.4% (P = 0.0352, n = 15), respectively, reflecting the transition to the more complex non-SLE shown in Figure 12C2–4.

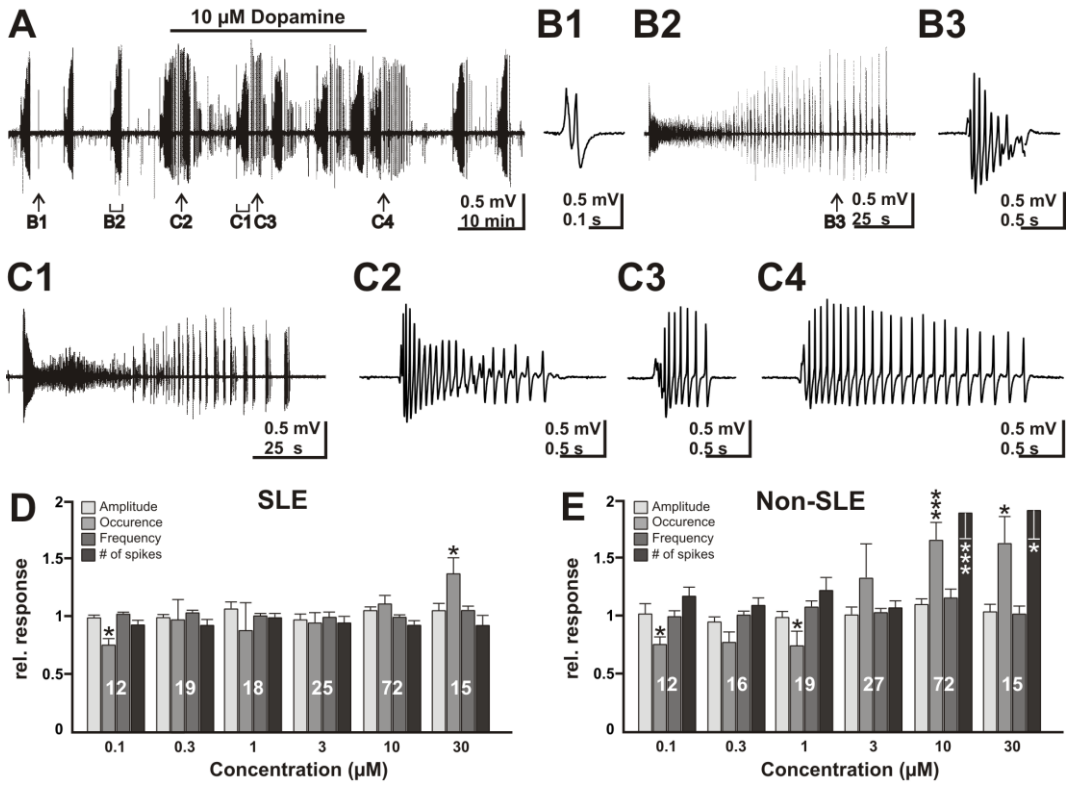

*Figure 12. Effect of DA on the discharge pattern of low-Mg^{2+}-induced epileptiform activity. A: Field potential recording in the CA3 region of an intact CHF from a P4 mouse illustrating the effect of bath application of 10 µM DA. Typical discharge pattern of this recording, as marked in A, shown in higher temporal resolution in the following. B1: One interictal event. B2: Seizure-like event (SLE). B3: One spindle-like discharge of the clonic phase of the SLE, as marked in B2. C1: SLE, recorded after application of 10 µM DA. C2–4: Representative short discharge patterns induced by the bath application of 10 µM DA. D: Statistical analysis of the effect of different DA concentrations on different parameters of SLE. E: Statistical analysis of the DA effect on non- SLE. Bars represent mean ± SEM. Number of experiments is given in the bars. Statistically significant differences are marked by *P < 0.05 and ***P < 0.001.*

In summary, these results indicate that DA strongly influenced low-Mg^{2+} induced epileptiform activity. Low DA concentrations attenuated epileptiform activity, most probably by reducing the number of SLE and non-SLE. In contrast, DA at concentrations of >10 µM exaggerated epileptiform activity, mainly by increasing the occurrence of non-SLE and the number of spikes within these discharges (Sharopov et al., 2012).

4.2.3 Dopamine effect after blockade of adrenergic receptors

It has been demonstrated that DA at higher concentrations is an agonist of adrenergic receptors (Malenka and Nicoll, 1986), and these receptors are known to

influence epileptiform activity (Mueller and Dunwiddie, 1983; Stanton et al., 1987). Therefore, it has been investigated whether the pro- and anticonvulsive effect of high and low DA concentrations were influenced by interactions with the adrenergic system. For this purpose, we completely blocked α- and β-adrenergic receptors by the combined application of 10µM phentolamine and 10µM propranolol, respectively. Under this condition, application of 10µM DA increased the occurrence of non-SLE by 331% ± 35.1% (n = 13), which was significantly (P = 0.00001) greater than in the absence of phentolamine and propranolol (Fig. 13). The anticonvulsant effect of low DA concentrations was unaffected in the presence of phentolamine and propranolol. Bath application of 0.1 µM DA under these conditions significantly reduced the occurrence of non-SLE by 22% ± 11.8% (n = 10) and the occurrence of SLE by 11% ± 7% (n = 6). In summary, these results indicate that the proconvulsive effect of 10 µM DA was even underestimated due to the anticonvulsive effect that high DA concentrations exert via adrenergic receptors (Mueller and Dunwiddie, 1983).

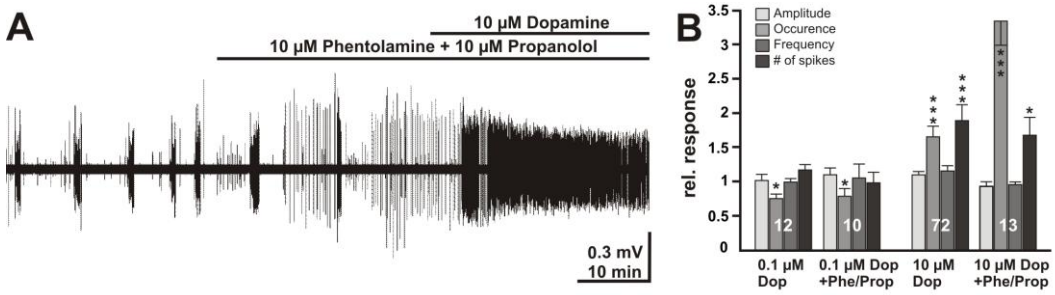

Figure 13. Effect of adrenergic antagonists on the DA effects. A: Field potential recording of epileptiform activity induced by low- Mg^{2+} ACSF in the CA3 region of an intact CHF from a P4 mouse. Application of 10 µM DA in the presence of 10 µM phentolamine and 10 µM propranolol enhanced epileptiform activity. B: Statistical analysis of the effect of 0.1 µM and 10 µM DA on different parameters of non-SLE revealed that, in the presence of phentolamine and propranolol, the anticonvulsive effect of 0.1 µM DA was unaltered and the proconvulsive effect of 10 µM DA was enhanced (Sharopov et al., 2012).

4.2.4 Modulation of low-Mg^{2+} - induced epileptiform activity by DAergic agonists and antagonists

To disclose which receptor subtypes underlie the DA effect on epileptiform activity, we first used subtype-specific agonists. Application of the D1-agonist

SKF38393 (Ruskin et al., 1998) enhanced epileptiform activity (Fig. 14A). The power of low-Mg^{2+} -induced epileptiform activity was significantly increased in the presence of 10 µM SKF38393 in alpha (125.9% ± 3.5%, P = 0.0005, n = 12), beta (129.9% ± 2.8%, P = 0.0005), and gamma (133.3% ± 1.5%, P = 0.0386) frequency ranges, whereas the power in the delta frequency range was not altered. A detailed analysis of the properties of SLE and non-SLE revealed that 10 µM SKF38393 had no significant effect on SLE (n = 12) but significantly (P = 0.0063) increased the occurrence of non-SLE by 119.3% ± 27.5% (n = 12). Amplitude and frequency of non-SLE and number of spikes per non- SLE remained unaffected (Fig. 14B).

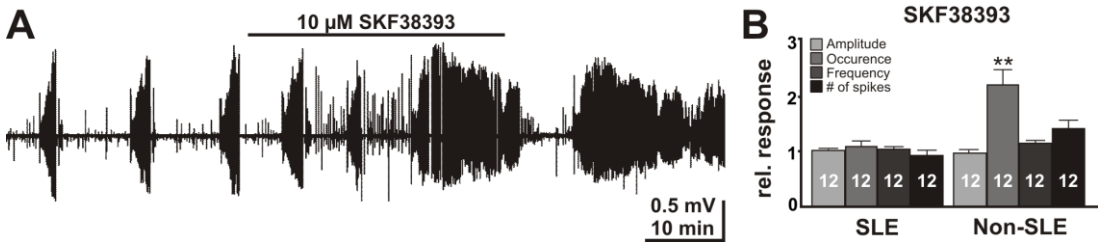

Figure 14. Application of D1-agonist SKF38393 enhanced epileptiform activity. A: Field potential recording in the CA3 region of an intact CHF from a P3 mouse. Application of the D1 agonist SKF38393 enhanced epileptiform activity. B: Statistical analysis of SKF38393 effects on different parameters of SLE and non-SLE. Note that the properties of SLE were unaltered by 10 µM SKF38393 application, whereas the occurrence of non-SLE was significantly increased.

Application of the D2-like receptor agonist quinpirole (Koller et al., 1987) did not have such clear effects on low-Mg^{2+} -induced epileptiform activity (Fig. 15A). In the presence of 10 µM quinpirole, neither occurrence nor amplitude, nor frequency, nor number of spikes per SLE was significantly affected (n = 24; Fig. 15B). Quinpirole significantly (P = 0.026) reduced the occurrence of non-SLE by 10% ± 7.2% (n = 24; Fig. 15B). In contrast, application of 1 µM GSK-789472, a partial D2 agonist that antagonizes D3 receptors (Holmes et al., 2010), had no significant effect on epileptiform activity (Fig. 15C). Neither occurrence (89% ± 10.9%, n = 17 and 105% ± 9.4%, n = 17) nor amplitude (109% ± 4.2% and 109% ± 6.7%), nor frequency (98% ± 2.4% and 100% ± 5.2%), nor number of spikes per event (110% ± 10 and 114% ± 18.3%) of SLE and non-SLE was significantly affected (Fig. 15D).

Coapplication of 10 µM SKF38393 and 10 µM quinpirole enhanced epileptiform activity similarly to the effect of 10 µM SKF38393 alone (data not shown). In the presence of SKF38393 and quinpirole, the occurrence of non-SLE significantly (P =

0.0005) increased by 136.5% ± 33.5% (n = 16), whereas the properties of SLE were unaffected. Accordingly, the power of epileptiform activity was significantly (P < 0.001) increased in alpha (124.7% ± 3.5%, n = 16), beta (135.5% ± 2.7%), and gamma (168.9% ± 3.5%) frequency ranges.

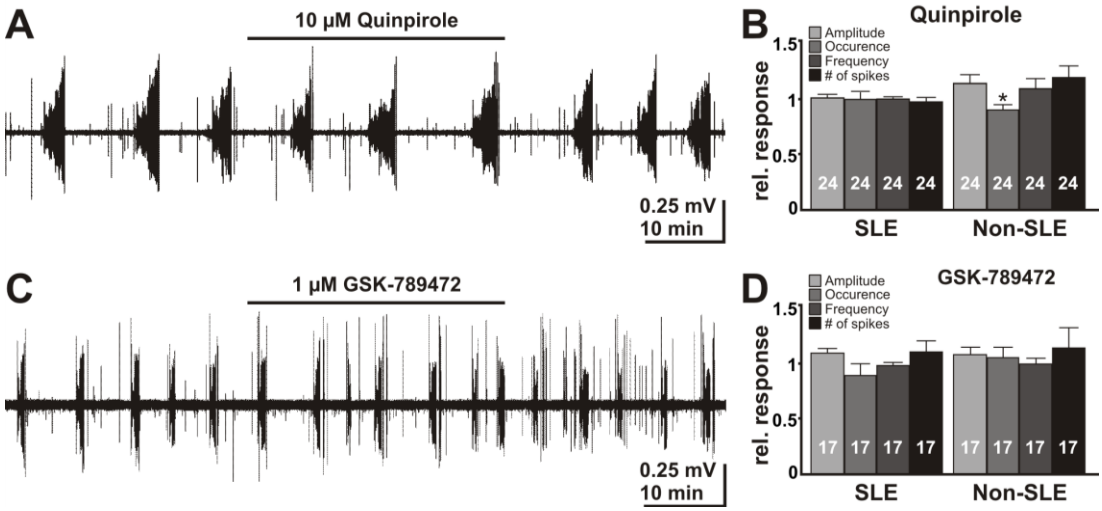

*Figure 15. Effect of subtype-specific DA receptor agonists on low-Mg^{2+}-induced epileptiform activity. A: Field potential recording in the CA3 region of an intact CHF from a P3 mouse. Field potential recording from a P4 mouse CHF illustrating the effect of the D2-like receptor agonist quinpirole. B: Statistical analysis of the effect of 10 µM quinpirole on different parameters of SLE and non-SLE revealed that only the occurrence of non-SLE was significantly reduced. C: Field potential recording from a P3 mouse CHF illustrating the effect of the D2 agonist GSK-789427. D: Statistical analysis of the effect of 1 µM GSK-789427 revealed that neither SLE nor non-SLE was affected. Bars represent mean 6 SEM. Number of experiments is given in the bars. Statistically significant differences are marked by *P < 0.05 and **P < 0.01.*

In summary, these results demonstrate a strong proconvulsive effect of the D1 agonist SKF38393, which mainly increased the occurrence of non-SLE. The D2- like receptor agonist quinpirole mediated a slight anticonvulsive effect, whereas the D2-specific agonists GSK-789472 did not increase epileptiform activity, suggesting that D2 receptors are not directly involved in the anticonvulsant effect.

Next, we tested the effect of SCH39166, an antagonist of D1-like receptors, on low-Mg^{2+} -induced epileptiform activity. Application of 2 µM SCH39166 itself had no significant effect on the properties of SLE or non-SLE, but SCH39166 prevented the proconvulsive effect of DA. In the presence of 2 µM SCH39166, bath application of 10 µM DA did not enhance the power of epileptiform activity, and the DA-induced increase in the occurrence of non-SLE (Fig. 16A,F) and the number of

spikes per non-SLE event was abolished. In addition, the anticonvulsive effect of 0.1 µM DA was also completely blocked in the presence of 2 µM SCH39166 (Fig. 16G).

Bath application of the D2 receptor antagonist L- 741626 (50 nM) did not have a significant effect on the properties of SLE and non-SLE events. In the presence of L-741626, bath application of 10 µM DA increased the occurrence of SLE by 133% ± 46.7% (n = 9), which was not significantly (P = 0.178) different from the DA effect under control conditions (Fig. 16B, F). In contrast, the anticonvulsive effect of 0.1 µM DA was completely suppressed in the presence of 50 nM L-741626 (Fig. 16C, G). Neither the occurrence of SLE (108% ± 14.8%, n = 11) nor that of non-SLE (103% ± 12.1%, n = 15) was significantly reduced when 0.1 µM DA was applied in the presence of 50 nM L-741626.

Bath application of the D3 antagonist SB-277011- A (100 nM) had no effect on low-Mg^{2+} -induced epileptiform activity, except for a significant (P = 0.006) reduction in the number of spikes per non-SLE by 42.4% ± 7.9% (n = 12). In the presence of 100 nM SB-277011-A, bath application of 10 µM DA enhanced the epileptiform activity (Fig. 16D). The proepileptic effect of 10 µM DA on the occurrence and number of spikes per non-SLE was not significantly affected in the presence of 100 nM SB-277011-A (Fig. 16F). In contrast, the anticonvulsive effect of 0.1 µM DA was abolished in the presence of 100 nM SB- 277011-A (n = 12; Fig. 16G) These results suggest that D3 receptors were not involved in the proconvulsive effect of higher DA concentrations but might mediate the anticonvulsive effect of low DA concentrations.

Bath application of the D2-like receptor antagonist sulpiride (10 µM) did not significantly affect the properties of SLE and non-SLE, except for a slight increase in the frequency of spikes within non-SLE by 14.7% ± 5.6% (n = 22; P = 0.004). Surprisingly, the proepileptic DA effect was completely abolished by 10 µM sulpiride (Fig. 16E). In the presence of sulpiride, bath application of 10 µM DA influenced neither the average power of epileptiform activity nor the occurrence and number of spikes per non-SLE (Fig. 16E, F). Sulpiride (10 µM) also suppressed the anticonvulsive effect of 0.1 µM DA (Fig. 16G).

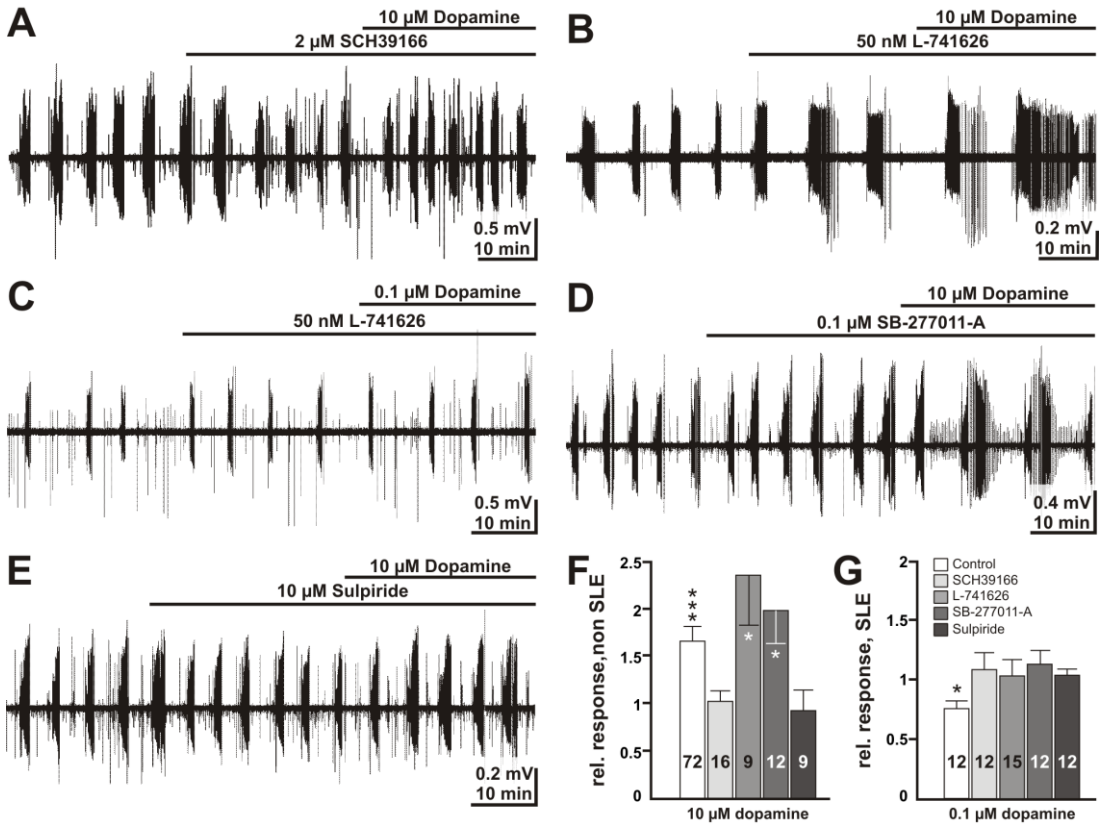

*Figure 16. Influence of DA receptor antagonists on the proconvulsive and anticonvulsive effect of DA. A: Field potential recording from a P3 mouse illustrating that the D1-like receptor antagonist SCH39166 suppressed the proconvulsive effect of 10 µM DA. B: Field potential recording from a P4 mouse illustrating that the D2 antagonist L-741626 had no effect on the proconvulsive effect of 10 µM DA. C: Field potential recording from a P3 mouse illustrating that the D2 antagonist L-741626 suppressed the anticonvulsive effect of 0.1 µM DA. D: Field potential recording from a P4 mouse demonstrating that the D3-antagonist SB- 277011-A did not influence the proconvulsive effect of DA. E: Field potential recording from a P3 mouse demonstrating that the D2-like receptor antagonist sulpiride suppressed the proconvulsive effect of DA. F: Statistical analysis of the influence of DArgic antagonists on the 10 µM DA induced proconvulsive effect. The key to columns is displayed in G. G: Statistical analysis of the influence of DArgic antagonists on the 0.1 µM DA induced anticonvulsive effect. Bars represent mean ± SEM. Number of experiments is given in the bars. Statistically significant differences are marked by *P < 0.05 and ***P < 0.001.*

To investigate whether the unexpected effect of sulpiride was caused by an interaction with D1-like receptors, we specifically activated D1-like receptors with SKF-38393. The proconvulsive effect of SKF- 38393 was virtually unaffected in the presence of 10 µM sulpiride (Fig. 17A). Under this condition, 10 µM SKF- 38393 significantly (P = 0.0078) increased the frequency of non-SLE by 151% ± 30.4%. In contrast, 10 µM SKF-38393 had, as expected, no significant effect on epileptiform

activity in the presence of the D1-like receptor antagonist SCH39166 (2 µM, Fig. 17B). These experiments illustrate that the paradoxical effect of sulpiride was most probably not mediated by an interaction with D1-like receptors.

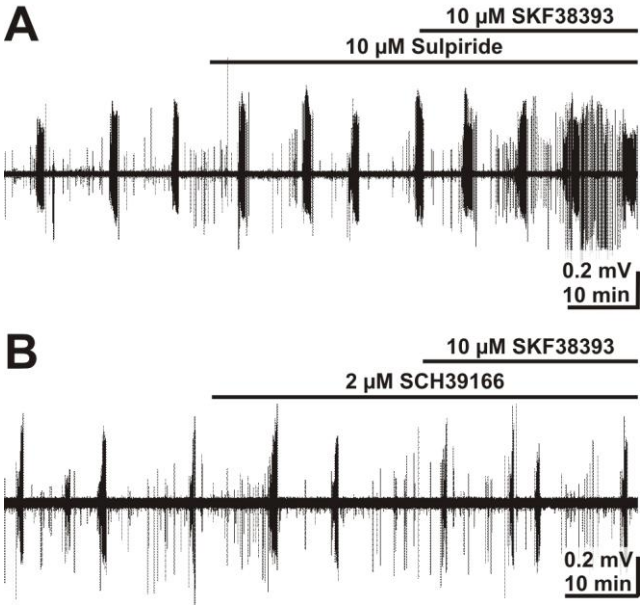

Figure 17. Effect of the D2-like receptor antagonist sulpiride and the D1-like receptor antagonist SCH 39166 on the proconvulsive SKF38393 effect. A: Field potential recording from a P3 mouse illustrating that 10 µM sulpiride had no effect on the proconvulsive effect of 10 µM SKF38393. B: Field potential recording from a P3 mouse illustrating that 2 µM SCH39166 suppressed the proconvulsive effect of 10 µM SKF38393.

In summary, these results suggest that the proconvulsive effect of high DA concentrations was suppressed by D1-like receptor antagonists, but also by sulpiride. The anticonvulsive effect of low DA concentrations was suppressed by D1-like, D2, and D3 receptor antagonists. These results support the hypothesis that D1-like receptors mediate the proconvulsive effect of DA, but the impact of the different DA receptors on the anticonvulsive effect is less clear (Sharopov et al., 2012).

4.2.5 Identification of synaptic targets mediating the DAergic proconvulsive effect

Finally, we investigated the question of whether the proconvulsive DA effect requires particular synaptic receptors. Bath application of the $GABA_A$ antagonist gabazine (3 µM) to the low-Mg^{2+} solution considerably changed the discharge pattern and led to an activity pattern that consisted of repetitive discharges of 7.6 ± 1.8 spikes (n = 9) at a frequency of 16 ± 3.5 Hz (Fig. 18A). These discharges occurred at a frequency of 0.07 ± 0.01 Hz (n = 9). Under this condition, bath application of 10 µM DA changed the discharge pattern (Fig. 18A). The occurrence of epileptiform discharges significantly (P = 0.0391) increased from 0.07 ± 0.01 Hz to 0.12 ± 0.02 Hz (n = 9), but the number of spikes per discharge was significantly (P

= 0.0391) reduced from 31 ± 8 to 22 ± 4 (Fig. 18B). In accordance with these divergent effects, no significant effect of 10 µM DA on the average power of epileptiform activity was observed in the presence of gabazine.

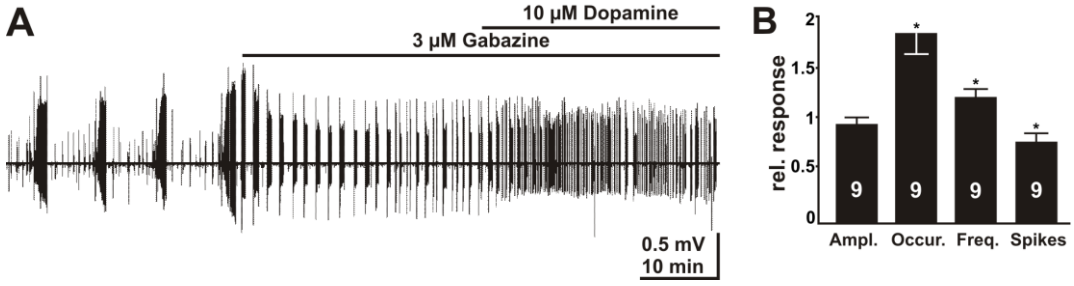

Figure 18. Effect of 10 µM DA after pharmacological blockade of GABA receptors. A: Field potential recording from a P4 mouse illustrating that blockade of $GABA_A$ receptors with 3 µM gabazine transforms epileptiform activity into a repetitive discharge pattern. Coapplication of 10 µM DA leads to modification of activity pattern. B: Statistical analysis of the effect of 10 µM DA on different parameters of epileptiform discharges in the presence of 3 µM gabazine.

Inhibition of AMPA/kainate receptors with CNQX attenuated both SLE and non-SLE. In the presence of 10 µM CNQX, the occurrence and amplitude of SLE were significantly ($P < 0.001$) reduced by 41% ± 3.8% and 81% ± 3.1% (n = 16), respectively (Fig. 19A). CNQX reduced the occurrence and amplitude of non-SLE by 40% ± 6.6% and 36% ± 5.0% (n = 17), respectively. In the continuous presence of CNQX, bath application of 10 µM DA significantly (n = 0.049) increased the occurrence of non-SLE by 32% ± 12.5% (n = 17). On the other hand, the other properties of non-SLE such as amplitude and number of spikes per discharge were unaffected (Fig. 19B), indicating that, in the absence of functional AMPA/kainate receptors, 10µM DA did not mediate the typical transition to more complex non-SLE discharges. Selective blockade of AMPA receptors with GYKI 52466 (Paternain et al., 1995) provoked effects on epileptiform activity similar to those of CNQX. Bath application of 50 µM GYKI 52466 reduced the occurrence and amplitude of SLE (by 24% ± 13% and 24% ± 6%; n = 13) and non- SLE (by 61% ± 4.4% and 39% ± 4.4%; n = 16). In the continuous presence of 50 µM GYKI 52466, bath application of 10 µM DA induced a significant ($P = 0.035$) increase in the occurrence of non-SLE by 44% ± 20.7% (n = 16), whereas other properties of non-SLE such as amplitude and number of spikes per discharge were again unaffected (Fig. 19B). DA did not significantly alter the occurrence and properties of SLE in the presence of either

CNQX or GYKI 52466, except for a significant (P = 0.0034) reduction in the number of spikes per discharge by 35% ± 6.6% (n = 13) in the presence of CNQX.

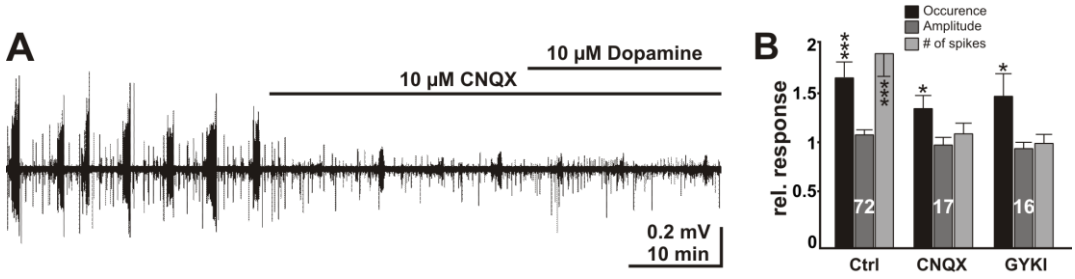

Figure 19. Effect of DA in the presence of functional blocked AMPA/kainate receptors. C: Field potential recording from a P3 mouse illustrating that the AMPA/kainate receptor antagonist CNQX massively reduced the amplitude of SLE and non-SLE. D: Statistical analysis of the effect of 10 µM DA on different parameters of non-SLE under control conditions (left bars) and in the presence of 10 µM CNQX (middle bars) or 50 µM GYKI 52466 (right bars).

In summary, these results demonstrate that, even in the absence of functional AMPA receptors, DA can exert a proconvulsive effect. On the other hand, the typical DA-induced transition of non-SLE to more complex discharge patterns was suppressed in the presence of both CNQX and GYKI 52466, indicating that AMPA receptor may play an important role in this proconvulsive component.

Blockade of NMDA receptors with 60 µM (±) - APV completely abolished SLE in all 10 CHF tested (Fig. 20A) and significantly (P = 0.0215) reduced the amplitude, frequency, and number of spikes of non-SLE, but not their occurrence. Bath application of 10 µM DA in the presence of APV provoked massive epileptiform activity with an SLE-like appearance in all 10 CHF tested (Fig. 20A). In addition, the occurrence of non-SLE was significantly (P = 0.0215) increased by 29% ± 7.5% (n = 10). Accordingly, the average power of field potential activity was significantly (all P = 0.0039) increased in all frequency bands (Fig. 20B). It has been shown that the prolonged application of low-Mg^{2+} solution can lead to APV-insensitive epileptiform discharges (Quilichini et al., 2002), so we performed additional control experiments. These experiments revealed that, during 1 hr of APV application, a stable anticonvulsive effect was maintained. No SLE could be observed during this interval, and the properties of non- SLE were not significantly different between 15–30 min and 45–60 min of APV application, indicating that the proconvulsive effect of DA was not overestimated by a gradual loss of APV sensitivity during this interval.

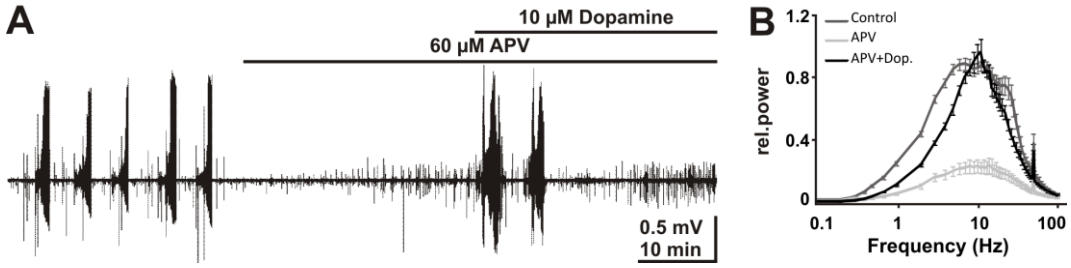

*Figure 20. Effect of DA in the presence of functional blocked NMDA receptors. A: Field potential recording from a P3 mouse illustrating that the NMDA receptor antagonist APV completely blocked SLE. Note that 10 µM DA induced SLE in the presence of APV. B: Power spectrogram of epileptiform activity shown in A before (dark gray line) and during (light gray line) the application of 60 µM APV and after application of DA 10 µM in APV (black line). Bars represent mean ± SEM. Number of experiments is given in the bars. Statistically significant differences are marked by *P < 0.05 and ***P < 0.001.*

In summary, these results suggest that DA can provoke a proepileptic effect in the absence of functional NMDA receptors. Overall, these experiments indicate that DA mediates a reliable proconvulsive effect even after inhibition of AMPA, NMDA, and $GABA_A$ receptors, suggesting that DA enhances the excitability by influencing various neurotransmitter systems (Sharopov et al., 2012).

4.3 Proepileptic effect of methylxanthines on the immature hippocampal formation

4.3.1 Proepileptic effect of methylxanthines in a whole-hippocampus preparation of immature rats

In human preterm infants, the methylxanthines caffeine and theophylline are routinely administered to stabilize respiration. In order to investigate, whether these substances promote epileptic seizures, we performed field-potential recordings from the CA1 region of *in-toto* hippocampal preparations of P1-4 and P8-10 rats. In this preparation we analyzed the effect of theophylline and caffeine on postsynaptic field potential responses (FP), evoked by electrical stimulation in the CA3 region, and tested also whether they can induce spontaneous epileptiform discharges.

4.3.2 Effect of theophylline on the whole-hippocampus preparation

In this preparation we first investigated the effect of theophylline on postsynaptic field-potential responses (FP-PSPs) recorded in the CA1 region upon electrical stimulation in CA3 (Fig.21A, B). Electrical stimulation in CA1 evoked in all of the control experiments (n=6 in P1-4 animals and n=9 in P8-10 animals) monosynaptic FP-PSPs. In hippocampi from P1-4 rats no significant effect of bath applied theophylline on the amplitude of the FP-PSPs could be observed (Fig.21A, C). In the P8-10 hippocampal preparations 2.5 mM, 5 mM and 10 mM theophylline significantly ($p= 0.024$, 0.037 and 0.0087) potentiated the FP-PSPs by 35 ± 13% (n=8), 29 ± 11 % (n=7) and 23 ± 5% (n=8), respectively. Additional polysynaptic components appeared in the FP responses at theophylline concentration ≥ 2.5 mM for P1-4 and ≥ 0.5 mM for P8-10 rats. This polysynaptic activity was in most cases accompanied by electrically evoked epileptiform discharges (Fig.21D, E). In the P1-4 hippocampi such evoked epileptiform activity could be observed at theophylline concentration ≥ 2.5 mM. While at 2.5 mM theophylline only 33% (n=8) of FP-PSCs were followed by epileptiform activity, in the presence of 5 mM theophylline epileptiform activity was observed already in 83 % (n=7) of the recordings (Fig. 21D, F1). Neither the duration (Fig.21F2) of evoked epileptiform activity, nor the frequency of discharges within such epileptiform bursts (Fig.21 F3) showed a significant dependency on the theophylline concentration. In the P8-10 hippocampi

evoked epileptiform activity could be observed already at a theophylline-concentration of 1 mM in 29 % (n=7) of the recordings (Fig.21E, F1). In the presence of 2.5 mM theophylline evoked epileptiform discharges were observed already in 88% (n=8) of the recordings (Fig.21C, E). While the frequency of discharges within epileptiform bursts did not reveal a significant dependency on the theophylline concentrations, the duration of these events significantly (p=0.034) increased at a theophylline concentration of 10 mM (Fig.21F2).

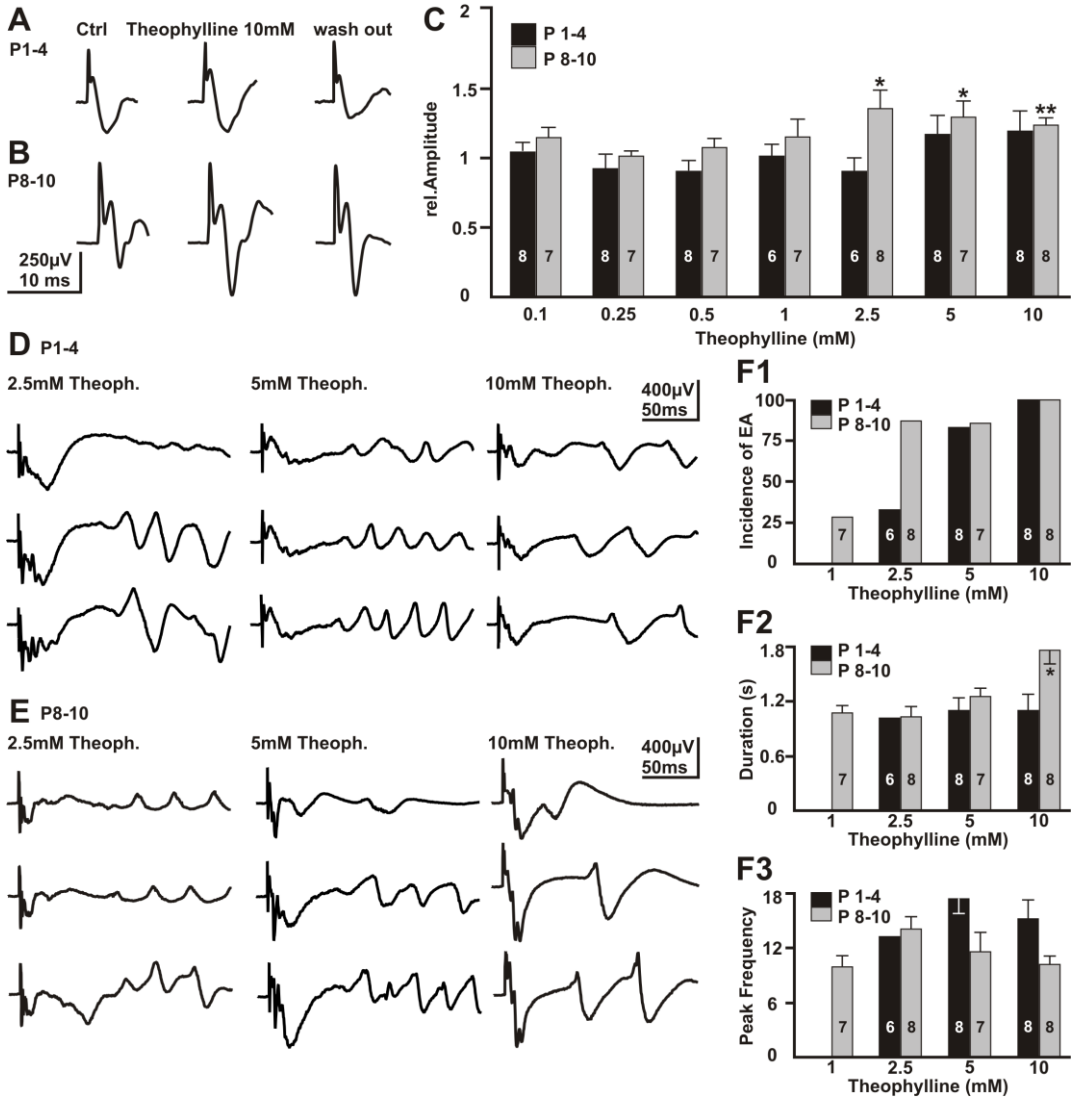

Figure 21. Effect of theophylline on the whole-hippocampus preparation upon electrical stimulation. A, B: Field-potential recordings in CA1 upon electrical stimulation of CA3 illustrating the effect of theophylline on FP-PSPs in a P4 (A) and a P8 (B) hippocampus. C: Statistical analysis of the theophylline effect on the amplitude of FP-PSPs. D, E: Representative FP recordings in CA1 illustrating that theophylline promote the occurrence of polysynaptic responses and epileptiform discharges after electrical stimulation in a P4 (D) and P9 (E) hippocampus. F: Statistical analysis illustrating the effect of theophylline on incidence, duration and frequency of the evoked epileptiform

*discharges. Bars represent mean ± SEM, number of experiments are given in the bars, * and ** indicated significance levels of 0.05 and 0.01, respectively, calculated by students t-test.*

In summary, these results indicate that high theophylline concentrations enhanced the amplitude of postsynaptic events at concentrations above 1 mM only in the P8-10 age group, but reliably promote evoked epileptiform activity in the whole hippocampus preparation at both age groups at concentrations above 1 mM.

In the presence of theophylline we also observed spontaneous epileptiform discharges with an ictal-like appearance (Fig.22A, B). Such ictal-like epileptiform discharges also occurred in the experiments with electrical stimulation (Fig.22C, D). In P1-4 hippocampi spontaneous ictal-like epileptiform activity was observed in 81% of 16 preparations at theophylline concentrations of 5 mM and 10 mM (Fig. 22E1). The occurrence of ictal-like events increased from 0.07 ± 0.007 min^{-1} (n=13 preparations) at 5 mM to 0.11 ± 0.01 min^{-1} (n=13 preparations) at 10 mM theophylline (Fig. 22E2). Neither the amplitude nor the duration or the frequency of discharges within such an ictal-like event showed a significant dependency on the concentration (Fig. 22E3-5). In P8-10 hippocampi spontaneous ictal-like epileptiform activity was already observed in 25 % of 12 preparations at a theophylline concentration of 1 mM. With increasing theophylline concentrations the incidence of ictal-like events was enhanced and the occurrence of ictal-like events also significantly increased (Fig.22E1-2). Neither the amplitude nor the duration or the frequency of discharges within such an ictal-like event showed a significant dependency on the concentration (Fig. 22E3-5).

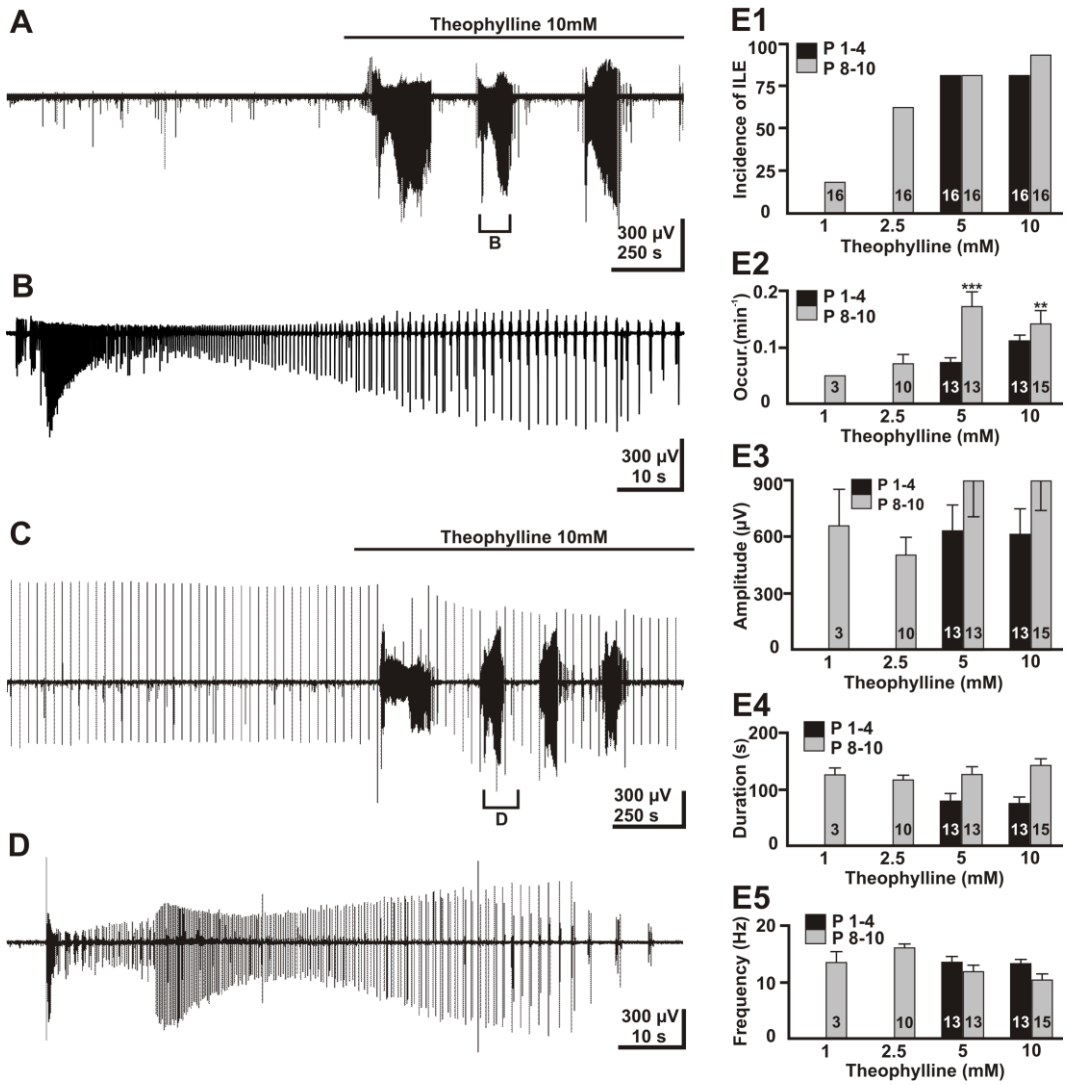

*Figure 22. Theophylline induces spontaneous epileptiform activity. A: Representative field-potential recording illustrating that bath application of 10 mM theophylline induces spontaneous ictal-like epileptiform discharges (ILE). B: One ILE at a higher temporal resolution as indicated in A. D: Representative field-potential recording illustrating that spontaneous ILE also occurred under stimulated conditions in the presence of 10 mM theophylline. D: One ILE shown in C at a higher temporal resolution. E: Statistical analysis illustrating the effect of theophylline on incidence of ILE, occurrence of ILE, amplitude of discharges, duration of an ILE, and the frequency of discharges within an ILE. Bars represent mean ± SEM, numbers are given in the bars, ** and *** indicated significance levels of 0.01 and 0.001, respectively, calculated by students t-test.*

In summary, these results indicate that theophylline at concentration ≥ 1 mM can provoke epileptiform activity in the isolated hippocampus preparation, with the P1-4 hippocampus being less susceptible.

4.3.3 Effect of caffeine on the whole-hippocampus preparation

Next the effect of caffeine on monosynaptic FP-PSPs in the CA1 region was investigated (Fig. 23A, B). In hippocampi from P1-4 rats 2.5 mM caffeine significantly ($p=0.018$) reduced the amplitude of FP-PSPs by 17 ± 7% ($n=8$), while 5 mM and 10 mM caffeine reduced FP-PSPs by 31 ± 6% ($n=8$, $p=0.002$) and 38 ± 6% ($n=8$, $p=0.0005$), respectively (Fig. 23A, C). In the P8-10 hippocampal preparations no significant effect of caffeine on the amplitude of the FP-PSPs were observed. Additional polysynaptic components were observed after electrical stimulation at caffeine concentration ≥ 5 mM for P1-4 (Fig. 23D) and ≥ 1 mM for P8-10 rats (Fig. 23E). These polysynaptic PSPs were in the P8-10 rats usually accompanied by electrically evoked epileptiform discharges (Fig. 23E), while in the P1-4 hippocampi ($n=8$) no epileptiform activity could be observed. In the P8-10 hippocampi such evoked epileptiform activity occurred already at a caffeine-concentration of 1 mM (Fig.23E, F1). At 1 mM and 2.5 mM only 33 % ($n=8$) and 14% ($n=8$) of FP-PSCs were followed by evoked epileptiform activity (Fig.23F1). With increasing caffeine concentrations the incidence increased to 44% and 77% at 5 mM and 10 mM, respectively (Fig. 23F1). No obvious dependence between the caffeine concentration and the duration of evoked epileptiform activity or the frequency of discharges within such epileptiform bursts were observed (Fig. 23F2,3).

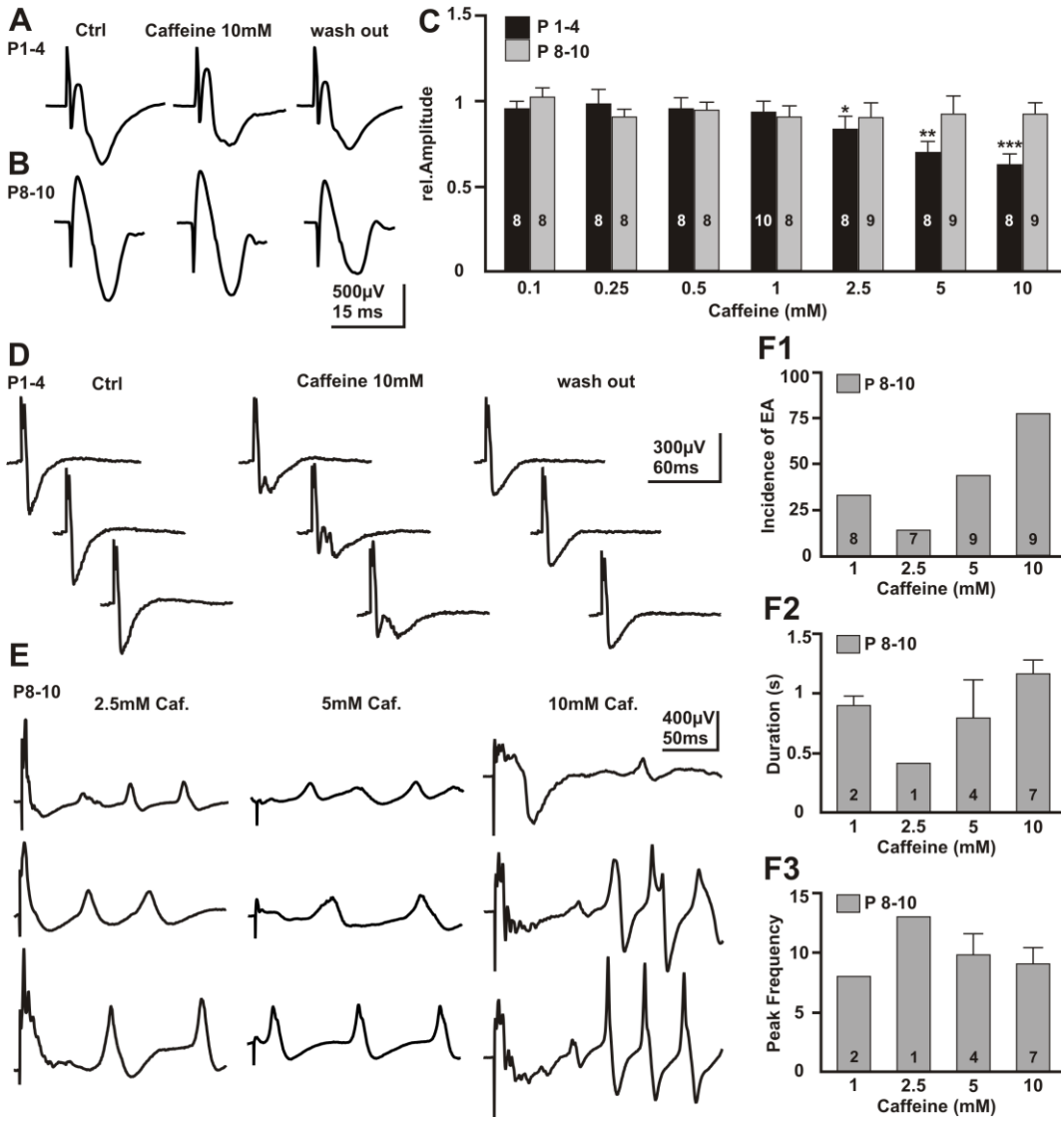

*Figure 23. Effect of caffeine on the whole-hippocampus preparation upon electrical stimulation. A, B: Field-potential recordings in CA1 illustrating the effect of caffeine on FP-PSPs upon electrical stimulation of CA3 in a P4 (A) and P8 (B) hippocampus. C: Statistical analysis of the caffeine effects on the amplitude of electrically evoked FP-PSPs. D: Three consecutive FP recordings illustrating that caffeine promotes the occurrence of polysynaptic PSPs in P1-4 hippocampi, while no epileptiform discharges are observed. E: In P8-10 hippocampi caffeine promotes epileptiform discharges after electrical stimulation. F: Statistical analysis illustrating the effect of caffeine on incidence, duration and frequency of the evoked epileptiform discharges. Bars represent mean ± SEM, numbers are given in the bars, *, ** and *** indicated significance levels of 0.05, 0.01 and 0.001 calculated by students t-test.*

In summary, these results indicate that high caffeine concentrations promote epileptiform discharges only in the P8-10 age group, but reduced the amplitude of evoked postsynaptic responses in the P1-4 age group.

We also observed spontaneous epileptiform activity with an ictal-like appearance upon caffeine application (Fig. 24A, B). Such ictal-like events could also be observed in experiments with electrical stimulation (Fig.24C, D). In P1-4 hippocampi spontaneous ictal-like epileptiform activity was observed only in 6% of 16 preparations at caffeine concentration of 10 mM (Fig. 24E1). At this concentration the occurrence of ictal-like events was 0.05 min^{-1} (n=1 preparation, Fig. 24E2). In P8-10 hippocampi spontaneous ictal-like epileptiform activity was already observed at a caffeine concentration of 2.5 mM in 6% of 16 preparations. With increasing of caffeine concentrations the incidence of ictal-like events was enhanced and the occurrence of ictal-like event also increased (Fig. 24E1-4). Neither the amplitude nor the duration or the frequency of discharges within such an ictal-like event showed a significant dependency on the concentration.

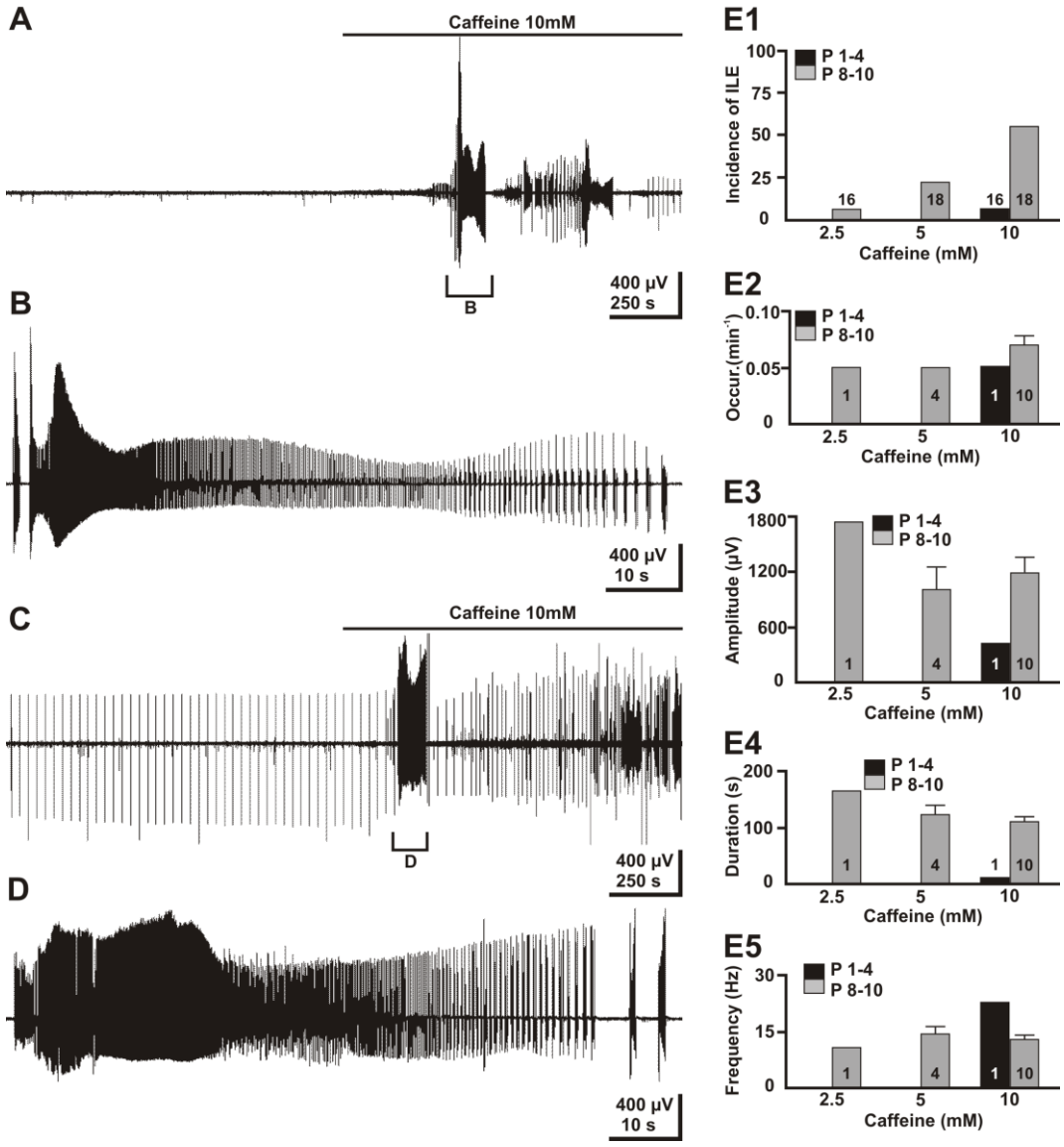

Figure 24. Caffeine induces spontaneous epileptiform activity. A: Representative field-potential recording illustrating that bath application of 10 mM caffeine induces spontaneous ictal-like epileptiform discharges (ILE). B: The ILE shown in A at a higher temporal resolution. C: Representative field-potential recording illustrating that spontaneous ILE also occurred under stimulated conditions upon bath application of 10 mM caffeine. D: The ILE shown in C at a higher temporal resolution. E: Statistical analysis illustrating the effect of theophylline on incidence of ILE, occurrence of ILE, amplitude of discharges, duration of an ILE, and the frequency of discharges within an ILE. Bars represent mean ± SEM, numbers are given in the bars.

In summary these results indicate that caffeine at concentration ≥ 2.5 mM can provoke epileptiform activity in the isolated hippocampus preparation of the P8-10 age group, while in the P1-4 hippocampi caffeine concentrations below 10 mM failed to provoke epileptiform discharges.

4.3.4 Role of the disinhibitory capacity of methylxanthines.

We finally performed experiments to determine whether a partial antagonism of $GABA_A$ receptors by caffeine and theophylline (Roca et al., 1988) contributes to the induction of epileptiform activity. Therefore we first investigated the effect of theophylline and caffeine in the presence of 30 µM Picrotoxin, a noncompetitive inhibitor of $GABA_A$ receptors. Bath application of 30 µM Picrotoxin already induced epileptiform discharges, suggesting a disinhibitory action of this drug (Fig. 25A, C). Addition of 10 mM caffeine under this condition significantly (p=0.009) enhanced the frequency of epileptiform discharges by 29 ± 8% (n=8) (Fig. 25A). A similar result was also observed with theophylline (Fig. 25B). Bath application of 5 mM theophylline in the presence of 30 µM Picrotoxin also significantly (p=0.041) increased the frequency of epileptiform activity by 29 ± 12 % (n=8). These results suggest that the epileptogenic effect of caffeine and theophylline is independent from an antagonistic action on $GABA_A$ receptors. To support this suggestion we performed an additional set of experiments, in which we first demonstrated that 30 µM Picrotoxin can fully revert the antiepileptic effect promoted by the bath application of the $GABA_A$ agonist Muscimol (Fig. 25C, D). Under this condition, both 10 mM caffeine (Fig 25C) or 5 mM theophylline (Fig. 25D) were capable to massively enhance the frequency of epileptiform discharges, again suggesting that the proepileptic capacity of both substances is independent of $GABA_A$ receptors.

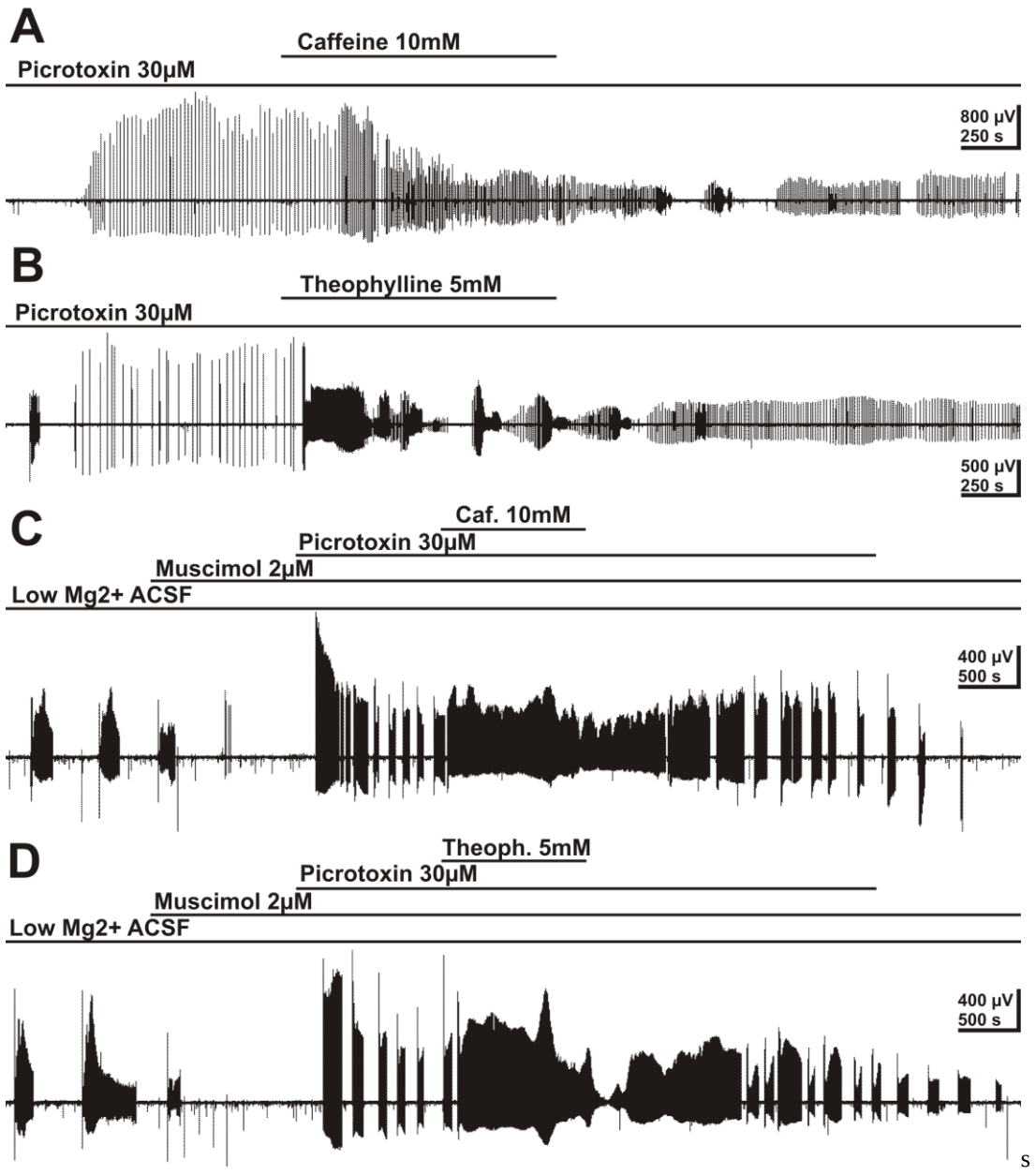

Figure 25. Role of the disinhibitory capacity of methylxanthines. A: Typical recording in the CA1 region of a P8 hippocampus illustrating the 30 µM picrotoxin induces spontaneous epileptiform discharges and that the addition of 10 mM caffeine massively augmented epileptiform activity. B: Representative registration of a P9 hippocampus demonstrating that 5 mM theophylline also augmented the epileptiform activity induced by 30 µM Picrotoxin. C: Representative recording in the CA1 region of a P8 hippocampus. The epileptiform discharges induced in low Mg^{2+} solution were abolished by bath application of the $GABA_A$ agonist muscimol. Addition of 30 µM picrotoxin led to the reappearance of epileptiform activity, which was augmented by 10 mM caffeine. D: Similar experimental paradigm demonstrating that 5 mM theophylline also augmented the epileptiform activity observed in the presence of 30 µM picrotoxin and 2 µM muscimol under low-Mg^{2+} conditions.

In summary, these results indicate that epileptiform activity induced by 10mM caffeine and 5 mM theophylline does not require the inhibition of $GABA_A$ receptors.

5 Discussion

In the projects summarized in my thesis I addressed the questions to study i) the effects of GABAergic antagonists and modulators possessing different affinities to synaptic and extrasynaptic GABAergic receptors that mediate phasic and tonic currents in the developing hippocampus. ii) The role of DAergic receptors in epileptiform activity of the immature hippocampus and iii) the effects of the methylxanthines theophylline and caffeine and their possible adverse effects on the neuronal activity in the hippocampus. In summary, these results demonstrate that i) $GABA_A$ receptors can mediate both pro- and anticonvulsive effects on the immature hippocampus, depending on the mode of transmission, that ii) DA can promote both pro- and anticonvulsant effects in the immature hippocampus, and iii) methyxanthines provoke epileptiform discharges also in the hippocampus of mice corresponding to the developmental status of preterm babies, with caffeine being less ictogenic than theophylline. These findings may contribute to our understanding of mechanisms that modulate the excitability of the immature hippocampus and support clinical studies for the use of substances acting on the GABAergic and DArgic system and methylxanthines in pre- and interm babies.

5.1 Phasic $GABA_A$ – receptor activation is required to suppress epileptiform activity

It has already been demonstrated that activation of the GABAergic system in the immature hippocampus can either mediate depolarization (Ben-Ari et al., 1989; Luhmann and Prince, 1991) of the cell membrane or inhibition by shunting the excitatory inputs (Edwards, 1990b; Kolbaev et al., 2011 ; Staley and Mody, 1992). This controversial situation does not clarify the role of GABAergic transmission in seizure induction of the immature nervous system, which requires further detailed investigation.

For this purpose, the field potential recordings were used in acute hippocampal slices of P4-P7 rats to investigate the role of phasic and tonic GABAergic currents on immature hippocampus excitability. Our results reveal that low concentrations of gabazine and picrotoxin, which antagonize only synaptic GABAergic recdeptors, induce spontaneous epileptiform activity, while activation of extrasynaptic receptors

with THIP promotes epileptiform activity. Therefore, we conclude from these results that synaptic GABAergic activity is needed to control excitability, whereas tonic GABAergic currents can augment excitability in the immature hippocampus (Kolbaev et al., 2012).

5.1.1 GABAergic activation mediates inhibitory action in the immature hippocampus

It has already been shown that GABA activation can induce depolarizing membrane responses during the first postnatal week in the rodent hippocampus (e.g., Ben-Ari et al., 1989; Mueller et al., 1984), which can lead to excitation in the immature hippocampus (Ben-Ari et al., 1989; Sipila et al., 2005). Consequently, recent studies demonstrated that depolarizing GABA responses can trigger the generation of epileptiform activity at the early postnatal period (Dzhala and Staley, 2003; Dzhala et al., 2005). However, such depolarizing GABAergic membrane responses are not always excitatory, but can also contribute to inhibitory effects, for instance, by decreasing the membrane resistance and then as a result shunting excitatory inputs (Edwards, 1990b; Kolbaev et al., 2011 ; Staley and Mody, 1992). Shunting inhibition can be one explanation for why GABA antagonists provoke epileptiform activity in the immature CNS, which is in agreement with different studies that also found a proepileptic effect of $GABA_A$-receptor antagonists in the immature CNS (Baram and Snead, III, 1990; Wells et al., 2000). And in fact, shunting of excitatory inputs by depolarizing GABA responses was demonstrated in the immature hippocampus (Lamsa et al., 2000). In the publication of the presented results (Kolbaev et al., 2012) we also observed that activation of $GABA_A$ receptors attenuates epileptiform discharges, which is supported by many studies indicating the inhibitory action of $GABA_A$-mediated responses (Kolbaev et al., 2012).

5.1.2 Phasic and tonic GABAergic activity exerts a different effect

In our experiments we demonstrate that in the immature hippocampus picrotoxin soothes phasic currents at lower concentrations and has no effect on tonic currents. In contrast, such an effect was observed in the mature hippocampus (Semyanov et al., 2003) emphasizing that no pharmacologic agents are currently available to clearly distinguish between tonic and phasic currents. Activation of extrasynaptic

GABAergic receptors and their mediated tonic current with 1µM THIP promotes epileptiform discharges, indicating that tonic currents can mediate an excitatory influence on the immature hippocampus. Inhibition of NKCC1 using bumetanide or furosemide abolished the proconvulsive effect of 1µM THIP supporting the hypothesis that membrane depolarization mediate this proconvulsive effect of tonic GABAergic currents. Since formation of dendritic synapses in the immature hippocampus occurs before perisomatic synapses (Tyzio et al., 1999), an additionally attenuation of dendritic propagation of synaptic inputs by membrane shunting is observed (Andersen et al., 1980; Staley and Mody, 1992). The control by such dendritic shunting effects may, therefore, clarify why the synaptic GABAergic responses mediate a general inhibitory action in the immature hippocampus. Experiments in slightly older animals (P13–18), in the CA3 region of the hippocampus in which more negative reversal potential of GABAergic currents is observed (Tyzio et al., 2007), THIP soothes epileptiform activity (Gonzalez-Sulser et al., 2011), indicating that at this age tonic $GABA_A$ receptors mediate the expected inhibitory action (Walker and Semyanov, 2008). In the presence of the α5-specific antagonist L-655,708 this proconvulsive THIP effect is attenuated (Caraiscos et al., 2004) suggesting that α5-subunit–containing $GABA_A$ receptors are involved in the proconvulsive effect of 1µM THIP. On the other hand, L-655,708 had no significant effect on the basal tonic current, indicating that α5- subunit–containing $GABA_A$ receptors do not contribute considerably to tonic GABAergic currents under in vitro experimental conditions. Probably remaining extracellular GABA, originating from synaptic and extrasynaptic sources (Marchionni et al., 2007), act on a population of extrasynaptic $GABA_A$ receptors different from the externally applied agonist THIP (Kolbaev et al., 2012).

5.2 DA modulates epileptiform activity in the immature hippocampus

While the role of DA on epileptogenesis in the juvenile and adult hippocampus has already been investigated, very poor information is available about its action on the immature hippocampus. Particularly, because of possible anticonvulsive actions of D2-like receptor agonists (Cepeda et al., 1999) and the high pharmacological resistance of immature seizures (Silverstein and Jensen, 2007) D2-like receptor may

be interesting as a potential therapeutical target for researchers. The main aim of this part of the study was to investigate the role of the DAergic system in epileptiform activity in the intact CHF of the immature hippocampus (Sharopov et al., 2012).

The main results can be summarized as follows. 1) Depending on the used concentration DA mediated anti- and proconvulsive effects on low-Mg^{2+}-induced epileptiform activity in the intact CHF of P3–4 mice. DA applied at low concentrations (<0.3 µM) attenuated and concentrations >3 µM augmented epileptiform activity. 2) Application of the D1-agonist SKF38393 promoted the proconvulsive effect, and the D1-like receptor antagonist SCH39166 abolished the DA-induced proconvulsive effect. 3) Quinpirole, a D2-like agonist, exerts a weak anticonvulsive effect, however, antagonists of D1-like, D2, and D3 receptors are able to abolish the anticonvulsive effect of low DA concentrations. 4) In presence of non-functional AMPA, NMDA or $GABA_A$ receptors DA still has a proconvulsive effect. These experiments revealed that DA influences epileptiform activity at early developmental stages and can bidirectionally influence the excitability of the hippocampus. Presumably, D1-like receptors and modifications of GABAergic and glutamatergic synapses are responsible for the proconvulsive effect of high DA concentrations, but the mechanism by which low DA concentrations exert their anticonvulsive effect is less clear. Our study demonstrates that DA can mediate pro- and anticonvulsive effects in the immature hippocampus, in agreement with a variety of investigations that demonstrate pro- and anticonvulsive effects of DA in the mature hippocampus stages (Bo et al., 1995; Haas et al., 1984; Suppes et al., 1985). It has been suggested that DA plays an important role in the functional state controlling of adult hippocampus (Jay, 2003). Thus, the functional expression of D1- and D2-like receptors in the hippocampus during early developmental periods observed in our study also suggests that DA most likely influences hippocampal functions already at early postnatal stages (Sharopov et al., 2012).

Investigations with subtype-specific DAergic agonists and antagonists have already shown that activation of D1-like receptors has a proconvulsive effect in many epilepsy models (Alam and Starr, 1992; Barone et al., 1990; Burke et al., 1990; Loscher and Czuczwar, 1986; O'Sullivan et al., 2008; for reviews see Starr, 1996; Weinshenker and Szot, 2002). Our study demonstrated that the D1 agonist SKF38393 mimics the proconvulsive DA effect and that the D1-like antagonist

SCH39166 abolished the proconvulsive effect of 10 µM DA, which strongly suggests that D1 receptors mediate a proconvulsive effect already in the immature hippocampus and that D1-like receptors are essential for the proconvulsive DA effect. A contribution of synergistic activation of D4 receptors can also not be excluded since the D2-like antagonist sulpiride also attenuates the proconvulsive DA effect, and D2 and D3 specific antagonists had no effect. In contrast to in vivo studies (Altajir et al., 1990; Barone et al., 1990), application of the D1- like antagonist SCH39166 alone had only a slight anticonvulsive effect on epileptiform activity, suggesting that endogenous activation of D1-like receptors did not considerably affect the excitability under our experimental conditions. In addition, our study provides interesting experimental evidence that the proepileptic DA effect was enhanced after blockade of adrenergic receptors. Therefore, in the hippocampal CA3 region, the strong proconvulsive effect of higher DA concentrations seems to be counterbalanced by the anticonvulsive effect of DA mediated via adrenergic receptors (Mueller and Dunwiddie, 1983). In contrast, in the mature DG, activation of β-adrenergic receptors can even exert proconvulsive effect (Stanton et al., 1987), which complicates an estimation of the physiological influence of adrenergic receptor activation by DA (Sharopov et al., 2012).

In the adult CNS, D2-like receptors mediate an anticonvulsive effect (Alam and Starr, 1992; Bozzi et al., 2000; Burke et al., 1990; Clinckers et al., 2004; for reviews see Starr, 1996; Weinshenker and Szot, 2002). In our experimental conditions, the influence of D2-like receptors on epileptiform activity is less obvious. Since the D2-like agonist quinpirole reduced the occurrence of non-SLE, this indicates that D2-like receptors might also mediate a minor anticonvulsive effect at early postnatal life. D3 and/or D4 receptors may be more appropriate candidates mediating anticonvulsive action because the specific D2 agonist GSK-789472 had no anticonvulsive effect. Alternatively, the D2-like antagonist sulpiride, the D2-specific antagonist L-741626, and the D3-specific antagonist SB-277011-A, including D1-like antagonist SCH39166, absolutely suppressed the anticonvulsive effect of low DA concentration. These results suggest that D3 and/or D4 receptors may mediate an anticonvulsive effect but that the anticonvulsive effect of low DA concentration requires the activation of diverse DA receptors, which may be located on different post- and presynaptic elements of the hippocampal network (Sharopov et al., 2012).

The observation that applied DA receptor antagonists alone had no significant effect on epileptiform activity, indicate that under our experimental conditions an intrinsic activation of DA receptors does influence the epileptiform activity. In mature brain structures with strong DAergic projections, the stimulation of the striatum or the nucleus accumbens leads to DA release up to 0.5 µM, although the basal levels are most probably in the low nanomolar concentrations (Garris and Wightman, 1994; Kawagoe et al., 1992; Schultz, 2007). Probably, such extracellular DA concentrations in the hippocampus are even lower (Mokler et al., 2007) and might not be sufficient to mediate even the anticonvulsive effect. However, bath application of the DA uptake inhibitor nomifensine promotes a significant anticonvulsive effect suggesting that an intrinsic release of DA can, potentially mediate an antiepileptic effect, even in reduced preparations without the VTA, as used in our experimental conditions. A nomifensine - induced anticonvulsive effect has also been demonstrated in the adult CNS (but see Edwards and Glenbott, 1987; Warter et al., 1988). During stress situations (Barr et al., 2009), unpredicted reward (Schultz, 2007) and during epileptic seizures (Dazzi et al., 1997), the DA levels increase and may even reach concentrations sufficient to stimulate D1-like receptors (Sharopov et al., 2012).

DA can influence the membrane properties of the neurons and thus neuronal excitability by directly changing membrane currents, input resistance and action potential threshold (see, e.g., Chen and Yang, 2007; Gulledge and Jaffe, 1998; Podda et al., 2010; Stanzione et al., 1984). The hippocampal glutamate release is increased by D1 receptor activation (Bouron, 2001; Kobayashi and Suzuki, 2007), whereas D2 receptor activation decreases glutamate release (Hsu, 1996). The GABAergic release has also been shown to be altered by DA receptors (Cameron and Williams, 1993; Radnikow and Misgeld, 1998). DA enhances NMDA currents via activation of D1 receptors (Flores-Hernandez et al., 2002; Gonzalez-Islas and Hablitz, 2003; Tseng and O'Donnell, 2004), on the contrary D2 receptors decrease these currents (Flores-Hernandez et al., 2002; Kotecha et al., 2002). However, despite to these reports indicating the prominent role of NMDA receptors in mediating DAergic alterations of excitability, our study revealed that DA has a prominent proconvulsive action after blocking of NMDA receptors. With this experiment we strongly suggest the involvement of other neurotransmitter receptors in the DA- exerted proconvulsive

effect. DA also contributes to enhancement of AMPA receptor function via D1 receptor activation (Gonzalez-Islas and Hablitz, 2003; O'Sullivan et al., 2008; Umemiya and Raymond, 1997). In contrast, our experiments showed that inhibition of AMPA receptors with either the AMPA/kainate antagonists CNQX or the specific AMPA receptor antagonist GYKI 52466 did not alter the proconvulsive effect of DA. Instead, it prevented the DA-induced increase in spike number per non-SLE, indicating that a DAergic effect on AMPA receptors may be involved in the transition to more complex non-SLE discharges. The $GABA_A$ receptor antagonist gabazine considerably changed the pattern of epileptiform discharges, indicating the significant role of GABAergic synaptic connections in controlling excitability in the immature hippocampus (Farrant and Kaila, 2007). Although a direct comparison of the DAergic effect among the different epileptiform patterns is not possible, our observation that DA enhanced epileptiform activity in the presence of inactivated GABA receptors indicates that DA - induced changes in glutamate receptor functions are sufficient to induce epileptiform activity. On the other hand, the proconvulsive effect of DA on epileptiform activity was only moderate, indicating that a decrease in GABAergic inhibition might also considerably contribute to the strong proconvulsive effect of DA. Generally, the experiments revealed that the effect of DA on excitability is not mediated by a single postsynaptic receptor; most probably, at least two postsynaptic receptor systems act synergistically to mediate the proconvulsive effect of higher DA concentrations (Sharopov et al., 2012).

Overall, the results of this part of the study demonstrate that higher DA concentrations have a proepileptic effect in the immature hippocampus via D1-like receptor activation, whereas lower DA concentrations exert a moderate anticonvulsant effect. Therefore, in general the effect of DA on the excitability of the immature hippocampus is comparable to the DAergic actions in the adult hippocampus (Sharopov et al., 2012).

5.3 Methylxanthines exert proconvulsive effects in the immature hippocampal formation

In this part of the thesis the effects of the methylxanthines theophylline and caffeine on isolated in-toto hippocampal preparations were analyzed to investigate whether these substances at therapeutically used and non-therapeutically

concentrations have detrimental effects on neuronal activity in this brain region. In our study the following main results were obtained: 1) Theophylline at concentrations < 1 mM and caffeine at concentrations < 2.5 mM did not evoke epileptiform activity in the immature hippocampus. Brain preparations resembling the premature situation are even less sensitive for methylxanthines. 2) Theophyline at concentrations > 1 mM and caffeine at concentrations > 2.5 mM induce epileptiform activity and additional pharmacological experiments revealed that both caffeine and theophylline enhanced the frequency of epileptiform discharges also in the presence of the noncompetitive $GABA_A$ antagonist picrotoxin 3) The effects on postsynaptic responses and spontaneous epileptiform discharges were less pronounced for caffeine and in the concentrations of 2.5mM, 5mM and 10mM caffeine even significantly reduced the amplitude of evoked FP-PSPs. 5) In contrast, theophylline at the same concentrations potentiated the amplitude of evoked FP-PSPs. We conclude from these experiments that at higher concentrations application of caffeine and theophylline results in a pronounced epileptiform activity, and that these methyxanthines exert their proconvulsive effect also in the presence of inactive $GABA_A$ receptors.

Clinical studies have already shown that in view of its lower toxicity, once-a-day dosing, more predictable plasma concentration and earlier onset of action caffeine could be the preferred drug for the treatment of apnoea (Gannon, 2000; Henderson-Smart and De Paoli, 2010), which is in best agreement with very recent studies suggesting caffeine, because of its safer therapeutic range, as a very important and effective medication in the neonatal intensive care units (NICU) to date (Spitzer, 2012). Interestingly, a study by Bory and colleagues noted that in neonates given theophylline for the treatment of apnoea approximately the third of the dose was converted to caffeine (Bory et al., 1979). This active metabolic pathway seemed to be present only during the first months of life, and was not seen in adults (Spitzer, 2012).

The mechanism by which methyxanthines induce seizures is still not completely understood. However, it is very likely to us that the proconvulsive role of methylxanthines is based on antagonism of the brain's own adenosine system. It is well recognized that endogenous adenosine is an anticonvulsant and may regulate the brain activity (Boison, 2005; Dunwiddie and Masino, 2001). The hippocampal

formation is one of the most seizure-susceptible areas of the brain, where adenosine receptors are located in very high density (Lee et al., 1983; Lewis et al., 1981; Murray and Cheney, 1982). Methylxanthine-induced inhibition of adenosine A1 receptors (A_1Rs) can directly have proconvulsive effects, however under certain circumstances can also suppress excitation. First, it happens due to the chronic drug intakes, which may inverse and alter the gene expression (Svenningsson et al., 1999). Second, antagonism of adenosine A2 (A_{2A}Rs) by methylxanthines may have direct anticonvulsant and neuroprotective consequences (Boison, 2011).

Methylxanthines can also block the binding site of $GABA_A$Rs for benzodiazepines (Marangos et al., 1981), which might contribute to a proconvulsive role of high or toxic doses of methylxanthines (Boison, 2011). The affinities of theophylline and caffeine for diazepam – binding sites associated with the $GABA_A$ receptor channel however, are very low and might play only a minor role in the action of convulsant doses of caffeine and theophylline (Tchekalarova et al., 2007). But our experiments have demonstrated that EA induced by theophylline and caffeine does not require the inhibition of $GABA_A$ receptors. Some authors indicate the convulsant action of methylxanthines due to an inhibitory effect on PDE, which results in an increase of intracellular cyclic adenosine monophosphate (cAMP) levels (Chu, 1981; Yarnell and Chu, 1975). In contrast, other studies suggest that a rise in brain tissue cAMP is antiepileptic and that it is, in fact, cyclic guanosine monophosphate (cGMP) that is involved in producing neuronal epileptic discharges (Gross and Ferrendelli, 1979; Nakada et al., 1983; Phillis, 1977). This can be also in our case since 10 to 100 times higher than therapeutic methylxanthine concentrations of 10-100μM are needed to inhibit PDE or even $GABA_A$Rs (Fredholm et al., 1999). Another mechanism that can contribute to seizure induction is a change in Ca^{2+} homeostasis and subsequent constant increases of intracellular Ca^{2+} concentration (DeLorenzo et al., 2005). It was also suggested that IBMX another methylxanthine exerts membrane currents via activation of Ca^{2+} regulated K^+ channels and direct inhibition of tetraethylammonium (TEA) - sensitive K^+ channels (Usachev et al., 1995). Ryanodine receptors (RyR) may also be involved by calcium–induced calcium release (CICR) that plays a crucial role in regulations of intracellular calcium concentrations (Boison, 2011; Pal et al., 2001). RyRs in the brain are sensitive to caffeine and mediate the caffeine- induced mobilization of Ca^{2+} from

internal stores (Mcpherson et al., 1991; Usachev et al., 1993). Through these mechanisms neurons become more prone to excitotoxicity and expression of seizures (Chan et al., 2000; Verkhratsky, 2005). A role of free radicals in methylxanthine-induced seizures was recently discussed (Gulati et al., 2005; Gulati et al., 2007). In the underlying studies aminophylline (a mixture of theophylline and ethylenediamine) dose-dependently induced convulsions and mortality in rats. These complications were attenuated by antioxidants and by nitric oxide (NO) synthesis inhibitors (Boison, 2011). Harinath et al. demonstrated that both caffeine and theophylline dose–dependently inhibit hTREK-1 channels. The TREK-1 channel family plays an important role in regulating the resting membrane potential of neurons, which make their role crucial in controlling neuronal excitability (Honore, 2007). Inhibition of TREK-1 channels may increase membrane excitability that could contribute to the excitotoxic side effects of caffeine and theophylline (Harinath and Sikdar, 2005).

In summary, our experiments suggest that in a whole hippocampus preparation of immature rats caffeine and theophylline exert proconvulsive effect at higher concentrations. Both methylxanthines were able to induce seizures also in the presence of inactive $GABA_A$ receptors. The proconvulsive action of caffeine was less pronounced and caffeine suppressed the amplitude of FP-PSPs in the hippocampus.

5.4 Clinical implication of the projects

5.4.1 Efficacy of AEDs with higher specificity to synaptic $GABA_A$ receptors

Epileptic seizures in neonates often require immediate therapeutic intervention, since they may represent a critical insult and are a major risk factor for the manifestation of epilepsy (Holmes, 1997; Silverstein and Jensen, 2007). However, the therapeutic efficacy of standard AEDs, including positive modulators of $GABA_A$ receptors such as midazolam or phenobarbital, appears to be limited at this age (Booth and Evans, 2004; Silverstein and Jensen, 2007). Our observation that $GABA_A$ receptors, depending on the mode of GABAergic transmission, can mediate both anticonvulsive and proconvulsive effects may explain the heterogeneity of the responsiveness to these AEDs. In addition, our in vitro study suggests that AEDs with a higher specificity to synaptic $GABA_A$ receptors may be more effective in

controlling epileptic seizures in neonates, although it should be considered that the composition of $GABA_A$ receptors, and thus their pharmacologic profile, significantly differs between neonatal and adult brains (Taketo and Yoshioka, 2000) (Kolbaev et al., 2012)

5.4.2 Use of D1-like antagonists in controlling seizures in children

The experiments in the second part of my thesis demonstrate that higher DA concentrations have a proconvulsant effect in the immature hippocampus via D1-like receptor activation, whereas lower DA concentrations exert a moderate anticonvulsant influence. "This effect of DA on the excitability of the immature hippocampus is very similar to the DArgic actions in the mature hippocampus. It may suggest a potential use of D1-like antagonists in controlling seizures in children, however, possible adverse developmental effects of DArgic agonists and antagonists must be considered. For instance, it has been shown that DA controls neuronal proliferation (Zhang et al., 2005), migration (Crandall et al., 2007), and differentiation (Song et al., 2002) in the immature brain. These important functions of DA may complicate or exclude a pharmacological intervention with DA antagonists or agonists in the immature brain (Sharopov et al., 2012).

5.4.3 Caffeine as a drug of choice for the treatment of apnoea of prematurity

Preterm babies are routinely treated with methylxanthines to stabilize the respiratory rhythm and avoid other complications (Comer et al., 2001; Delanty et al., 1998; Fredholm et al., 1999; Lee et al., 1996). However, the possible adverse and long term effects of these drugs have not been systematically investigated yet. The experiments performed in the third part of the thesis revealed that the proconvulsive action of caffeine was less pronounced and caffeine suppressed the amplitude of FP-PSPs in the hippocampus, which is in line with many clinical and experimental studies suggesting caffeine as the drug of choice, and makes caffeine more desirable drug for the treatment of apnea of prematurity.

6 Summary

Epileptic seizures during early life represent a severe neurological situation due to the fact that they are a major risk factor for the manifestation of epilepsy and show a poor pharmacological responsiveness. In my PhD thesis I therefore concentrated on the question how different neurotransmitter systems and clinically used substances affect epileptiform discharges in the perinatal hippocampus.

In the first part of my project I studied the effect of GABAergic antagonists and modulators that differentiate between receptors that mediate phasic and tonic GABAergic currents on field potential activity of hippocampal slices. These experiments revealed that in the immature hippocampus synaptic GABAergic activity is required to limit excitability, whereas tonic GABAergic currents can increase excitability. This may suggest that AEDs with a higher specificity for synaptic $GABA_A$ receptors may be more effective in controlling epileptic seizures in neonates.

To unravel whether DA has an effect on the excitability of the immature hippocampus I investigated in the second part of my work the effect of different DA concentrations and specific agonists and antagonists of DA receptor subtypes on epileptiform discharges. These experiments revealed that low DA concentrations promote an anticonvulsant effect that can be mimicked by the D2-like receptor agonist quinpirole, while higher DA concentration evoke a proconvulsive effect via activation of D1-like receptors. Although our study may suggest a potential use of D2-like agonists in seizure control in children, possible adverse developmental effects of DAergic agonists and antagonists must be considered.

In the third part of my work I investigated at which concentrations methylxanthines promote epileptic seizures and affect synaptic transmission in the perinatal hippocampus. These experiments revealed that both theophylline and caffeine at higher concentrations modify basal synaptic transmission in the CA1 region of the hippocampus and provoke epileptiform discharges. The effects on postsynaptic responses and spontaneous epileptiform discharges were less pronounced for caffeine, suggesting that this substance may be potentially advantageous for therapeutic applications in preterm infants.

In summary, the results of my study substantially augmented our knowledge about the mechanisms underlying epileptiform activity in the immature hippocampus and the therapeutical use of methylxanthines and pharmaceuticals acting on the GABAergic and DArgic systems in the perinatal CNS.

Zusammenfassung

Während des frühen Lebens stellen epileptische Anfälle schwere neurologische Zustände dar, weil sie ein großer Risikofaktor für die Manifestation der Epilepsie sind und eine hohe pharmakologische Resistenz zeigen. In meiner Doktorarbeit konzentrierte ich mich auf die Frage, wie verschiedene Neurotransmitter-Systeme und klinisch verwendete Medikamente epileptiforme Entladungen im perinatalen Hippocampus beeinflussen.

Im ersten Teil meines Projektes untersuchte ich die Wirkung von GABA-Antagonisten und Modulatoren, die zwischen phasischen und tonischen GABAergen Strömen differenzieren, auf Feldpotentialaktivität in Hippocampusschnitten. Diese Experimente zeigten, dass im unreifen Hippocampus synaptische GABAerge Aktivität benötigt wird, um die Erregbarkeit zu begrenzen, während tonische GABAerge Ströme die Erregbarkeit verstärken können. Dies könnte darauf hinweisen, dass Antiepileptika mit einer höheren Spezifität für synaptische $GABA_A$-Rezeptoren wirksamer zur Behandlung von epileptischen Anfällen bei Neugeborenen sein können.

Um den Einfluss von Dopamin auf die Erregbarkeit des unreifen Hippocampus herauszufinden, untersuchte ich im zweiten Teil meiner Arbeit die Wirkung von verschiedenen Dopaminkonzentrationen und spezifische Agonisten und Antagonisten der Dopamin-Rezeptor-Subtypen auf epileptiforme Entladungen. Diese Experimente zeigten, dass niedrige Dopamin Konzentrationen eine antikonvulsive Wirkung haben, welche vom D2-ähnliche-Rezeptor-Agonisten Quinpirol nachgeahmt werden kann, während höhere Dopamin-Konzentrationen eine prokonvulsive Wirkung über Aktivierung von D1-ähnlichen Rezeptoren hervorrufen. Obwohl unsere Untersuchungen eine mögliche Verwendung von D2-ähnlichen Rezeptor-Agonisten zur Kontrolle epileptischer Anfälle in Neugeborenen nahelegen, müssen mögliche negative Auswirkungen von DAergen Agonisten und Antagonisten auf die neuronale Entwicklung berücksichtigt werden.

Im dritten Teil meiner Arbeit untersuchte ich welche Konzentrationen von Methylxanthinen epileptische Anfälle in Hippocampuspreparationen auslösen die synaptische Übertragungen verändern können. Diese Experimente zeigten, dass

sowohl Theophyllin als auch Koffein in höheren Konzentrationen die basale synaptische Übertragungen in der CA1-Region des Hippocampus modifizieren und epileptiforme Entladungen provozieren. Die Auswirkungen auf die postsynaptischen Antworten und spontanen epileptiformen Entladungen durch Koffein waren weniger ausgeprägt, was darauf hindeutet, dass diese Substanz potentiell vorteilhafter für therapeutische Anwendungen bei Frühgeborenen sein kann.

Zusammenfassend bereichern die Ergebnisse meiner Studie erheblich unser Wissen über die zugrunde liegenden Mechanismen epileptiformer Aktivität im unreifen Hippocampus und den therapeutischen Einsatz von Methylxanthinen und Pharmaka, die auf das GABAerge und DArge System einwirken.

References

Reference List

Ackman JB, Aniksztejn L, Crépel V, Becq H, Pellegrino C, Cardoso C, Ben-Ari Y, Represa A (Abnormal network activity in a targeted genetic model of human double cortex. J Neurosci 29:313-327.2009).

Alam AM, Starr MS (Dopaminergic Modulation of Pilocarpine-Induced Motor Seizures in the Rat - the Role of Hippocampal Dopamine-D(1) Receptors. Eur J Pharmacol 222:227-232.1992).

Alexiou M, Leese HJ (Purine utilisation, de novo synthesis and degradation in mouse preimplantation embryos. Development 114:185-192.1992).

Allene C, Cossart R (Early NMDA receptor-driven waves of activity in the developing neocortex: physiological or pathological network oscillations? Journal of Physiology-London 588:83-91.2010).

Altajir G, Chandler CJ, Starr BS, Starr MS (Opposite Effects of Stimulation of D1 and D2 Dopamine-Receptors on the Expression of Motor Seizures in Mouse and Rat. Neuropharmacology 29:657-661.1990).

Anden NE, Carlsson A, Dahlstrom A, Fuxe K, Hillarp NA, Larsson K (Demonstration and Mapping Out of Nigro-Neostriatal Dopamine Neurons. Life Sciences 3:523-530.1964).

Andersen P (2007) The Hippocampus Book. Oxford.

Andersen P, Dingledine R, Gjerstad L, Langmoen IA, Laursen AM (Two different responses of hippocampal pyramidal cells to application of gamma-amino butyric acid. J Physiol (Lond) 305:279-296.1980).

Angevine JB (Time of Neuron Origin in Hippocampal Region - An Autoradiographic Study in Mouse. Exp Neurol S:1-&.1965).

Aranda JV, Grondin D, Sasyniuk BI (Pharmacologic considerations in the therapy of neonatal apnea. Pediatr Clin North Am 28:113-133.1981).

Aranda JV, Turmen T (Methylxanthines in apnea of prematurity. Clin Perinatol 6:87-108.1979).

Arnold SE, Trojanowski JQ (Human fetal hippocampal development .1. Cytoarchitecture, myeloarchitecture, and neuronal morphologic features. J Comp Neurol 367:274-292.1996).

Aronica E, Boer K, Redeker S, Spliet WG, van Rijen PC, Troost D, Gorter JA (Differential expression patterns of chloride transporters, Na+-K+-2Cl--cotransporter and K+-Cl--cotransporter, in epilepsy-associated malformations of cortical development. Neuroscience 145:185-196.2007).

Ashihara H, Suzuki T (Distribution and biosynthesis of caffeine in plants. Frontiers in Bioscience 9:1864-1876.2004).

Bairam A, Boutroy MJ, Badonnel Y, Vert P (Theophylline versus caffeine: comparative effects in treatment of idiopathic apnea in the preterm infant. J Pediatr 110:636-639.1987).

Ballanyi K, Onimaru H, Homma K (Respiratory network function in the isolated brainstem-spinal cord of newborn rats. Prog Neurobiol 59:583-634.1999).

Baram TZ, Snead OC, III (Bicuculline induced seizures in infant rats: Ontogeny of behavioral and electrocortical phenomena. Dev Brain Res 57:291-295.1990).

Barone P, Parashos SA, Palma V, Marin C, Campanella G, Chase TN (Dopamine-D1 Receptor Modulation of Pilocarpine-Induced Convulsions. Neuroscience 34:209-217.1990).

Barr GA, Moriceau S, Shionoya K, Muzny K, Gao PH, Wang SN, Sullivan RM (Transitions in infant learning are modulated by dopamine in the amygdala. Nat Neurosci 12:1367-1369.2009).

Beaulieu JM, Gainetdinov RR (The Physiology, Signaling, and Pharmacology of Dopamine Receptors. Pharmacological Reviews 63:182-217.2011).

Behar TN, Schaffner AE, Scott CA, Greene CL, Barker JL (GABA receptor antagonists modulate postmitotic cell migration in slice cultures of embryonic rat cortex. Cereb Cortex 10:899-909.2000).

Ben Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215-1284.2007).

Ben-Ari Y (Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 3:728-739.2002).

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa J-L (Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol (Lond) 416:303-325.1989).

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215-1284.2007).

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL ($GABA_{A,}$ NMDA and AMPA receptors: a developmentally regulated 'menage a trois'. Trends in Neurosciences 20:523-529.1997).

Benedetti F, Bernasconi A, Pontiggia A (Depression and neurological disorders. Current Opinion in Psychiatry 19:14-18.2006).

Berg AT, Berkovic SF, Brodie M, Buchhalter J, Cross JH, Boas WV, Engel J, French J, Glauser TA, Mathern GW, Moshe SL, Nordli D, Plouin P, Scheffer IE (Revised Terminology and Concepts for Organization of Seizures and Epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005-2009. Aktuelle Neurologie 37:120-130.2010).

Bhatia J (Current options in the management of apnea of prematurity. Clinical Pediatrics 39:327-336.2000).

Blume WT (The progression of epilepsy. Epilepsia 47:71-78.2006).

Bo P, Soragna D, Marchioni E, Candeloro E, Albergati A, Savoldi F (Role of Dopamine D-1 and D-2 Antagonists in A Model of Focal Epilepsy Induced by Electrical-Stimulation of Hippocampus and Amygdala in the Rabbit. Progress in Neuro-Psychopharmacology & Biological Psychiatry 19:917-930.1995).

Boison D (Adenosine and epilepsy: From therapeutic rationale to new therapeutic strategies. Neuroscientist 11:25-36.2005).

Boison D (Methylxanthines, seizures, and excitotoxicity. Handb Exp Pharmacol:251-266.2011).

Bonifazi P, Goldin M, Picardo MA, Jorquera I, Cattani A, Bianconi G, Represa A, Ben Ari Y, Cossart R (GABAergic hub neurons orchestrate synchrony in developing hippocampal networks. Science 326:1419-1424.2009).

Booth D, Evans DJ (Anticonvulsants for neonates with seizures. Cochrane Database Syst Rev:CD004218.2004).

Bory C, Baltassat P, Porthault M, Bethenod M, Frederich A, Aranda JV (Metabolism of Theophylline to Caffeine in Premature Newborn-Infants. Journal of Pediatrics 94:988-993.1979).

Bouron A (Modulation of spontaneous quantal release of neurotransmitters in the hippocampus. Prog Neurobiol 63:613-635.2001).

Bozzi Y, Vallone D, Borrelli E (Neuroprotective role of dopamine against hippocampal cell death

22824. J Neurosci 20:8643-8649.2000).

Briggs SW, Galanopoulou AS (Altered GABA Signaling in Early Life Epilepsies. Neural Plasticity.2011).

Brouard C, Moriette G, Murat I, Flouvat B, Pajot N, Walti H, de GE, Relier JP (Comparative efficacy of theophylline and caffeine in the treatment of idiopathic apnea in premature infants. Am J Dis Child 139:698-700.1985).

Burke K, Chandler CJ, Starr BS, Starr MS (Seizure Promotion and Protection by D-1 and D-2 Dopaminergic Drugs in the Mouse. Pharmacology Biochemistry and Behavior 36:729-733.1990).

Cameron DL, Williams JT (Dopamine D1 receptors facilitate transmitter release. Nature 366:344-347.1993).

Cancedda L, Fiumelli H, Chen K, Poo MM (Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo. J Neurosci 27:5224-5235.2007).

Caraiscos VB, Elliott EM, You T, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:3662-3667.2004).

Cepeda C, Li Z, Cromwell HC, Altemus KL, Crawford CA, Nansen EA, Ariano MA, Sibley DR, Peacock WJ, Mathern GW, Levine MS (Electrophysiological and morphological analyses of cortical neurons obtained from children with catastrophic epilepsy: dopamine receptor modulation of glutamatergic responses. Dev Neurosci 21:223-235.1999).

Chan SL, Mayne M, Holden CP, Geiger JD, Mattson MP (Presenilin-1 mutations increase levels of ryanodine receptors and calcium release in PC12 cells and cortical neurons. Journal of Biological Chemistry 275:18195-18200.2000).

Chang BS, Lowenstein DH (Mechanisms of disease - Epilepsy. New England Journal of Medicine 349:1257-1266.2003).

Chen L, Yang XL (Hyperpolarization-activated cation current is involved in modulation of the excitability of rat retinal ganglion cells by dopamine. Neuroscience 150:299-308.2007).

Chinta SJ, Andersen JK (Dopaminergic neurons. International Journal of Biochemistry & Cell Biology 37:942-946.2005).

Chu NS (Caffeine-Induced and Aminophylline-Induced Seizures. Epilepsia 22:85-94.1981).

Clemens B (Dopamine Agonist Treatment of Self-Induced Pattern-Sensitive Epilepsy - A Case-Report. Epilepsy Res 2:340-343.1988).

Clinckers R, Smolders I, Meurs A, Ebinger G, Michotte Y (Anticonvulsant action of hippocampal dopamine and serotonin is independently mediated by D-2 and 5-HT1A receptors. Journal of Neurochemistry 89:834-843.2004).

Comer AM, Perry CM, Figgitt DP (Caffeine citrate: a review of its use in apnoea of prematurity. Paediatr Drugs 3:61-79.2001).

Commission of ILAE 1 (Commission on Classification and Terminology of the International League Against Epilepsy, 1981. Proposal for revised clinical and electroencephalographic classification of epileptic seizures. Epilepsia 22:489-501.1981).

Commission of ILAE 1 (Commission on Classification and Terminology of the International League Against Epilepsy, 1989. Proposal for revised classification of epilepsies and epileptic syndromes. Epilepsia 30:389-399.1989).

Crandall JE, McCarthy DM, Araki KY, Sims JR, Ren JQ, Bhide PG (Dopamine receptor activation modulates GABA neuron migration from the basal forebrain to the cerebral cortex. J Neurosci 27:3813-3822.2007).

Dahlstroem A, Fuxe K (EVIDENCE FOR THE EXISTENCE OF MONOAMINE-CONTAINING NEURONS IN THE CENTRAL NERVOUS SYSTEM. I. DEMONSTRATION OF MONOAMINES IN THE CELL BODIES OF BRAIN STEM NEURONS. Acta Physiol Scand Suppl:SUPPL-55.1964).

Daly JW (Caffeine analogs: biomedical impact. Cellular and Molecular Life Sciences 64:2153-2169.2007).

Daly JW, Jacobson KA, Ukena D (Adenosine receptors: development of selective agonists and antagonists. Prog Clin Biol Res 230:41-63.1987).

Dazzi L, Serra M, Porceddu ML, Sanna A, Chessa MF, Biggio G (Enhancement of basal and pentylenetetrazol (PTZ)-stimulated dopamine release in the brain of freely moving rats by PTZ-induced kindling. Synapse 26:351-358.1997).

Delanty N, Vaughan CJ, French JA (Medical causes of seizures. Lancet 352:383-390.1998).

DeLorenzo RJ, Sun DA, Deshpande LS (Cellular mechanisms underlying acquired epilepsy: The calcium hypothesis of the induction and maintainance of epilepsy. Pharmacology & Therapeutics 105:229-266.2005).

Denter DG, Heck N, Riedemann T, White R, Kilb W, Luhmann HJ (GABA(C) receptors are functionally expressed in the intermediate zone and regulate radial migration in the embryonic mouse neocortex. Neuroscience 167:124-134.2010).

Dunwiddie TV, Masino SA (The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci 24:31-55.2001).

Dzhala VI, Brumback AC, Staley KJ (Alteration of CI--transport enhances efficacy of barbiturates in early-life seizures. Epilepsia 47:13.2006).

Dzhala VI, Staley KJ (Excitatory actions of endogenously released GABA contribute to initiation of ictal epileptiform activity in the developing hippocampus. J Neurosci 23:1840-1846.2003).

Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ (NKCC1 transporter facilitates seizures in the developing brain. Nat Med 11:1205-1213.2005).

Eccles JC, Magni F, WILLIS WD (Depolarization of central terminals of Group I afferent fibres from muscle. J Physiol 160:62-93.1962).

Edwards DH (Mechanisms of depolarizing inhibition at the crayfish giant motor synapse. I. Electrophysiology. J Neurophysiol 64:532-540.1990a).

Edwards DH (Mechanisms of depolarizing inhibition at the crayfish giant motor synapse. II. Quantitative reconstruction. J Neurophysiol 64:541-550.1990b).

Edwards JG, Glenbott M (Nomifensine and Convulsive Seizures. Human Toxicology 6:247-249.1987).

Eldridge FL, Millhorn DE, Waldrop TG, Kiley JP (Mechanism of Respiratory Effects of Methylxanthines. Respiration Physiology 53:239-261.1983).

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (Neurogenesis in the adult human hippocampus. Nature Med 4:1313-1317.1998).

Essayan DM (Cyclic nucleotide phosphodiesterases. Journal of Allergy and Clinical Immunology 108:671-680.2001).

Eteng MUEEUAEOAMAaACY (Recent advances in caffeine and theobromine toxicities: a review. Plant Foods for Human Nutrition 51:231-243.1997).

Farrant M, Kaila K (The cellular, molecular and ionic basis of GABA(A) receptor signalling. Prog Brain Res 160:59-87.2007).

Farrant M, Nusser Z (Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6:215-229.2005).

Feldman JL, Del Negro CA (Looking for inspiration: new perspectives on respiratory rhythm. Nat Rev Neurosci 7:232-242.2006).

Fisher RS, Boas WV, Blume W, Elger C, Genton P, Lee P, Engel J (Epileptic seizures and epilepsy: Definitions proposed by the International League against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). Epilepsia 46:470-472.2005).

Flores-Hernandez J, Cepeda C, Hernandez-Echeagaray E, Calvert CR, Jokel ES, Fienberg AA, Greengard P, Levine MS (Dopamine enhancement of NMDA currents in dissociated medium-sized striatal neurons: Role of D1 receptors and DARPP-32. J Neurophysiol 88:3010-3020.2002).

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE (Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacological Reviews 51:83-133.1999).

Freeman JM (1995) A Clinician's Look at the Developmental Neurobiology of Epilepsy. In: Brain Development and Epilepsy (Philip A.Schwartzkroin SLMJLNJWS, ed), pp 9-29 New York: Oxford University Press.

Freeman JM (Seizures, EEG events, and the ketogenic diet. Epilepsia 50:329-330.2009).

Fritschy JM, Paysan J, Enna A, Mohler H (Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. J Neurosci 14:5302-5324.1994).

Gannon BA (Theophylline or caffeine: which is best for apnea of prematurity? Neonatal Netw 19:33-36.2000).

Garaschuk O, Linn J, Eilers J, Konnerth A (Large-scale oscillatory calcium waves in the immature cortex. Nat Neurosci 3:452-459.2000).

Garris PA, Wightman RM (Different Kinetics Govern Dopaminergic Transmission in the Amygdala, Prefrontal Cortex, and Striatum - An In-Vivo Voltammetric Study. J Neurosci 14:442-450.1994).

Gattereau A, Vezina J, Rousseau S, Bielmann P (Hyperprolactinemia and Temporal-Lobe Epilepsy in A Woman - Concomitant and Persistent Prolactin Suppression and Temporal-Lobe Epilepsy Relief. Journal of Endocrinological Investigation 13:247-249.1990).

Gonzalez-Islas C, Hablitz JJ (Dopamine enhances EPSCs in layer II-III pyramidal neurons in rat prefrontal cortex. J Neurosci 23:867-875.2003).

Gonzalez-Sulser A, Wang J, Motamedi GK, Avoli M, Vicini S, Dzakpasu R (The 4-aminopyridine in vitro epilepsy model analyzed with a perforated multi-electrode array. Neuropharmacology 60:1142-1153.2011).

Goodman CS, Shatz CJ (Developmental mechanisms that generate precise patterns of neuronal connectivity. Neuron 10 Suppl.:77-98.1993).

Gross RA, Ferrendelli JA (Effects of Reserpine, Propranolol, and Aminophylline on Seizure Activity and Cns Cyclic-Nucleotides. Ann Neurol 6:296-301.1979).

Guerrini R, Dravet C, Raybaud C, Roger J, Bureau M, Battaglia A, Livet MO, Gambarelli D, Robain O (Epilepsy and Focal Gyral Anomalies Detected by Mri - Electroclinico-Morphological Correlations and Follow-Up. Developmental Medicine and Child Neurology 34:706-718.1992).

Guerrini R, Sicca F, Parmeggiani L (Epilepsy and malformations of the cerebral cortex. Epileptic Disord 5 Suppl 2:S9-26.2003).

Gulati K, Ray A, Pal G, Vijayan VK (Possible role of free radicals in theophylline-induced seizures in mice. Pharmacology Biochemistry and Behavior 82:241-245.2005).

Gulati K, Ray A, Vijayan VK (Free radicals and theophylline neurotoxicity : An experimental study. Cellular and Molecular Biology 53:42-52.2007).

Gulledge AT, Jaffe DB (Dopamine decreases the excitability of layer V pyramidal cells in the rat prefrontal cortex. J Neurosci 18:9139-9151.1998).

Haas HL, Jefferys JGR, Slater NT, Carpenter DO (Modulation of low calcium induced field bursts in the hippocampus by monoamines and cholinomimetics

1222. Pflügers Arch 400:28-33.1984).

Hanganu-Opatz IL (Between molecules and experience: Role of early patterns of coordinated activity for the development of cortical maps and sensory abilities. Brain Res Rev 64:160-176.2010).

Harinath S, Sikdar SK (Inhibition of human TREK-1 channels by caffeine and theophylline. Epilepsy Res 64:127-135.2005).

Hauser WA, Annegers JF, Kurland LT (Incidence of Epilepsy and Unprovoked Seizures in Rochester, Minnesota - 1935-1984. Epilepsia 34:453-468.1993).

Haydar TF, Wang F, Schwartz ML, Rakic P (Differential modulation of proliferation in the neocortical ventricular and subventricular zones. J Neurosci 20:5764-5774.2000).

Henderson-Smart DJ, De Paoli AG (Methylxanthine treatment for apnoea in preterm infants. Cochrane Database Syst Rev:CD000140.2010).

Henderson-Smart DJ, Steer PA (Caffeine versus theophylline for apnea in preterm infants. Cochrane Database Syst Rev:CD000273.2010).

Holmes GL (Epilepsy in the developing brain: Lessons from the laboratory and clinic. Epilepsia 38:12-30.1997).

Holmes GL (The Long-Term Effects of Neonatal Seizures. Clinics in Perinatology 36:901-+.2009).

Holmes GL, Ben-Ari Y (The neurobiology and consequences of epilepsy in the developing brain. Pediatr Res 49:320-325.2001).

Holmes GL, Khazipov R, Ben Ari Y (New concepts in neonatal seizures

4. Neuroreport 13:A3-A8.2002).

Holmes IP, Blunt RJ, Lorthioir OE, Blowers SM, Gribble A, Payne AH, Stansfield IG, Wood M, Woollard PM, Reavill C, Howes CM, Micheli F, Di Fabio R, Donati D, Terreni S, Hamprecht D, Arista L, Worby A, Watson SP (The identification of a selective dopamine D(2) partial agonist, D(3) antagonist displaying high levels of brain exposure. Bioorganic & Medicinal Chemistry Letters 20:2013-2016.2010).

Honore E (The neuronal background K-2P channels: focus on TREK1. Nat Rev Neurosci 8:251-261.2007).

Hsu KS (Characterization of dopamine receptors mediating inhibition of excitatory synaptic transmission in the rat hippocampal slice. J Neurophysiol 76:1887-1895.1996).

Humphrey T (The development of the human hippocampal fissure. J Anat 101:655-676.1967).

Jay TM (Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. Prog Neurobiol 69:375-390.2003).

Kaila K (Ionic basis of $GABA_A$ receptor channel function in the nervous system. Prog Neurobiol 42:489-537.1994).

Kawagoe KT, Garris PA, Wiedemann DJ, Wightman RM (Regulation of Transient Dopamine Concentration Gradients in the Microenvironment Surrounding Nerve-Terminals in the Rat Striatum. Neuroscience 51:55-64.1992).

Khalilov I, Holmes GL, Ben-Ari Y (In vitro formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures. Nat Neurosci 6:1079-1085.2003).

Khazipov R, Khalilov I, Tyzio R, Morozova E, Ben-Ari Y, Holmes GL (Developmental changes in GABAergic actions and seizure susceptibility in the rat hippocampus. Eur J Neurosci 19:590-600.2004).

Khazipov R, Luhmann HJ (Early patterns of electrical activity in the developing cerebral cortex of human and rodents. Trends in Neurosciences 29:414-418.2006).

Kilb W (Development of the GABAergic System from Birth to Adolescence. Neuroscientist 18:613-630.2012).

Kilb W, Dierkes PW, Sykova E, Vargova L, Luhmann HJ (Hypoosmolar conditions reduce extracellular volume fraction and enhance epileptiform activity in the CA3 region of the immature rat hippocampus. J Neurosci Res 84:119-129.2006).

Kilb W, Sinning A, Luhmann HJ (Model-specific effects of bumetanide on epileptiform activity in the in-vitro intact hippocampus of the newborn mouse. Neuropharmacology 53:524-533.2007).

Kobayashi K, Suzuki H (Dopamine selectively potentiates hippocampal mossy fiber to CA3 synaptic transmission. Neuropharmacology 52:552-561.2007).

Kolbaev SN, Achilles K, Luhmann HJ, Kilb W (Effect of depolarizing GABA(A)-mediated membrane responses on excitability of Cajal-Retzius cells in the immature rat neocortex. J Neurophysiol 106:2034-2044.2011).

Kolbaev SN, Sharopov S, Dierkes PW, Luhmann HJ, Kilb W (Phasic GABAA-receptor activation is required to suppress epileptiform activity in the CA3 region of the immature rat hippocampus. Epilepsia 53:888-896.2012).

Koller W, Herbster G, Anderson D, Wack R, Gordon J (Quinpirole Hydrochloride, A Potential Anti-Parkinsonism Drug. Neuropharmacology 26:1031-1036.1987).

Korpi ER, Kuner T, Seeburg PH, Luddens H (Selective antagonist for the cerebellar granule cell-specific gamma-aminobutyric acid type A receptor

29. Mol Pharmacol 47:283-289.1995).

Kotecha SA, Oak JN, Jackson MF, Perez Y, Orser BA, Van Tol HH, MacDonald JF (A D2 class dopamine receptor transactivates a receptor tyrosine kinase to inhibit NMDA receptor transmission

15676. Neuron 35:1111-1122.2002).

Kotsopoulos IAW, van Merode T, Kessels FGH, De Krom MCTF, Knottnerus JA (Systematic review and meta-analysis of incidence studies of epilepsy and unprovoked seizures. Epilepsia 43:1402-1409.2002).

Kuhn HG, DickinsonAnson H, Gage FH (Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027-2033.1996).

Lamsa K, Palva JM, Ruusuvuori E, Kaila K, Taira T (Synaptic GABA(A) activation inhibits AMPA-kainate receptor-mediated bursting in the newborn (P0-P2) rat hippocampus. J Neurophysiol 83:359-366.2000).

Lee KS, Reddington M, Schubert P, Kreutzberg G (Regulation of the Strength of Adenosine Modulation in the Hippocampus by A Differential Distribution of the Density of A1 Receptors. Brain Res 260:156-159.1983).

Lee TC, Charles BG, Steer PA, Flenady VJ (Saliva as a valid alternative to serum in monitoring intravenous caffeine treatment for apnea of prematurity. Therapeutic Drug Monitoring 18:288-293.1996).

Leinekugel X, Medina I, Khalilov I, Ben Ari Y, Khazipov R (Ca2+ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. Neuron 18:243-255.1997).

Lewis ME, Patel J, Edley SM, Marangos PJ (Autoradiographic Visualization of Rat-Brain Adenosine Receptors Using N6-Cyclohexyl [H-3]Adenosine. Eur J Pharmacol 73:109-110.1981).

Li H, Tornberg J, Kaila K, Airaksinen MS, Rivera C (Patterns of cation-chloride cotransporter expression during embryonic rodent CNS development. Eur J Neurosci 16:2358-2370.2002).

Lindquist CE, Ebert B, Birnir B (Extrasynaptic GABA(A) channels activated by THIP are modulated by diazepam in CA1 pyramidal neurons in the rat brain hippocampal slice. Mol Cell Neurosci 24:250-257.2003).

Lipka LJ, Lathers CM (Psychoactive Agents, Seizure Production, and Sudden-Death in Epilepsy. Journal of Clinical Pharmacology 27:169-183.1987).

Lombroso CT (Neonatal seizures: Historic note and present controversies. Epilepsia 37:5-13.1996).

Loscher W, Czuczwar SJ (Studies on the Involvement of Dopamine D-1 and D-2 Receptors in the Anticonvulsant Effect of Dopamine Agonists in Various Rodent Models of Epilepsy. Eur J Pharmacol 128:55-65.1986).

LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR (GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 15:1287-1298.1995).

Luhmann HJ, Prince DA (Postnatal maturation of the GABAergic system in rat neocortex. J Neurophysiol 65:247-263.1991).

Luo AH, Tahsili-Fahadan P, Wise RA, Lupica CR, Aston-Jones G (Linking Context with Reward: A Functional Circuit from Hippocampal CA3 to Ventral Tegmental Area. Science 333:353-357.2011).

Malenka RC, Nicoll RA (Dopamine decreases the calcium-activated afterhyperpolarization in hippocampal CA1 pyramidal cells. Brain Res 379:210-215.1986).

Manent JB, Demarque M, Jorquera I, Pellegrino C, Ben-Ari Y, Aniksztejn L, Represa A (A noncanonical release of GABA and glutamate modulates neuronal migration. J Neurosci 25:4755-4765.2005).

Marangos PJ, Martino AM, Paul SM, Skolnick P (The benzodiazepines and inosine antagonize caffeine-induced seizures. Psychopharmacology (Berl) 72:269-273.1981).

Marchionni I, Omrani A, Cherubini E (In the developing rat hippocampus a tonic GABAA-mediated conductance selectively enhances the glutamatergic drive of principal cells. J Physiol 581:515-528.2007).

Mcpherson PS, Kim YK, Valdivia H, Knudson CM, Takekura H, Franziniarmstrong C, Coronado R, Campbell KP (The Brain Ryanodine Receptor - A Caffeine-Sensitive Calcium Release Channel. Neuron 7:17-25.1991).

Mienville JM (Persistent depolarizing action of GABA in rat Cajal-Retzius cells. J Physiol (Lond) 512:809-817.1998).

Miller MW (Generation of neurons in the rat dentate gyrus and hippocampus: effects of prenatal and postnatal treatment with ethanol. Alcohol Clin Exp Res 19:1500-1509.1995).

Mokler DJ, Torres OI, Galler JR, Morgane PJ (Stress-induced changes in extracellular dopamine and serotonin in the medial prefrontal cortex and dorsal hippocampus of prenatally malnourished rats. Brain Res 1148:226-233.2007).

Moser J, Kilb W, Werhahn KJ, Luhmann HJ (Early developmental alterations of low-Mg2+ -induced epileptiform activity in the intact corticohippocampal formation of the newborn mouse in vitro. Brain Res 1077:170-177.2006).

Mueller AL, Dunwiddie TV (Anti-Convulsant and Proconvulsant Actions of Alpha-Noradrenergic and Beta-Noradrenergic Agonists on Epileptiform Activity in Rat Hippocampus Invitro. Epilepsia 24:57-64.1983).

Mueller AL, Taube JS, Schwartzkroin PA (Development of hyperpolarizing inhibitory postsynaptic potentials and hyperpolarizing response to gamma-aminobutyric acid in rabbit hippocampus studied in vitro. J Neurosci 4:860-867.1984).

Murray TF, Cheney DL (Neuronal Location of N6-Cyclohexyl[H-3]Adenosine Binding-Sites in Rat and Guinea-Pig Brain. Neuropharmacology 21:575-580.1982).

Nakada T, Kwee IL, Lerner AM, Remler MP (Theophylline-Induced Seizures - Clinical and Pathophysiologic Aspects. Western Journal of Medicine 138:371-374.1983).

Neves G, Cooke SF, Bliss TV (Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. Nat Rev Neurosci 9:65-75.2008).

Nowakowski RS (Development of the Hippocampal-Formation in Mutant Mice. Drug Development Research 15:315-336.1988).

Nowakowski RS, Rakic P (The Site of Origin and Route and Rate of Migration of Neurons to the Hippocampal Region of the Rhesus-Monkey. J Comp Neurol 196:129-154.1981).

Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP (Pitx3 is required for development of substantia nigra dopaminergic neurons. Proc Natl Acad Sci USA 100:4245-4250.2003).

O'Sullivan GJ, Dunleavy M, Hakansson K, Clementi M, Kinsella A, Croke DT, Drago J, Fienberg AA, Greengard P, Sibley DR, Fisone G, Henshall DC, Waddington JL (Dopamine D(1) vs D(5) receptor-dependent induction of seizures in relation to DARPP-32, ERK1/2 and GluR1-AMPA signalling. Neuropharmacology 54:1051-1061.2008).

Owens DF, Boyce LH, Davis MB, Kriegstein AR (Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci 16:6414-6423.1996).

Pal S, Sun D, Limbrick D, Rafiq A, DeLorenzo RJ (Epileptogenesis induces long-term alterations in intracellular calcium release and sequestration mechanisms in the hippocampal neuronal culture model of epilepsy. Cell Calcium 30:285-296.2001).

Panayiotopoulos CP (2005) Neonatal Seizures and Neonatal Syndromes. Oxfordshire (UK): Bladon Medical Publisher.

Paternain AV, Morales M, Lerma J (Selective antagonism of AMPA receptors unmasks kainate receptor- mediated responses in hippocampal neurons. Neuron 14:185-189.1995).

Payne JA, Rivera C, Voipio J, Kaila K (Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci 26:199-206.2003).

Phillis JW (Role of Cyclic Nucleotides in Cns. Canadian Journal of Neurological Sciences 4:153-195.1977).

Plotkin MD, Snyder EY, Hebert SC, Delpire E (Expression of the Na-K-2Cl cotransporter is developmentally regulated in postnatal rat brains: A possible mechanism underlying GABA's excitatory role in immature brain. J Neurobiol 33:781-795.1997).

Podda MV, Riccardi E, D'Ascenzo M, Azzena GB, Grassi C (Dopamine D1-Like Receptor Activation Depolarizes Medium Spiny Neurons of the Mouse Nucleus Accumbens by Inhibiting Inwardly Rectifying K(+) Currents Through A Camp-Dependent Protein Kinase A-Independent Mechanism. Neuroscience 167:678-690.2010).

Prakash N, Wurst W (Development of dopaminergic neurons in the mammalian brain. Cellular and Molecular Life Sciences 63:187-206.2006).

Prayson RA, Estes ML, Morris HH (Coexistence of Neoplasia and Cortical Dysplasia in Patients Presenting with Seizures. Epilepsia 34:609-615.1993).

Psarropoulou C, Descombes S (Differential bicuculline-induced epileptogenesis in rat neonatal, juvenile and adult CA3 pyramidal neurons in vitro. Brain Res Dev Brain Res 117:117-120.1999).

Quattlebaum TG (Benign Familial Convulsions in the Neonatal-Period and Early Infancy. Journal of Pediatrics 95:257-259.1979).

Quilichini PP, Diabira D, Chiron C, Ben Ari Y, Gozlan H (Persistent epileptiform activity induced by low Mg2+ in intact immature brain structures. Eur J Neurosci 16:850-860.2002).

Quilichini PP, Diabira D, Chiron C, Milh M, Ben-Ari Y, Gozlan H (Effects of antiepileptic drugs on refractory seizures in the intact immature corticohippocampal formation in vitro. Epilepsia 44:1365-1374.2003).

Radnikow G, Misgeld U (Dopamine D_1 receptors facilitate $GABA_A$ synaptic currents in the rat substantia nigra pars reticulata. J Neurosci 18:2009-2016.1998).

Richter D, Luhmann HJ, Kilb W (Intrinsic activation of GABA(A) receptors suppresses epileptiform activity in the cerebral cortex of immature mice. Epilepsia 51:1483-1492.2010).

Rickmann M, Amaral DG, Cowan WM (Organization of Radial Glial-Cells During the Development of the Rat Dentate Gyrus. J Comp Neurol 264:449-479.1987).

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K (The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-255.1999).

Roca DJ, Schiller GD, Farb DH (Chronic Caffeine Or Theophylline Exposure Reduces Gamma-Aminobutyric Acid Benzodiazepine Receptor-Site Interactions. Molecular Pharmacology 33:481-485.1988).

Ruangkittisakul A, Ballanyi K (Methylxanthine reversal of opioid-evoked inspiratory depression via phosphodiesterase-4 blockade. Respiratory Physiology & Neurobiology 172:94-105.2010).

Ruskin DN, Rawji SS, Walters JR (Effects of full D-1 dopamine receptor agonists on firing rates in the globus pallidus and substantia nigra pars compacta in vivo: Tests for D-1 receptor selectivity and comparisons to the partial agonist SKF 38393. Journal of Pharmacology and Experimental Therapeutics 286:272-281.1998).

Ryan SG, Wiznitzer M, Hollman C, Torres MC, Szekeresova M, Schneider S (Benign Familial Neonatal Convulsions - Evidence for Clinical and Genetic-Heterogeneity. Ann Neurol 29:469-473.1991).

Sanchez RM, Jensen FE (Maturational aspects of epilepsy mechanisms and consequences for the immature brain. Epilepsia 42:577-585.2001).

Sankar R, Wheless JW, Dravet C, Guerrini R, Medina MT, Bureau M, Genton P, Delgado-Escueta AV (Treatment of myoclonic epilepsies in infancy and early childhood. Adv Neurol 95:289-298.2005).

Scanlon JE, Chin KC, Morgan ME, Durbin GM, Hale KA, Brown SS (Caffeine or theophylline for neonatal apnoea? Arch Dis Child 67:425-428.1992).

Schmidt B, Roberts RS, Davis P, Doyle LW, Barrington KJ, Ohlsson A, Solimano A, Tin W (Caffeine therapy for apnea of prematurity. N Engl J Med 354:2112-2121.2006).

Schultz W (Multiple dopamine functions at different time courses. Annu Rev Neurosci 30:259-288.2007).

Schwartzkroin PA (Role of the Hippocampus in Epilepsy. Hippocampus 4:239-242.1994).

Schwartzkroin PASLMJLNJWS (1995) Brain Development and Epilepsy. New York, Oxford.

Semyanov A, Walker MC, Kullmann DM (GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 6:484-490.2003).

Shah KN, Rajadhyaksha SB, Shah VS, Shah NS, Desai VG (Experience with the International League Against Epilepsy Classifications of Epileptic Seizures (1981)

and Epilepsies and Epileptic Syndrome (1989) in Epileptic Children in A Developing-Country. Epilepsia 33:1072-1077.1992).

Sharopov S, Moser J, Chen R, Kolbaev SN, Bernedo VE, Werhahn KJ, Luhmann HJ, Kilb W (Dopaminergic modulation of low-Mg(2+) -induced epileptiform activity in the intact hippocampus of the newborn mouse in vitro. J Neurosci Res 90:2020-2033.2012).

Silverstein FS, Jensen FE (Neonatal seizures. Ann Neurol 62:112-120.2007).

Sipila ST, Huttu K, Soltesz I, Voipio J, Kaila K (Depolarizing GABA acts on intrinsically bursting pyramidal neurons to drive giant depolarizing potentials in the immature hippocampus. J Neurosci 25:5280-5289.2005).

Sipila ST, Kaila K (GABAergic Control of CA3-driven Network Events in the Developing Hippocampus. Results Probl Cell Differ 44:99-121.2008).

Smidt MP, Asbreuk CHJ, Cox JJ, Chen H, Johnson RL, Burbach JPH (A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3:337-341.2000).

Song ZM, Undie AS, Koh PO, Fang YY, Zhang L, Dracheva S, Sealfon SC, Lidow MS (D1 dopamine receptor regulation of microtubule-associated protein-2 phosphorylation in developing cerebral cortical neurons

3. J Neurosci 22:6092-6105.2002).

Spitzer AR (Evidence-Based Methylxanthine Use in the NICU. Clinics in Perinatology 39:137-+.2012).

Spitzer NC (Electrical activity in early neuronal development. Nature 444:707-712.2006).

Staley KJ, Mody I (Shunting of excitatory input to dentate gyrus granule cells by a depolarizing GABAA receptor-mediated postsynaptic conductance. J Neurophysiol 68:197-212.1992).

Stanton PK, Jones RSG, Mody I, Heinemann U (Epileptiform activity induced by lowering extracellular [Mg^{++}] in combined hippocampal-entorhinal cortex slices: modulation by receptors for norepinephrine and N-methyl-D-aspartate. Epilepsy Res 1:53-62.1987).

Stanzione P, Calabresi P, Mercuri N, Bernardi G (Dopamine Modulates Ca1 Hippocampal-Neurons by Elevating the Threshold for Spike Generation - An Invitro Study. Neuroscience 13:1105-1116.1984).

Starr MS (The role of dopamine in epilepsy. Synapse 22:159-194.1996).

Suppes T, Kriegstein AR, Prince DA (The influence of dopamine on epileptiform burst activity in hippocampal pyramidal neurons. Brain Res 326:273-280.1985).

Svenningsson P, Nomikos GG, Fredholm BB (The stimulatory action and the development of tolerance to caffeine is associated with alterations in gene expression in specific brain regions. J Neurosci 19:4011-4022.1999).

Taketo M, Yoshioka T (Developmental change of GABA(A) receptor-mediated current in rat hippocampus. Neuroscience 96:507-514.2000).

Tchekalarova J, Kubova H, Mares P (Effects of postnatal caffeine exposure on seizure susceptibility in developing rats. Brain Res 1150:32-39.2007).

Tseng KY, O'Donnell P (Dopamine-glutamate interactions controlling prefrontal cortical pyramidal cell excitability involve multiple signaling mechanisms

20154. J Neurosci 24:5131-5139.2004).

Tyzio R, Holmes GL, Ben Ari Y, Khazipov R (Timing of the developmental switch in GABA(A) mediated signaling from excitation to inhibition in CA3 rat hippocampus using gramicidin perforated patch and extracellular recordings. Epilepsia 48 Suppl 5:96-105.2007).

Tyzio R, Represa A, Jorquera I, Ben-Ari Y, Gozlan H, Aniksztejn L (The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. J Neurosci 19:10372-10382.1999).

Umemiya M, Raymond LA (Dopaminergic modulation of excitatory postsynaptic currents in rat neostriatal neurons. J Neurophysiol 78:1248-1255.1997).

Usachev Y, Kostyuk P, Verkhratsky A (3-Isobutyl-1-Methylxanthine (Ibmx) Affects Potassium Permeability in Rat Sensory Neurons Via Pathways That Are Sensitive and Insensitive to [Ca2+](In). Pflugers Archiv-European Journal of Physiology 430:420-428.1995).

Usachev Y, Shmigol A, Pronchuk N, Kostyuk P, Verkhratsky A (Caffeine-Induced Calcium-Release from Internal Stores in Cultured Rat Sensory Neurons. Neuroscience 57:845-859.1993).

Verkhratsky A (Physiology and pathophysiology of the calcium store in the endoplasmic reticulum of neurons. Physiological Reviews 85:201-279.2005).

Walker MC, Semyanov A (Regulation of excitability by extrasynaptic GABA(A) receptors. Results Probl Cell Differ 44:29-48.2008).

Wallen A, Perlmann T (Transcriptional control of dopamine neuron development. Parkinson'S Disease: the Life Cycle of the Dopamine Neuron 991:48-60.2003).

Wang C, Shimizu-Okabe C, Watanabe K, Okabe A, Matsuzaki H, Ogawa T, Mori N, Fukuda A, Sato K (Developmental changes in KCC1, KCC2, and NKCC1 mRNA expressions in the rat brain. Brain Res Dev Brain Res 139:59-66.2002).

Wang DD, Kriegstein AR (GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. J Neurosci 28:5547-5558.2008).

Wang DD, Kriegstein AR (Blocking early GABA depolarization with bumetanide results in permanent alterations in cortical circuits and sensorimotor gating deficits. Cereb Cortex 21:574-587.2011).

Warter JM, Vergnes M, Depaulis A, Tranchant C, Rumbach L, Micheletti G, Marescaux C (Effects of Drugs Affecting Dopaminergic Neurotransmission in Rats with Spontaneous Petit-Mal-Like Seizures. Neuropharmacology 27:269-274.1988).

Weinshenker D, Szot P (The role of catecholamines in seizure susceptibility: new results using genetically engineered mice. Pharmacol Ther 94:213-233.2002).

Wells JE, Porter JT, Agmon A (GABAergic inhibition suppresses paroxysmal network activity in the neonatal rodent hippocampus and neocortex. J Neurosci 20:8822-8830.2000).

Wheless JW, Clarke DF, Arzimanoglou A, Carpenter D (Treatment of pediatric epilepsy: European expert opinion, 2007. Epileptic Disord 9:353-412.2007).

Wong M, Yamada KA (Developmental characteristics of epileptiform activity in immature rat neocortex: a comparison of four in vitro seizure models. Dev Brain Res 128:113-120.2001).

Yamada J, Okabe A, Toyoda H, Kilb W, Luhmann HJ, Fukuda A (Cl- uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1. J Physiol 557:829-841.2004).

Yarnell PR, Chu NS (Focal seizures and aminophylline. Neurology 25:819-822.1975).

Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (Dopamine neuron agenesis in Nurr1-deficient mice. Science 276:248-250.1997).

Zhang L, Bai J, Undie AS, Bergson C, Lidow MS (D1 dopamine receptor regulation of the levels of the cell-cycle-controlling proteins, cyclin D, P27 and Raf-1, in cerebral cortical precursor cells is mediated through cAMP-independent pathways. Cerebral Cortex 15:74-84.2005).