Kainate-type glutamate receptors modulating network activity in developing hippocampus

Juuso Juuri

Faculty of Biological and Environmental Sciences
Neuroscience Center and Department of Biosciences
University of Helsinki

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Supervisors
Professor Tomi Taira
University of Helsinki, Finland
and
Docent Sari Lauri
University of Helsinki, Finland

Pre-examiners
Professor Bryndis Birnir
Uppsala University, Sweden
and
Professor Ari Koskelainen
Aalto University, Finland

Opponent
Professor Karri Lämsä
University of Szeged, Hungary

Custodian
Professor Juha Voipio
University of Helsinki, Finland

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Men of good fortune very often can't do a thing
While men of poor beginnings often can do anything

Lou Reed, *Men of Good Fortune*, 1973

We dissect and analyze, probe and examine, slide the earth under a microscope lens hoping to find a code for miracles. Meanwhile, the sun shines and rivers rush and trout rise, and every hour of every day there is a real magic show of light and shadow and the dance of time.

Harry Middleton, *On the Spine of Time*, 1991

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Original publications

This thesis is based on the following publications and a manuscript referred hereafter with corresponding Roman numerals:

I. Segerstråle M, **Juuri J**, Lanore F, Piepponen P, Lauri SE, Mulle C & Taira T, 2010, High firing rate of neonatal hippocampal interneurons is caused by attenuation of afterhyperpolarizing potassium currents by tonically active kainate receptors, Journal of Neuroscience, 30(19):6507-14.

II. **Juuri J**, Clarke VRJ, Lauri SE & Taira T, 2010, Kainate receptor induced ectopic spiking of CA3 pyramidal neurons initiates network bursts in neonatal hippocampus, Journal of Neurophysiology, 104(3):1696-706.

III. **Juuri J**, Velusamy R, Lauri SE & Taira T, Ethanol modulates spontaneous network activity in the developing hippocampus in an age-dependent manner (manuscript).

Author's contribution to the studies above:

I. The author conducted all the recordings of network activity and analyzed them, participated in recordings and analysis of afterhyperpolarizing currents and participated in manuscript writing.

II. The author participated in designing the experiments, conducted most of the experiments, analysed the data and wrote most of the manuscript.

III. The author participated in designing the experiments, conducted most of the electrophysiological recordings, analysed the data and wrote most of the manuscript.

Abbreviations

ACET	(S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenyl-thiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione
AHP	afterhyperpolarization
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	adenosine triphosphate
ATPA	(S)-2-amino-3-(5-tert-butyl-3-hydroxy-4-isoxazolyl)propionic acid
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CA	*cornu ammonis*
CGP 55845	(2S)-3-[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid
CNQX	6-cyano-7-nitroquinoxaline
CNS	central nervous system
CsOH	cesium hydroxide
D-AP5	D-(-)-2-amino-5-phosphonopentanoic acid
DCG-IV	(2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine
Dlg1	Drosophila disc large tumor suppressor
EGTA	ethylene glycol tetraacetic acid
EPSC	excitatory postsynaptic current
EtOH	ethanol
E*X*	embryonic day *X*
GABA	gamma-aminobutyric acid
GDP	giant depolarizing potential
GDPbetaS	guanosine 5'-O-(2- thiophosphate)
GluK*X*-KA receptor	a kainate receptor containing subunit GluKX
glutamate	(*S*)-glutamate
GTP	guanosine triphosphate
GYKI53655	1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylenedioxy-5H-2,3-benzodiazepine
HEPES	(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ImAHP	afterhyperpolarizing current of medium duration
IPSC	inhibitory postsynaptic current
KA	kainate
kainate	(2S,3S,4S)-3-carboxymethyl-4-isopropenylpyrrolidine-2-carboxylic acid
KOH	potassium hydroxide
LY382884	(3S,4aR,6S,8aR)-6-((4-carboxyphenyl)methyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid
mAHP	afterhyperpolarization of medium duration
mEPSC	miniature excitatory postsynaptic current
mIPSC	miniature inhibitory postsynaptic current

mPSC	miniature postsynaptic current
NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline
NMDA	N-methyl-D-aspartic acid
NS-102	6,7,8,9-tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione 3-oxime
PDZ	postsynaptic density protein 95, Drosophila disc large tumor suppressor, and zonula occludens-1 protein
PNW	postnatal week
PSC	postsynaptic current
PSD95	postsynaptic density protein 95
P*X*	postnatal day *X*
s. (sa.)	*stratum (strata)*
SK channel	small conductance calcium-activated potassium channel
SYM2081	(2S,4R)-4-methylglutamate
TTX	tetrodotoxin
WT	wild type
zo-1	zonula occludens-1 protein

Abstract

Kainate-type of ionotropic glutamate (KA) receptors are associated with the modulation of neuronal excitability, synaptic transmission, and activity of neuronal networks. They are believed to have an important role in the development of neuronal connections. In this thesis, the role of KA receptors in the early brain development was assessed by conducting *in vitro* electrophysiological recordings from individual neurons at CA3 region in acute slices of neonatal rodent hippocampi.

It was found that activation of separate KA receptor populations promoted action potential firing in both glutamatergic pyramidal neurons and GABAergic interneurons. The receptors in pyramidal neurons displayed a high affinity for agonist kainate, appeared to lack subunit GluK1, and promoted spontaneous firing of pyramidal neurons without depolarizing them. The receptors in interneurons contained subunit GluK1 and their activation suppressed afterhyperpolarizing current of medium duration (ImAHP). Receptors in both neuron types appeared to be activated tonically by ambient glutamate, suggesting that their physiological role may be to act as a modulatory mechanism sensitive to changes in extracellular glutamate concentration.

Changes in activity of neurons at CA3 by activation of KA receptors were reflected on the network level. Promotion of pyramidal cell firing by pharmacological activation of high-affinity KA receptors lead to enhanced glutamatergic drive and generation of network bursts in the CA3 region. The I_{mAHP} in interneurons was also suppressed by apamin, a blocker of SK potassium channels that mediate majority of this current, and apamin enhanced generation of network bursts. This suggests that also KA receptor mediated regulation of I_{mAHP} may modulate network activity. It was also found that there was an interaction between KA receptors and ethanol in the modulation of hippocampal network: ethanol decreased the occurrence of the network bursts at postnatal days 1 (P1) and P10, whereas it increased bursting at P5. The network effects of ethanol were partially or completely counteracted by specific pharmacological block of GluK1 subunit-containing KA receptors.

The findings disclose that via regulation of activity of individual neurons, KA receptors are capable of robust modulation of network activity in immature hippocampus. Additionally, exogenous agents affecting KA receptors may perturb activity dependent developmental processes that are central for the synaptic development. The results shed light on the mechanisms underlying development of hippocampal connectivity, and may help to understand early pathologies of the brain that have developmental origins.

1 Review of the literature

1.1 Introduction

The amino acid glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) (Collard *et al.* 1993; Yen *et al.* 1993; Behar *et al.* 1999). Its effects are mediated by metabotropic glutamate (mGlu) receptors that couple to guanosine nucleotide-binding proteins (G-proteins) (Conn and Pin 1997) and ionotropic glutamate (iGlu) receptors that are ligand gated ion channels (Traynelis *et al.* 2010). iGlu receptors are classified into three functionally distinct groups of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartic acid (NMDA) and kainate (KA) receptors named after their preferred agonists (Traynelis *et al.* 2010). AMPA receptors mediate the majority of fast synaptic transmission in the brain and NMDA receptors play a central role in the control of synaptic plasticity (Traynelis *et al.* 2010). Although the roles of the KA receptors remain less well understood, they seem to perform diverse functions acting in both pre- and postsynaptic locations to regulate neurotransmitter release and neuronal excitability (Pinheiro and Mulle 2006; Jane *et al.* 2009; Contractor *et al.* 2011; Sihra *et al.* 2013; Lerma and Marques 2013; Carta *et al.* 2014). These modulatory actions make KA receptors well suited for fine-tuning the activity and synchrony of neuronal networks (Lerma 2003; Pinheiro and Mulle 2006; Lauri and Taira 2012; Lerma and Marques 2013).

Coherent multi-neuronal activity patterns are a hallmark of neuronal networks (Buzsáki and Draguhn 2004). During the early stage of development, neuronal activity is generated spontaneously and the network activity patterns mature in a characteristic manner in parallel with the development of neuronal connectivity (Egorov and Draguhn 2013). The spontaneous activity is instrumental in guiding the formation of synaptic contacts by synaptic strengthening and elimination (Lauri *et al.* 2003; Huupponen *et al.* 2007; Huupponen *et al.* 2013). Thus, perturbations of early network activity may lead to disrupted development of cortical circuitries and to a variety of neurological dysfunctions manifesting later in life. In cortical regions, network activity presents as characteristic pattern of intermittent bursts of synchronous neuronal activity that emerges when the synaptic connections are established (Egorov and Draguhn 2013). This kind of activity is most extensively studied in the developing hippocampus (Ben-Ari *et al.* 2007).

Hippocampus is a cortical structure important for cognitive processes related to memory and spatial navigation, and associated with many neuropathological states such as epilepsy and Alzheimer's disease (Andersen *et al.* 2006). It is a widely used model system and studies of hippocampal neurons have given a wealth of in-

formation to base our understanding of cellular neurophysiology. Also the majority of insight into KA receptor function has been gained from the *in vitro* studies of rodent hippocampus (Carta *et al.* 2014). Although only few studies have addressed KA receptors in immature hippocampus, several lines of evidence indicate an important role for KA receptors in the early development of the CNS and in the maturation of synaptic networks (Pinheiro and Mulle 2006; Lauri and Taira 2011; Lauri and Taira 2012; Lerma and Marques 2013; Carta *et al.* 2014). Especially, KA receptors have been shown to display functions that enable them to modulate the early network activity patterns in the immature hippocampus (Lauri *et al.* 2005).

1.2 Development of hippocampal network

Hippocampus is an elongated cortical structure located in the medial temporal lobe (Figure 1 at page 5). It is functionally connected to related brain regions, the subiculum, presubiculum, parasubiculum, and entorhinal cortex that together comprise the hippocampal formation. Hippocampus receives polysensoral cortical information from the closely associated entorhinal cortex and modulatory input from septum and the serotonin, norepinephrine, and dopamine systems (Amaral *et al.* 2006). Hippocampus consists of two interlocked cortical infoldings, the dentate gyrus (DG) and *cornu ammonis* (CA) (also known as hippocampus proper or *Ammon's horn)* that belong to a phylogenetically old part of the cerebral cortex called archicortex. Archicortical structures display a three-layered structure, in which neuronal bodies and fibers are organized in distinct and highly organized laminae called *strata* (*sa.*) (Amaral *et al.* 2006).

The principal neurons of hippocampus use glutamate as a neurotransmitter and their somata are packed in distinct layers that are readily apparent in a transverse sections of hippocampus (Figure 1 at page 5). Principal neurons of the dentate gyrus are called granule cells and form the granule layer or *stratum (s.) granulosum*. Principal neurons of the CA are called pyramidal neurons (or pyramidal cells) and form the pyramidal layer or *s. pyramidale*. CA is further divided into subregions CA1 to CA3 based on the differences in connections, morphology, and molecular markers of the pyramidal neurons. Pyramidal neurons are generated between embryonic days 10 to 18 (E10-18) in mouse and E16-E18 in rat, and migrate in place from E10 (Angevine 1965; Bayer 1980; Altman and Bayer 1990). Granule cells are generated later than pyramidal neurons and their generation lasts much longer, well into postnatal development and, at reduced level, even into adulthood (Zhao *et al.* 2008; Piatti *et al.* 2011).

In the mature hippocampus, all the neuronal fibers are highly organized and information is processed through several parallel routes involving distinct subregions (Förster *et al.* 2006). The major synaptic connections in the hippocampus are glutamatergic (Amaral and Witter 1989). In the classic trisynaptic pathway, the information coming from the entorhinal cortex to DG via projection called perforant path (Steward 1976) is processed trough hippocampus via sequential exitatory projections between subregions. Accordingly, granule cells of DG project to CA3 via their axons called mossy fibers (MFs) (Spruston 2008), CA3 pyramidal neurons project to CA1 through their axons called Schaffer collaterals (Chronister and DeFrance 1979), and CA1 pyramidal neurons, in turn, project back to the entorhinal cortex (Köhler 1986). In addition to this major pathway, there are parallel and associative projections between and within hippocampal regions. For example, perforant path also targets CA3 as well as CA1, and CA3 pyramidal neurons project to other levels of the CA3 of the ipsi- and contralateral hippocampus via associational and commissural (A/C) fibers, respectively (Amaral *et al.* 2006).

The major glutamatergic projections of hippocampus are anatomically present already at early development. During the first stages of synaptogenesis, assembly of presynaptic specializations is guided by cellular and molecular events that are independent of neuronal activity (McAllister 2007; Jin and Garner 2008), but functional development occurs gradually in the early postnatal life in an activity dependent manner (Hsia *et al.* 1998; Fiala *et al.* 1998; Tyzio *et al.* 1999; Hanse *et al.* 2009). Initially, the synaptic connections are weak and labile due to characteristic pre- and postsynaptic features that are subject to change depending on the activity of the synapse (Isaac *et al.* 1995; Durand *et al.* 1996; Liao and Malinow 1996; Isaac *et al.* 1997; Gasparini *et al.* 2000; Xiao *et al.* 2004; Abrahamsson *et al.* 2007; Hanse *et al.* 2009). Presynaptically, synapses have a low probability of release, and therefore favor transmission with high-frequency (Gasparini *et al.* 2000; Lauri *et al.* 2006; Caiati *et al.* 2010). Additionally, mossy fibers display a peculiar property of being able to release both glutamate and GABA (Safiulina *et al.* 2006; Caiati *et al.* 2010; Safiulina *et al.* 2010). Postsynaptically, immature synapses have a labile AMPA receptor mediated component and they easily shift between an AMPA signaling and an AMPA silent state in an activity-dependent manner (Isaac *et al.* 1995; Durand *et al.* 1996; Xiao *et al.* 2004; Abrahamsson *et al.* 2005; Abrahamsson *et al.* 2008). Coincident pre- and postsynaptic activation of a synapse enhances and stabilizes the AMPA receptor-mediated component in the postsynaptic responses and increases the probability of transmitter release (Hanse *et al.* 2009; Lauri and Taira 2012; Hanse *et al.* 2013). In contrast, AMPA silencing occurs in response to asynchronous activity (Xiao *et al.* 2004; Hanse *et al.* 2009). Thus, immature gluta-

matergic synapses are strengthened by synchronous and weakened by asynchronous neuronal activity (Huupponen *et al.* 2013). In addition to changes in synapse function, average synaptic connectivity between CA3 and CA1 pyramidal neurons increases from single connections in the neonatal rat to multiple connections in the young adult rat (Durand *et al.* 1996; Liao and Malinow 1996; Hsia *et al.* 1998; Gasparini *et al.* 2000; Hanse and Gustafsson 2001). In rodents, glutamatergic transmission attains adult-like properties around the end of the second postnatal week (PNW) paralleling the establishment of adult-like computational operations of the hippocampal network (Amaral and Dent 1981; Marchal and Mulle 2004).

The interneurons of hippocampus are a morphologically and functionally heterogenous group of locally projecting neurons that use γ-aminobutyric acid (GABA) as their main neurotransmitter. They are relatively sparsely distributed and mostly situate outside of principal cell layers (Freund and Buzsáki 1996). By contacting the nearby neurons in a complex manner, interneurons form local microcircuits that contribute crucially to the processing of information in the hippocampus. Interneurons are generated and migrate to hippocampus earlier than principal neurons (Bayer 1980; Amaral and Kurz 1985; Lübbers *et al.* 1985; Soriano *et al.* 1989; Dupuy and Houser 1997; Dupuy-Davies and Houser 1999). Also anatomical maturation of connections of interneurons precedes that of principal neurons: functional GABAergic synapses are established before glutamatergic ones and the maturation of synaptic afferents occurs later in pyramidal neurons than in interneurons (Hollrigel and Soltesz 1997; Hollrigel *et al.* 1998; Tyzio *et al.* 1999; Khazipov *et al.* 2001; Hennou *et al.* 2002; Groc *et al.* 2003). Additionally, multiple transmitter release site connectivity from interneurons to CA1 pyramidal neurons is established on first postnatal days and this degree of connectivity remains stable into adulthood (Groc *et al.* 2003).

In many neurons, a functional shift occurs in the GABAergic transmission as it matures: initially depolarizing GABAergic responses become mainly hyperpolarizing or shunting. This is believed to be due to ontogenetical changes in neuronal chloride regulation (Ben-Ari *et al.* 2007). Activation of $GABA_A$ receptors, the main ionotropic GABA receptor in the brain, opens a channel permeable mainly to chloride and the current generated thus depends on the intracellular chloride concentration ($[Cl^-]_i$) . Neuronal $[Cl^-]_i$ is controlled by two chloride transporters, sodium potassium chloride cotransporter (NKCC1) that imports chloride into the cell and potassium-chloride cotransporter 2 (KCC2) that extrudes chloride from the cell (Payne *et al.* 2003). Immature neurons predominantly express NKCC1 (Yamada *et al.* 2004) but during development its expression decreases (Dzhala *et al.* 2005) and that of KCC2 increases (Rivera *et al.* 1999; Dzhala *et al.* 2005) leading to grad-

ually lower $[Cl^-]_i$. Thus, immature neurons have a relatively high $[Cl^-]_i$ and activation of $GABA_A$ receptors leads to an efflux of chloride anions and depolarization (Cherubini *et al.* 1991; Owens *et al.* 1996; Leinekugel *et al.* 1997; Ben-Ari *et al.* 1997; Rivera *et al.* 1999; Ben-Ari 2002; Sipilä *et al.* 2006; Valeeva *et al.* 2013). In contrast, GABAergic currents are hyperpolarizing or shunting due to influx of chloride in mature neurons with low $[Cl^-]_i$ (Owens and Kriegstein 2002). In rodents, this developmental shift in GABAergic responses occurs by the end of the second PNW in pyramidal neurons (Ganguly *et al.* 2001; Tyzio *et al.* 2007). However, in granule cells the GABAergic currents are hyperpolarizing and inhibitory already at P3 (Holter *et al.* 2010).

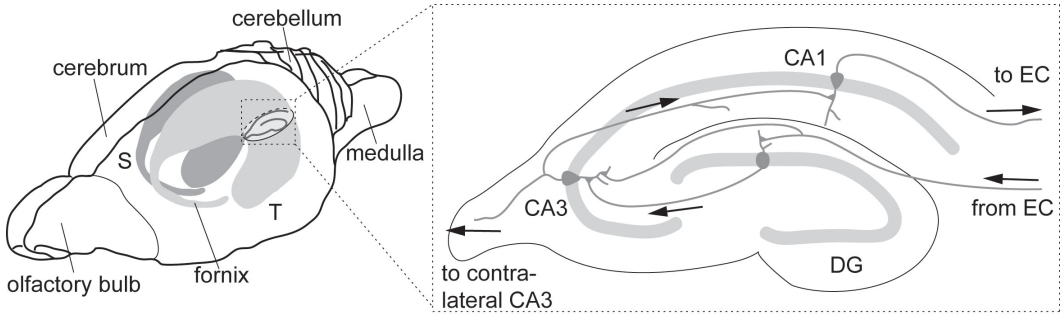

Figure 1 Hippocampal structure and major connections
Left Schematical presentaiton of hippocampi and their location in a rat brain with major anatomical brain structures depicted. S and T denote septal and temporal poles of hippocampus, respectively. ***Right*** Schematical presentation of transverse section of hippocampus with principal neurons and their major projection pathways illustrated. DG, dentate gyrus; CA, *cornu ammonis*; EC, entorhinal cortex. Figure modified from Amaral and Witter (1989).

1.3 Properties of kainate receptors

1.3.1 Structure

Like other ionotropic glutamate receptors, KA receptors are integral membrane proteins formed by four subunits surrounding a central ion channel pore (Hollmann and Heinemann 1994; Collingridge *et al.* 2009; Traynelis *et al.* 2010; Lerma and Marques 2013). They are composed of varying combinations from five subunits, GluK1 to GluK5 (previously known as GluR5, GluR6, GluR7, KA1 and KA2) (Collingridge *et al.* 2009; Traynelis *et al.* 2010). Each subunit displays a similar architecture of extracellular, transmembrane, and intracellular parts. The large extracellular part consists of an amino terminal domain involved in subunit recog-

nition and a ligand-binding site. The transmembrane ion-channel domain consists of three transmembrane a-helices and a pore-forming re-entrant pore-loop. The intracellular carboxy-terminal regions are highly variable and involved in receptor trafficking and interaction with intracellular partner proteins (see figure 2 on page 7) (Mayer 2006).

The subunits are divided into two subclasses, GluK1-3 and GluK4-5, that share only 45% sequence homology and differ in functional properties. The subunits GluK1-3 share 75-80% sequence homology, are able to form functional homomeric channels in recombinant systems, and bind glutamate and KA with low affinity at micromolar range. The subunits GluK4 and GluK5 share 68% sequence homology, only form functional receptors as heteromers with subunits GluK1-3, and bind glutamate and KA with high-affinity at nanomolar range (Bettler *et al.* 1990; Egebjerg *et al.* 1991; Werner *et al.* 1991; Bettler *et al.* 1992; Sakimura *et al.* 1992; Herb *et al.* 1992; Sommer *et al.* 1992; Wisden and Seeburg 1993; Kamboj *et al.* 1994; Howe 1996; Schiffer *et al.* 1997; Cui and Mayer 1999; Mott *et al.* 2010). Additionally, GluK1-3 have several variants with alternatively spliced intracellular carboxyterminal region probably conferring distinct modes of cellular trafficking and signalling with intracellular partners (Bettler *et al.* 1990; Gregor *et al.* 1993; Schiffer *et al.* 1997; Jaskolski *et al.* 2005). Further, mRNA of GluK1 and GluK2 may undergo editing at the channel pore-forming P-loop region (Egebjerg *et al.* 1991; Sommer *et al.* 1991; Egebjerg and Heinemann 1993; Köhler *et al.* 1993; Gregor *et al.* 1993; Hollmann and Heinemann 1994; Bettler and Mulle 1995; Seeburg 1996; Swanson *et al.* 1996; Schiffer *et al.* 1997). The exact subunit composition of native KA receptors is still under investigation, but based on overlapping gene expression patterns it is assumed that neuronal KA receptors are heteromers with potentially diverse properties due to distinct properties of the component subunits and their variants (Christensen *et al.* 2004; Ruiz *et al.* 2005).

The diversity of KA receptors is further increased by association with interacting proteins (Laezza *et al.* 2007; Tomita and Castillo 2012; Copits and Swanson 2012). For example, recently identified auxiliary subunits neuropilin- and tolloid-like proteins 1 and 2 (NETO1/2) alter the trafficking, gating and pharmacology of the KA receptors (Zhang *et al.* 2009; Copits *et al.* 2011; Straub *et al.* 2011; Fisher and Mott 2012; Fisher and Mott 2013; Wyeth *et al.* 2014). Other interacting proteins modulating KA receptors include, for example, the PDZ (post synaptic density protein 95 [PSD95], Drosophila disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]) proteins PICK1 (protein interacting with C kinase-1) and GRIP (glutamate receptor interacting protein) that appear to be important for stabilizing KA receptors at synapses and the BTB/kelch-domain protein KRIP6

(KA receptor interacting protein for GluR6) that directly modulates channel gating (Hirbec *et al.* 2003; Laezza *et al.* 2007; reviewed by Copits and Swanson 2012). Supposedly, organisms are able to "fine-tune" receptor functions by combining various subunits in different cell types and with different interacting proteins in different stages of development.

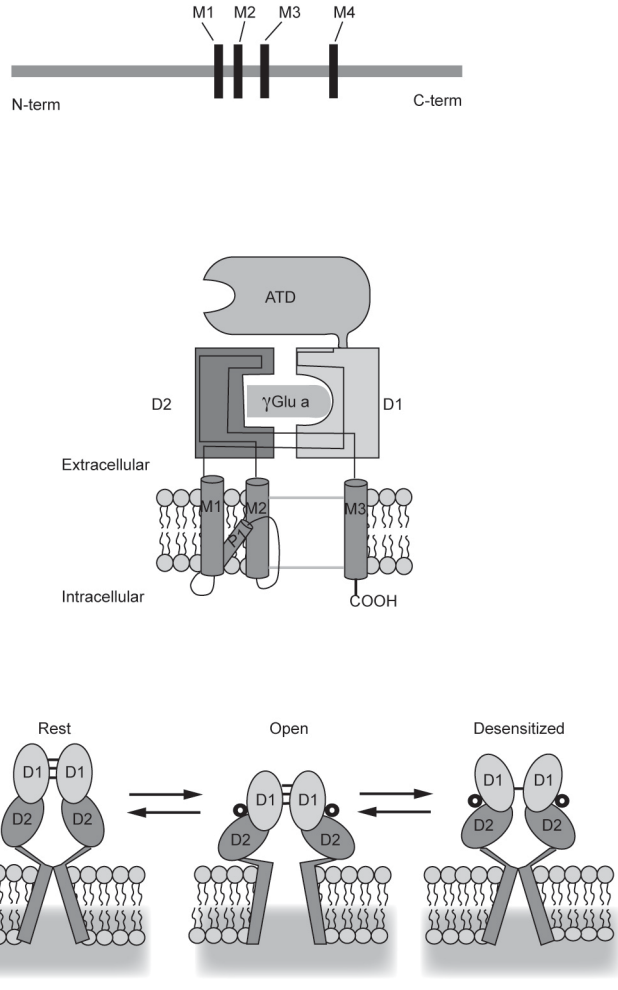

Figure 2 Structure and activation of kainate receptors

Top Schematic representation of a KA receptor subunit with the amino-terminal domain (ATD), the ligand-binding core formed by two separate domains (D1 and D2), the ion-channel domain with three membrane-spanning segments (M1 to M3) and a pore-loop (P), as well as the the intracellular C terminus.

Bottom Schematic representation of the resting, activated and desensitized states of a KA receptor to illustrate how domain closure is linked to separation of the ligand-binding core dimer domain 2 segments.

Figures modified after Mayer (2006).

1.3.2 Signaling

As their name implies, iGlu receptors exert cellular effects by ionotropic action, which is due to increased cationic conductance upon opening of the channel pore. In addition to this well characterised ionotropic signaling, activation of iGlu receptors may in some cases also induce a non-conventional metabotropic action mediated by G-protein activation (Lerma *et al.* 2001; Schenk and Matteoli 2004; Takago *et al.* 2005; Bowie 2010; Nabavi *et al.* 2013; Lerma and Marques 2013). KA receptors have been shown to exert their effect by both of these operation modes. Although considered likely, it is still uncertain whether a given receptor molecule may simultaneously display both of these functions.

The ionotropic function is activated when an agonist binds to the ligand binding core and triggers a conformational change in the receptor protein resulting in opening of the ion channel pore. Additionally, it triggers desensitization, a state which develops more slowly than activation and in which the channel pore closes and receptor becomes unresponsive to ligands (see figure 2 on page 7). The flow of ions through the pore down their electrochemical gradient results in transmembrane current, and the time course of the activation-desensitization sequence dictates the opening time of the channel pore. Because KA receptors, like other iGlu receptors, are mostly permeable to monovalent cations such as sodium and potassium, the reversal potential of the current mediated by them in neurons is typically close to 0 mV and thus depolarizes the cell from the negative resting potential. In addition to monovalent cations, most KA receptor channels are moderately permeable to calcium (Köhler *et al.* 1993; Burnashev *et al.* 1995; Perrais *et al.* 2009). The RNA editing of KA receptor subunits GluK1 and GluK2, however, makes channels impermeable to calcium (Burnashev *et al.* 1995). KA receptors desensitize rapidly by agonists and recover from desensitization slowly (Heckmann *et al.* 1996; Traynelis and Wahl 1997; Swanson *et al.* 1998; Perrais *et al.* 2009). The recovery from desensitized state depends on the subunit composition, presence of interacting proteins as well as the nature of agonist (Swanson *et al.* 1998; Paternain *et al.* 1998; Lerma *et al.* 2001; Perrais *et al.* 2010). Like other iGlu receptors, KA receptors are desensitized at lower agonist concentrations than they are activated (Jones *et al.* 1997; Paternain *et al.* 1998; Featherstone and Shippy 2008) and may therefore dwell in inactive state upon continuous agonist exposure. This feature has been argued to function as a means for physiological modulation of the size of the receptor pool susceptible to activation (Featherstone and Shippy 2008).

The magnitude and kinetics of the current mediated by KA receptors depend on the subunit composition as well as interactions with modulating proteins.

Most information on biophysical properties of KA receptors comes from studies of recombinant subunits (Perrais *et al.* 2010). Recombinant KA receptors have fast kinetics resembling those of AMPA receptors (Lerma 1997; Dingledine *et al.* 1999) whereas native KA receptors mediate slow currents with small amplitude (Castillo *et al.* 1997; Vignes and Collingridge 1997; Kidd and Isaac 1999; Cossart *et al.* 2002). This is apparently due to interactions with auxiliary subunits of Neto family (Zhang *et al.* 2009; Copits *et al.* 2011; Straub *et al.* 2011; Tang *et al.* 2011; Straub *et al.* 2011).

Accumulating evidence indicates that KA receptors also signal metabotropically via intracellular G protein-dependent transduction pathways. The molecular mechanisms underlying this are unclear especially as KA receptors do not share the prototypic seven transmembrane structure of G-protein coupled receptors and lack conventional G-protein coupling motifs in their intracellular C-terminal domains. However, a pertussis toxin-sensitive G protein activation leading to intracellular Ca^{2+} release and protein kinase C (PKC) activation has often been implicated with KA receptor activation (Rodríguez-Moreno and Lerma 1998; Cunha *et al.* 2000; Frerking *et al.* 2001; Melyan *et al.* 2002). Metabotrobic actions of KA receptors have been implicated in regulation of transmitter release (Rodríguez-Moreno and Sihra 2007) and neuronal excitability (Melyan *et al.* 2002; Melyan *et al.* 2004; Ruiz *et al.* 2005) as well as autoregulation of KA receptor surface expression (González-González and Henley 2013). There is evidence that the targets of KA receptor mediated metabotropic actions may be either voltage-activated calcium channels or calcium-activated potassium channels (Kamiya and Ozawa 1998; Frerking *et al.* 2001; Melyan *et al.* 2002; Rozas *et al.* 2003; Ruiz *et al.* 2005; Sallert *et al.* 2007; Salmen *et al.* 2012).

Physiological activation of KA receptors by glutamate can be either phasic or tonic. Phasic or transient activation is a result of exposure of postsynaptic receptors to transient elevation of glutamate after it is released from the presynaptic terminal. Despite the sequestration of glutamate by glutamate transporter, some ambient glutamate may be present in the extracellular space and activate KA receptors tonically. In the immature brain the glutamate transporter systems are less efficient and extracellular diffusion is less limited due to relatively larger volume of extracellular space than in mature brain (Danbolt 2001; Syková 2004; Diamond 2005). Therefore, the level of ambient glutamate may be higher and subject to more pronounced fluctuations in the developing brain. Accordingly, physiological tonic KA receptor activation has only been described in the immature hippocampus (Lauri *et al.* 2005; Lauri *et al.* 2006; Caiati *et al.* 2010). Thus far, only metabotropic signaling has been reported to be activated tonically. In principle, a

tonical ionotropic action is also possible since certain recombinant KA receptors have reported to pass tonic current when exposed to agonist concentrations high enough to open the channels but low enough to induce only partial desensitization (Paternain *et al.* 1998).

1.3.3 Pharmacology

For long, the overlapping agonist sensitivity made it impossible to pharmacologically distinguish KA and AMPA receptors and they were commonly referred together to as non-NMDA receptors. Only after the development of a series of selective 2,3-benzodiazepine antagonists for AMPA receptors such as 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-3,4-dihydro-7,8-methylene-dioxy-5H-2,3-benzodiazepine (GYKI53655) (Paternain *et al.* 1995; Wilding and Huettner 1995; Bleakman *et al.* 1996) was it possible to define the separate contributions of KA, AMPA, and NMDA receptors to glutamatergic currents (Castillo *et al.* 1997; Vignes and Collingridge 1997). As each KA receptor subunit has a distinct pharmacological profile, KA receptors present a challenging target for pharmacological modulation. Currently, few subunit spesific pharmacological tools are available and all of them target GluK1 due to the large size of its ligand binding pocket (Christensen *et al.* 2004).

Apart from glutamate and KA, many other agents act as KA receptor agonists (see table 1 on page 11). However, generally agonists show little specificity for KA receptors let alone individual subunits (Huettner 1990; Bleakman and Lodge 1998; Jane *et al.* 2009) and there are only few useful compounds that allow pharmacological discrimination of AMPA and KA receptors (Bleakman and Lodge 1998; Jane *et al.* 2009). The prototypic agonist KA and other structurally related natural products such as domoate activate KA but also AMPA receptors to a significant degree (Huettner 1990; Lerma *et al.* 1993; Wilding and Huettner 1997). More recently, some KA receptor selective agonists such as SYM2801 and GluK1 selective agonists 5-iodowillardiine, a derivative of an AMPA receptor agonist willardiine, and ATPA, a derivative of AMPA, have been developed (Zhou *et al.* 1997; Wilding and Huettner 1997; Jones *et al.* 1997; Clarke *et al.* 1997; Donevan *et al.* 1998; Paternain *et al.* 1998; Li *et al.* 1999).

Many KA receptor antagonists have been identified but only a small number of these are suitable for experimental tools in physiological settings (Jane *et al.* 2009) (see table 2 on page 12). As an antagonist is required for deduction of physiological function of a receptor, the lack of specific antagonists greatly limits the study of KA receptors. Compounds of the quinoxalinedione family, including the classic non-NMDA receptor antagonists CNQX and NBQX, act as competetive antagonists

on both AMPA and KA receptors with little selectivity. A group of decahydroisoquinolines display more selectivity towards KA receptors (Jane *et al.* 2009) and include LY382884 that selectively antagonizes the GluK1 subunit containing receptors (Simmons *et al.* 1998; Bortolotto *et al.* 1999; Alt *et al.* 2004). Recently, another and even more potent and selective antagonist of GluK1-containing KA receptors ACET (or UBP316) was derived from willardiine among a series of other less specific KA receptor antagonists (Dolman *et al.* 2005; Dargan *et al.* 2009). Another willardiine derivative UBP310 was originally developed as a GluK1 antagonist but has been shown to antagonize also GluK3-containing (Perrais *et al.* 2009) and heteromeric GluK2/GluK5 receptors (Pinheiro *et al.* 2013).

Table 1 Selected kainate receptor agonists

glutamate	Endogenous neurotransmitter and agonist for KA, AMPA, NMDA and mGlu receptors.
kainate *(2S,3S,4S)-3-carboxymethyl-4-isopropenylpyrrolidine-2-carboxylic acid*	KA receptor agonist but also activates AMPA receptors (Huettner 1990; Lerma *et al.* 1993).
domoate	KA receptor agonist that is more potent than KA and produces only partial desensitization but also activates AMPA receptors (Huettner 1990; Sommer *et al.* 1992; Lerma *et al.* 1993; Schiffer *et al.* 1997).
SYM2801 *2S-4R-methyl-glutamate*	Selective agonist for KA receptors that produces persistent desensitization and has been used as a functional agonist (Zhou *et al.* 1997; Wilding and Huettner 1997; Jones *et al.* 1997; Donevan *et al.* 1998; Paternain *et al.* 1998; Li *et al.* 1999).
(*S*)-5-iodowillardiine	An agonist that prefers subunit GluK1 (Wong *et al.* 1994; Swanson *et al.* 1998; Alt *et al.* 2004).
ATPA *(RS)-s-amino-3(3-hydroxy-5-tert-butylisoxazol-4-yl)propanoic acid*	An agonist selective for subunit GluK1 (Clarke *et al.* 1997), also partial agonist on GluK2 (Paternain *et al.* 2000; Alt *et al.* 2004).

KA receptor function is also modulated by extracellular ions. Activation of GluK2 containing KA receptors by ligands depends on external sodium and chloride ions (Bowie 2010), which is unique among ligand-gated ion channels. In principle, this can render KA receptor activation sensitive to fluctuations in extracellular ion concentrations (Bowie 2010), which are thought to occur during periods of high neuronal activity (Paternain *et al.* 2003).

Table 2 Selected kainate receptor antagonists

CNQX *6-cyano-7-nitroquinoxa-line-2,3-dione*	Competitive antagonist of AMPA and KA receptors
NBQX *6-nitro-7-sulfamoylbenzoquinoxaline-2,3-dione*	Competitive antagonist of AMPA and KA receptors, selective for KA receptors at low (1 μM) concentration (Rodríguez-Moreno *et al.* 1997; Mulle *et al.* 2000; Mayer *et al.* 2006).
LY382884 *(3S,4aR,6S,8aR)-6-((4-carboxyphenyl)methyl)-1,2,3,4,4a,5,6,7, 8,8a-decahydroisoquinoline-3-carboxylic acid*	Competitive antagonist selective for GluK1 but also a weak antagonist of AMPA receptors (Simmons *et al.* 1998; Bortolotto *et al.* 1999).
ACET *(S)-1-(2-amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione*	Selective antagonist of GluK1 (Dolman *et al.* 2007; Dargan *et al.* 2009).
NS-102 *6,7,8,9-Tetrahydro-5-nitro-1H-benz[g]indole-2,3-dione 3-oxime*	Antagonist selective for KA receptors but with limited solubility in physiological solutions (Verdoorn *et al.* 1994).
LY293558 *(3S, 4aR, 6R, 8aR)-6-[2-(1(2)H-tetrazol-5-yl)ethyl] decahydroisoquinoline-3-carboxylic acid*	Competitive antagonist selective for GluK1 but also antagonizes AMPA receptors (Bleakman *et al.* 1996).
LY294486 *(3SR, 4aRS, 6SR, 8aRS)-6-((((1H-tetrazol-5-yl)methyl)oxy)methyl)-1,2,3,4,4a,5,6,7,8,8a deca-hydroisoquinoline-3-carboxylic acid*	Competitive antagonist selective for GluK1 but also a weak AMPA receptor antagonist (Clarke *et al.* 1997).
LY377770	Active enantiomer of LY294486 (O'Neill *et al.* 2000).
UBP310	Antagonist of GluK1 as well as GluK3 containing KA receptors (Perrais *et al.* 2009), also antagonizes GluK2/GluK5 heteromers (Pinheiro *et al.* 2013).

1.4 Kainate receptors in immature hippocampus

1.4.1 Expression

KA receptor subunits are widely expressed throughout the CNS with heterogenous subunit specific expression patterns (Wisden and Seeburg 1993; Tölle *et al.* 1993; Bahn *et al.* 1994; Hollmann and Heinemann 1994; Bischoff *et al.* 1997; Belcher and Howe 1997; Stegenga and Kalb 2001; Ritter *et al.* 2002). The expression levels are particularly high in the early development (Bahn *et al.* 1994; Kask *et al.* 2000; Wilding and Huettner 2001; Lilliu *et al.* 2002; Ritter *et al.* 2002; Vesikansa *et al.* 2012) and patterned subunit specific changes in expression profiles take place during maturation of the brain (Bahn *et al.* 1994; Ritter *et al.* 2002; Vesikansa *et al.* 2012).

The distribution of functional KA receptors was first evaluated by assessing the binding of 3H-radiolabeled KA. In mature hippocampus, high affinity 3H-KA binding is almost exclusively restricted to CA3 with some binding at few sites of CA1 (Foster *et al.* 1981; Monaghan and Cotman 1982; Unnerstall and Wamsley 1983; Monaghan *et al.* 1986; Represa *et al.* 1987; Miller *et al.* 1990; Garcia-Ladona and Gombos 1993; Bahn *et al.* 1994). In contrast, 3H-KA binding is widespread in the early development. In rat hippocampus diffused binding is observed by embryonic day 17 (E17), the whole structure is strongly labeled by E19, and during the first two PNWs heightened expression of binding sites is detected throughout hippocampus (Miller *et al.* 1990; Garcia-Ladona and Gombos 1993; Bahn *et al.* 1994).

In situ hybridization analyses have confirmed the high expression of KA receptors during early development of rat hippocampus. The expression of subunits begins at E14 and all of them are initially expressed at high level that gradually declines during the early postnatal development (Bahn *et al.* 1994; Ritter *et al.* 2002; Vesikansa *et al.* 2012). The subunits have specific patterns and peaks in their developmental expression profile. GluK1 expression peaks on first PNW and begins to decline at P12 to a diminished but still detectable level in adults. It is initially expressed in both principal neurons and interneurons but in adults it is restricted to interneurons of CA and granule cells (Bettler *et al.* 1990; Bahn *et al.* 1994; Paternain *et al.* 2000; Ritter *et al.* 2002; Vesikansa *et al.* 2012). GluK1 exists as three splice variants GluK1a-c, of which the GluK1b is preferentially expressed in immature interneurons and GluK1c in pyramidal neurons (Vesikansa *et al.* 2012). GluK2 expression peaks in the end of the first PNW. It is preferentially expressed in pyramidal neuron layer with decreasing CA3 to CA1 gradient and low expression in dentate gyrus (Ritter *et al.* 2002). GluK3 expression peaks at birth and declines relatively steadily thereafter. It is preferentially expressed in the granule cell layer (Ritter *et al.* 2002). GluK4 expression peaks transiently on first PNW. It is initially expressed at all principal cell layers but in adults it is restricted to CA3 pyramidal neurons and some granule cells (Werner *et al.* 1991; Herb *et al.* 1992; Bahn *et al.* 1994; Kask *et al.* 2000; Ritter *et al.* 2002; Darstein *et al.* 2003; Vesikansa *et al.* 2012). Finally, GluK5 expression declines from birth to adult levels by second PNW; it is expressed in all hippocampal fields but preferentially at CA region, especially CA3 (Herb *et al.* 1992; Wisden and Seeburg 1993; Ritter *et al.* 2002; Vesikansa *et al.* 2012).

In addition to analysis of gene expression, certain KA receptor subunits have been localized with antibodies at postsynaptic membranes and in dendritic spines of pyramidal neurons in CA3 and CA1 regions of mature hippocampus (Huntley *et al.* 1993; Good *et al.* 1993; Petralia *et al.* 1994; Siegel *et al.* 1995). However, the cur-

rently available antibodies are quite unspecific, which limits the interpretations of these studies. Most of our knowledge on the subcellular localization and trafficking of KA receptor subunits relies on studies using tagged recombinant receptor subunits in neuronal culture. These studies have revealed that KA receptors are targeted to diverse subcellular domains where they serve distinct functional roles (Huettner 2003; Isaac *et al.* 2004; Jaskolski *et al.* 2005; Pinheiro and Mulle 2006; Contractor *et al.* 2011; Vesikansa *et al.* 2012; Lerma and Marques 2013).

1.4.2 Functions

While many KA receptor mediated functions have been described across the mature CNS, the peak of KA receptor expression in early development suggests that they have a role in the development of neuronal cirquits. In support for this assumption, KA receptors have been shown to be involved in the formation of glutamatergic synapses (Tashiro *et al.* 2003; Vesikansa *et al.* 2007). In the early stages of contact formation, activation of presynaptic KA receptors regulates motility of growth cones in primary cultures of hippocampal neurons (Ibarretxe *et al.* 2007) and mossy fiber axonal filopodia in organotypic cultures (Tashiro *et al.* 2003). Motility of these developmental structures is thought to enable the exploration of the synaptic targets. In mossy fibers, KA receptors appear to facilitate the formation and stabilisation of synaptic contacts (Tashiro *et al.* 2003). In cultured hippocampal slices pharmacological activation of GluK1-containing KA receptors leads to increased number of functional glutamatergic synapses whereas block of these receptors reduces the number of synapses (Vesikansa *et al.* 2007). Apart from this role in synapse formation, increasing data has uncovered the physiological functions of KA receptors during the early development of synaptic transmission, which are summarized in figure 3 on page 15 and discussed in more detail below.

Although KA receptors mediate excitatory postsynaptic currents (EPSCs) like other iGlu receptors, they do so only at surprisingly few central synapses compared to the wide expression of KA receptor subunits across CNS (Lerma and Marques 2013). Especially during the early postnatal development, KA receptor mediated postsynaptic currents appear to be virtually nonexistent in hippocampus. Where observed, KA receptors mediated EPSCs have slower kinetics and smaller amplitudes than those mediated by AMPA or NMDA receptors (Castillo *et al.* 1997; Vignes and Collingridge 1997; Cossart *et al.* 1998; Frerking *et al.* 1998; DeVries and Schwartz 1999; Li *et al.* 1999; Kidd and Isaac 1999) and they are believed to provide integrative capacity to glutamatergic synapses that express them (Frerking and Ohliger-Frerking 2002; Miyata and Imoto 2006; Goldin *et al.* 2007;

Barberis *et al.* 2008; Sachidhanandam *et al.* 2009; Perrais *et al.* 2010; Artinian *et al.* 2011; Pinheiro *et al.* 2013).

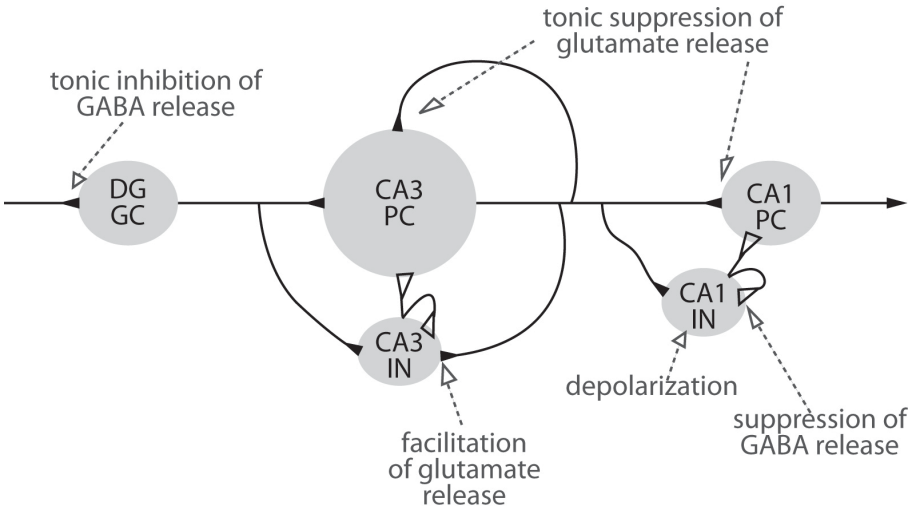

Figure 3 Summary of kainate receptor functions in the immature hippocampus
Reported KA receptor mediated actions located in a simplified wiring diagram of the hippocampus. Presynaptic KA receptors suppress release of GABA from mossy fibers (Caiati *et al.* 2010). Presynaptic KA receptors suppress GABAergic synapses onto CA1 interneurons (Maingret *et al.* 2005). Presynaptic KA receptors suppress release of glutamate from synapses onto pyramidal neuron in both CA3 (Lauri *et al.* 2005) and CA1 (Lauri *et al.* 2006). Presynaptic KA receptors enhance glutamate release from synapses onto CA3 interneurons (Lauri *et al.* 2005) Somatodendritic KA receptors depolarize interneurons in CA1 (Maingret *et al.* 2005). DG, dentate gyrus; GC, granule cell; CA1/CA3, cornu ammonis 1/3; PC, pyramidal cell; IN, interneurons

KA receptor mediated EPSCs were demonstrated for the first time in mature mossy fiber synapses onto CA3 pyramidal neurons (Castillo *et al.* 1997; Vignes and Collingridge 1997). However, a KA receptor mediated component of postsynaptic response does not emerge in this synapse until the end of first PNW after which it matures to attain adult-like properties, characterized by small amplitude and slow kinetics, by the 3rd PNW (Marchal and Mulle 2004). The maturation of mossy fiber synapse function is paralleled by increasing susceptibility of CA3 pyramidal neurons to depolarization by KA, which is due to activation of these postsynaptic KA receptors (Mulle *et al.* 1998; Khalilov *et al.* 1999). It appears that KA receptors are associated with the final maturation of mossy fiber transmission as both the

functional and morphological development of mossy fibre to CA3 pyramidal neuron synapses is delayed in transgenic mice deficient of the GluK2 subunit (Lanore *et al.* 2012). KA receptors mediate postsynaptic currents also in the interneurons of mature CA1 (Frerking *et al.* 1998; Cossart *et al.* 2002; Goldin *et al.* 2007; Wondolowski and Frerking 2009; Clarke *et al.* 2012) but no synaptically activated KA receptor mediated postsynaptic currents have been described in immature interneurons thus far. However, functional KA receptors are present at least in interneurons of immature CA1, in which pharmacologial activation of KA receptors induces a depolarising current (Maingret *et al.* 2005). Thus, postsynaptic KA receptors mediated currents appear to be a feature of mature glutamatergic synapses and the role of KA receptors as mediators of excitatory currents in hippocampus is seemingly minimal in the early development.

In contrast to postsynaptic KA receptors, presynaptic KA receptors appear to be a common feature of immature glutamatergic synapses in the hippocampus. KA receptors at or nearby presynaptic terminals regulate transmitter release in many regions of CNS by acting as autoreceptors or heteroreceptors that modulate transmitter release from glutamatergic or GABAergic terminals, respectively (Kullmann 2001; Lerma 2003; Huettner 2003; Pinheiro and Mulle 2008; Lauri and Taira 2012). In the immature hippocampus, activation of presynaptic KA receptors suppresses transmitter release from glutamatergic synaptic terminals onto pyramidal neurons in both CA3 and CA1 (Lauri *et al.* 2005; Lauri *et al.* 2006; Sallert *et al.* 2007) and facilitates release from glutamatergic terminals onto interneurons at CA3 (Lauri *et al.* 2005). In addition, it suppresses transmitter release from nascent mossy fiber terminals onto CA3 pyramidal neurons (Caiati *et al.* 2010) and from GABAergic terminals onto CA1 pyramidal neurons (Maingret *et al.* 2005). In all of these cases the KA receptors responsible for these actions contain subunit GluK1 (Lauri *et al.* 2005; Maingret *et al.* 2005; Lauri *et al.* 2006; Caiati *et al.* 2010). KA receptors are endogenously activated by ambient extracellular glutamate at immature glutamatergic synapses onto CA1 and CA3 pyramidal neurons and maintain a low release probability (Lauri *et al.* 2005; Lauri *et al.* 2006). This tonic KA receptor activation is developmentally downregulated and no longer observed by the end of second PNW (Lauri *et al.* 2005; Lauri *et al.* 2006). In CA1, the tonic activity of KA receptors is rapidly switched off in response to experimental induction of long-term potentiation by external stimulation of Schaffer collaterals by a mechanism controlled by the brain-derived neurotrophic factor (BDNF) (Lauri *et al.* 2006; Sallert *et al.* 2009). Tonic activation appears to be due to high agonist affinity of heteromeric receptors conferred by subunit GluK4, which co-localizes with GluK1c in pyramidal neurons (Vesikansa *et al.* 2012). Although in many cases

the exact molecular mechanism behind KA receptor-mediated regulation of transmitter release is unknown, there is evidence for both ionotropic and metabotropic mechanisms controlling the influx of calcium into presynaptic terminal (Pinheiro and Mulle 2008; Lauri and Taira 2012). In immature hippocampus, the suppression of glutamate release from CA3 pyramidal neurons and GABA release from granule cells is mediated metabotropically via G-protein coupled signaling pathway as it can be prevented by pertussis toxin (Lauri *et al.* 2005; Lauri *et al.* 2006; Caiati *et al.* 2010). In contrast, the facilitation of glutamate release from synapses onto interneurons at CA3 by GluK1-containing KA receptors is not dependent on G-protein signaling (Lauri *et al.* 2005). Rather, it may be due to direct influx of calcium via presynaptic calcium permeable KA receptors akin to the ionotropic presynaptic facilitation described in mature mossy fibers (Bernard *et al.* 1999; Lee *et al.* 2001; Lauri *et al.* 2003; Pinheiro *et al.* 2007). The regulation of transmitter release by KA receptors has been associated with dynamic short-term regulation as well as long-term plasticity of synaptic strenght (Bortolotto *et al.* 1999; Schmitz *et al.* 2001; Lauri *et al.* 2001; Lauri *et al.* 2001; Mellor 2006; Sihra *et al.* 2013). In immature hippocampus, tonic activation of presynaptic KA receptors sets the dynamic properties of developing synapses to favor transmission during frequent afferent activation (Lauri *et al.* 2006). Additionally, tonic KA receptor activity is critical for formation and maturation of CA3-CA1 glutamatergic synapses (Vesikansa *et al.* 2007).

Axonally located neurotransmitter receptors may influence the function of axons (Sasaki *et al.* 2011; Bucher and Goaillard 2011). KA receptor mediated modulation of axonal function has been documented in mature hippocampus for the mossy fibre input to CA3 (Kamiya and Ozawa 2000; Schmitz *et al.* 2000; Contractor *et al.* 2003) and for GABAergic interneuronal input to CA1 pyramidal neurons (Semyanov and Kullmann 2001). In immature hippocampus, KA receptors sensitive to GluK1 selective drugs regulate axonal excitability of interneurons at CA1 (Maingret *et al.* 2005). Additionally, KA receptors modulate excitability of thalamocortical axons to immature barrel cortex (Jouhanneau *et al.* 2011). The exact mechanism underlying these actions are unknown.

Metabotropic modulation of neuronal excitability by KA receptors has been demonstrated in pyramidal neurons of mature CA3 and CA1 where somatodendritic KA receptors control neuronal excitability by regulating calcium activated afterhyperpolarizing potassium currents (I_{AHP}s) (Melyan *et al.* 2002; Melyan *et al.* 2004; Fisahn 2005; Ruiz *et al.* 2005; Fernandes *et al.* 2009). I_{AHP}s are responsible for the afterhyperpolarizing potential (AHP) that follows bursts of action potentials and is an important regulator of neuronal firing dynamics (Gho *et al.* 1986; Ashwood *et*

al. 1986). In both mature and immature CA3 pyramidal neurons slow AHP (sAHP) is responsible for the accommodating firing response to depolarizing current injection and greatly limits the excitability of these neurons (Sipilä *et al.* 2006). In mature hippocampus, interneuronal firing is suppressed by afterhyperpolarization of medium duration (mAHP) mediated by apamin-sensitive calcium-activated SK potassium channels (Zhang and McBain 1995; Savić *et al.* 2001). Whether KA receptors modulate the excitability of neurons in neonatal hippocampus is not known.

1.4.3 Role in network activity

The many functions of KA receptors make them suitable for control of neuronal networks. Accordingly, pharmacological activation of KA receptors may induce epileptic activity in the hippocampus and nearby structures *in vivo* (Nadler 1981; Tremblay *et al.* 1984; Ben-Ari 1985; Ben-Ari and Cossart 2000) and trigger synchronous oscillations at gamma-frequency range in hippocampal slices (Ben-Ari and Cossart 2000; Fisahn *et al.* 2004).

The generation and control of synchronous neuronal network activity are thought to be crucial for functions of the mature brain (Buzsáki and Draguhn 2004). Primordial forms of synchronized activity precede the more organized mature activity pattern in most, if not all, regions of the developing CNS (O'Donovan 1999; Ben-Ari 2001; Corlew *et al.* 2004; McCabe *et al.* 2006; Pangratz-Fuehrer *et al.* 2007; Egorov and Draguhn 2013). In the immature hippocampus, intrinsic network activity presents as irregularly recurring bursts of synchronous neuronal activity that manifest in individual neurons as prolonged (200-600 ms) depolarization with superimposed action potentials (Ben-Ari *et al.* 1989; Leinekugel *et al.* 1998; Leinekugel *et al.* 2002; Mohajerani and Cherubini 2005). The bursts have often been referred to as giant depolarizing potentials (GDPs) after their manifestation in initial intracellular recordings (Ben-Ari *et al.* 1989). There is a constant asynchronous activity in the network and burst initiation occurs once a certain threshold of synaptic transmission and neuronal firing is exceeded (Menendez de la Prida and Sanchez-Andres 1999; Menendez de la Prida *et al.* 2006). All hippocampal regions can generate network bursts but they are most often initiated at CA3 (Strata *et al.* 1997; Garaschuk *et al.* 1998; Menendez de la Prida *et al.* 1998; Bolea *et al.* 1999; Menendez de la Prida and Sanchez-Andres 2000; Bolea *et al.* 2006; Marissal *et al.* 2012). Extensive pharmacological analysis has revealed that the bursts are generated by complex synaptic interaction of glutamatergic and GABAergic neurons. Excitation mediated by AMPA receptors is essential for burst generation as the bursts are prevented when AMPA receptors are blocked (Bolea *et al.* 1999; Sipilä *et al.*

2005). Burst initiation depends to a great deal on activity of CA3 pyramidal neurons apparently because a subpopulation of them preferably fires action potentials in bursts (Sipilä *et al.* 2005; Sipilä *et al.* 2006). Depolarizing currents mediated by GABAA receptors dominate the synaptic input to immature neurons (Lauri *et al.* 2005) and have a permissive role in burst generation by promoting the firing of pyramidal neurons and depolarization-dependent currents through NMDA receptors (Ben-Ari *et al.* 2007). Additionally, some interneurons are able to efficiently synchronize large neuron population during network bursts (Bonifazi *et al.* 2009). However, activation of GABAA receptors is not required for bursts generation as the network of immature hippocampus is able to generate spontaneous synaptically driven network bursts when GABAA receptors are blocked (Khalilov *et al.* 1999).

The depolarization during GDPs results in the activation of voltage-dependent calcium channels and NMDA receptors resulting in subsequent elevation of intracellular calcium (Leinekugel *et al.* 1997). The calcium waves are crucial for the structural refinement of the neuronal connectivity and the establishment of the adult neuronal circuit (Garaschuk *et al.* 2000; Voigt *et al.* 2005). Accordingly, bursts are required for the proper maturation of neurons and their synaptic connections and perturbations in early network activities may lead to disturbances in development (Holmes and Ben-Ari 1998; Lauri *et al.* 2003; Colin-Le Brun *et al.* 2004; Huupponen *et al.* 2007; Costa *et al.* 2010; Huupponen *et al.* 2013). For example, experimental lowering of network burst occurrence is associated with increased seizure susceptibility later on (Vargas *et al.* 2013).

Presynaptic KA receptors in hippocampus display developmentally restricted functions that allow them to control dynamic properties of glutamatergic synapses and modulate interactions of glutamatergic and GABAergic neurons. Accordingly, the network bursts in the immature hippocampus are highly sensitive to the level of KA receptor activation (Lauri *et al.* 2005). Both activation and block of GluK1-containing KA receptors with ATPA and LY382884, respectively, decrease the occurrence of network bursts (Lauri *et al.* 2005). This network effect of KA receptors appears to be mainly due to altered modulation of glutamate release as activation of KA receptors suppresses glutamate release from synapses onto pyramidal neurons and facilitates it from synapses onto interneurons in the CA3 region (Lauri *et al.* 2005). In the developing hippocampus, most of the endogenous glutamatergic activity occurs as high-frequency bursts during the intermittent periods of synchronous activity of the network (Lamsa *et al.* 2000). Tonically active GluK1-containing KA receptors suppress transmitter release from presynaptic terminals of all of the synapses in the classic trisynaptic pathway (Lauri *et al.* 2005; Lauri *et al.* 2006; Caiati *et al.* 2010). Because of this, presynaptic KA receptors act to suppress back-

ground activity and overexcitability and confer immature synapses of the trisynaptic circuit to allow transmission at high frequency during network bursts. It has been shown that asynchronous activity promotes silencing of immature glutamatergic synapses (Xiao *et al.* 2004) while synchronous activity increases synaptic efficacy and promotes synapse stabilization (Kasyanov *et al.* 2004; Mohajerani *et al.* 2007; Sivakumaran *et al.* 2009; Hanse *et al.* 2009; Huupponen *et al.* 2013). Taking this into account, it has been suggested that presynaptic KA receptors protect immature synapses from weakening and elimination (Lauri and Taira 2012). It is also interesting to note, that both experimental increase in the efficacy of and activity-dependent downregulation of tonic presynaptic KA receptor activity in CA3 to CA1 pyramidal neuron synapses depends on BDNF signaling (Mohajerani *et al.* 2007; Sallert *et al.* 2009).

Parenteral administration of subconvulsive doses of domoate in neonatal rats leads to disturbances in hippocampal functions later in life (Doucette *et al.* 2003; Doucette *et al.* 2004; Adams *et al.* 2008; Burt *et al.* 2008; Adams *et al.* 2009; Ryan *et al.* 2011; Marriott *et al.* 2012). Although the exact mechanisms underlying this remain unclear, alterations in the activity patterns guiding the proper maturation of hippocampal circuitry could explain the developmental disturbances by low doses of KA receptor agonists (Lauri *et al.* 2003; Colin-Le Brun *et al.* 2004; Huupponen *et al.* 2013).

The susceptibility to induction of epileptic activity by KA develops gradually and is generally lower in the immature brain (Nitecka *et al.* 1984; Tremblay *et al.* 1984; Stafstrom *et al.* 1992). Hippocampus, however, displays sensitivity to KA already at early stages of development and sufficiently high doses of exogenously applicated KA receptor agonist induce epileptic activity in the developing hippocampus (Tremblay *et al.* 1984; Stafstrom *et al.* 1992; Khalilov *et al.* 1999) agreeing with the high expression of KA binding sites and KA receptor subunits in the developing hippocampus. Studies *in vitro* have revealed that KA induced seizures are generated in the CA3 region and the convulsive action of KA stems from the GluK2 subunit dependent depolarization of CA3 pyramidal neurons both in mature and immature hippocampus (Westbrook and Lothman 1983; Khalilov *et al.* 1999; Ben-Ari and Cossart 2000; Mulle *et al.* 2000; Fisahn *et al.* 2004). During postnatal development, the susceptibility of hippocampus to KA-induced epileptic activity increases gradually in parallel with the emergence of KA receptor mediated postsynaptic currents in the CA3 pyramidal neurons (Khalilov *et al.* 1999). Although KA-induced paroxysmal discharges in the early development do not cause overt morphological consequences (Nitecka *et al.* 1984; Stafstrom *et al.* 1992), they lead to disturbances in hippocampal learning and plasticity later in life (Lynch *et al.* 2000).

1.5 Ethanol and the developing hippocampus

Consumption of ethanol during pregnancy can produce a wide range of cognitive, behavioral and physical anomalies collectively referred to as fetal alcohol spectrum disorder (FASD). FASD ranges from full blown fetal alcohol syndrome (FAS) with morphological anomalies accompanying severe mental retardation to alcohol-related neurodevelopmental disorder (ARND) manifesting as more subtle deficits in cognitive function (Jones and Smith 1975; Warren and Foudin 2001; Sokol *et al.* 2003). Neurological dysfunctions are common consequences of developmental ethanol exposure and include deficits in learning and memory functions that are thought to be a consequence of damage to the hippocampal formation (Berman and Hannigan 2000; Mattson *et al.* 2001). Accordingly, structural and functional alterations in the hippocampus have been demonstrated in animal models of developmental ethanol exposure (West *et al.* 1981; Farr *et al.* 1988; Farr *et al.* 1988; Swartzwelder *et al.* 1988; Tanaka *et al.* 1991; Sakata-Haga *et al.* 2003; Livy *et al.* 2003).

The mechanisms underlying hippocampal abnormalities associated with developmental ethanol exposure remain largely unclear (Goodlett and Horn 2001). However, the developing nervous system is especially vulnerable to the effects of ethanol during the period of intense synaptogenesis (Dikranian *et al.* 2005). Curiously, ethanol enhances spontaneous network bursting that dominates the activity in immature hippocampus during this period (Galindo *et al.* 2005). The enhancement persists during prolonged ethanol exposure (Galindo and Valenzuela 2006) and thus exposes the neurons in the network to increased calcium transients (Leinekugel *et al.* 1997; Galindo *et al.* 2005) and possibly to subsequent altrations in neuronal gene expression, development of synaptic connections, and regulation of neuronal apoptosis (Lauri *et al.* 2003; Aguado *et al.* 2003; Nuñez *et al.* 2003; Nuñez *et al.* 2003; Colin-Le Brun *et al.* 2004) that could lead to hippocampal dysfunction. In support for this, exposure to ethanol during the third semester equivalent of human gestation in the rat (P4-9) is known to lead to deficits in hippocampal functions (Wozniak *et al.* 2004; Popović *et al.* 2006) and structure (Livy *et al.* 2003). Some effects have been reported to appear at concentrations as low as those that can be achieved in blood after the ingestion of just 1–2 drinks (5–10 mM) (Parker *et al.* 1981; Carta *et al.* 2003).

In the adult brain, intoxicating and reinforcing actions of ethanol are thought to be the result of multiple effects of ethanol on synaptic transmission, involving enhancement of $GABA_A$ receptors and suppression of NMDA receptors (Lovinger 1997; Faingold *et al.* 1998; Tsai and Coyle 1998). Perhaps the most wide-

ly agreed effect of ethanol in the brain (Weiner and Valenzuela 2006) is promotion of GABAergic transmission both presynaptically and postsynaptically (Carta *et al.* 2003; Sanna *et al.* 2004; Ariwodola and Weiner 2004; Wu *et al.* 2005; Weiner *et al.* 2005). In the immature hippocampus, ethanol is shown to increase GABAergic transmission between interneurons at CA3, which might contribute to the enhancement of the early hippocampal network activity by ethanol (Galindo *et al.* 2005). Ethanol does not seem to affect immature $GABA_A$ receptors directly but likely acts presynaptically. In agreement, it has been observed that the susceptibility of GABA-A receptors to enhancement by ethanol is low in neonates and increases with age (Li *et al.* 2003; Li *et al.* 2006) (but see Everett *et al.* 2012).

Ethanol has also been shown to affect glutamate release in the hippocampus (Maldve *et al.* 2004; Hendricson *et al.* 2004; Mameli *et al.* 2005; Mameli and Valenzuela 2006) as well as other regions of CNS (Ziskind-Conhaim *et al.* 2003; Roberto *et al.* 2004). In the neonatal rat hippocampus, acute exposure to ethanol suppresses glutamate release from synapses on CA3 pyramidal neurons via inhibition of N-type presynaptic VGCC (Mameli *et al.* 2005). In contrast, release from glutamatergic synapses onto interneurons at CA3 is shown to be either unaffected or enhanced by ethanol (Galindo *et al.* 2005) while release to CA1 pyramidal neurons is reported to be enhanced (Mameli and Valenzuela 2006). Repeated ethanol administration leads to increased extracellular glutamate levels in the mature hippocampus (Chefer *et al.* 2011) but changes in ambient extracellular glutamate levels in the immature hippocampus have not been assessed.

In addition to effects on glutamate release, ethanol is able to directly inhibit ionotropic glutamate receptors (Lovinger 1997; Faingold *et al.* 1998; Tsai and Coyle 1998; Möykkynen and Korpi 2012). The sensitivity of ionotropic glutamate receptors to ethanol varies by receptor type, by brain region as well as developmentally (Swartzwelder *et al.* 1995; Li *et al.* 2002). Although it is widely accepted that inhibition of NMDA receptors is one of the major action of ethanol in the mature brain (Lovinger 1997), sensitivity of NMDA receptors to ethanol appears to be significantly lower in the early development. Accordingly, in rodent hippocampus NMDA receptors are acutely inhibited by ethanol in juvenile and mature (Weiner *et al.* 1999) but relatively insensitive to ethanol in the neonatal stage (Gordey *et al.* 2001; Mameli *et al.* 2005; Puglia and Valenzuela 2010). Despite lack of direct mechanistic explanation, this may be due to changes in NMDA receptor subunit composition, phosphorylation state, or association with interacting proteins (Chu *et al.* 2002; Yasuda *et al.* 2003; Elias *et al.* 2008). Compared to NMDA receptors, the sensitivity of AMPA receptors is apparently regulated in an opposite manner as ethanol inhibits postsynaptic AMPA receptors in the neonatal CA1 (Mameli *et al.*

2005; Puglia and Valenzuela 2010) but not in the juvenile or mature hippocampus (Lovinger *et al.* 1989; Lovinger *et al.* 1990; Weiner *et al.* 1999; Carta *et al.* 2003; Ariwodola *et al.* 2003; Hendricson *et al.* 2003).

KA receptors formed by homomeric or heteromeric combinations of recombinantly expressed subunits GluK1, GluK2, GluK4, and GluK5 are inhibited to a fairly similar degree by ethanol (Dildy-Mayfield and Harris 1992; Dildy-Mayfield and Harris 1995; Valenzuela *et al.* 1998; Valenzuela and Cardoso 1999). Suppression of synaptic currents mediated by native KA receptors has been observed in the mature hippocampus (Weiner *et al.* 1999; Crowder *et al.* 2002; Carta *et al.* 2003), amygdala (Läck *et al.* 2008; Läck *et al.* 2009) and cerebellum (Valenzuela *et al.* 1998). No studies have addressed the functional consequences of acute ethanol exposure to KA receptors in the neonatal brain. However, prenatal ethanol exposure has been reported to decrease KA binding sites in CA3 (Farr *et al.* 1988).

2 Aims

The aims of this thesis are to study (1) the consequences of activation of KA receptors with high agonist affinity for the spontaneous network activity in the developing hippocampus, (2) how KA receptors containing subunit GluK1 influence the function of interneurons and how this affects hippocampal synchronization, and (3) whether GluK1-containing KA receptors are involved in the network effects of ethanol in the immature CA3.

3 Materials and methods

3.1 Preparation of acute hippocampal slices

All the experiments in this work were conducted on acutely prepared slices of rodent hippocampus. Wistar rats were used in studies I and III and wild type C57/BL/6 mice in Study II. All experiments were done in accordance with the guidelines given by the ethics committee for animal research at the University of Helsinki. The hippocampi were dissected with a vibratome (Vibratome, www.vibratome.com) to 350 to 400-µm-thick slices cut parasagittally in an ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM) 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26 $NaHCO_3$, 15 D-glucose, 2 $CaCl_2$ (saturated with 5% CO_2/95% O_2) after which the slices were stored in aCSF at room temperature for at least 1 hour before recordings. The recordings were conducted within 6 hours of slice preparation.

3.2 Electrophysiological recordings

For recordings, slices were placed on a nylon mesh (blind setup) or a glass plate (visualized setup) in a submerged recording chamber and superfused at a rate of 1–2 ml/min with aCSF resulting in near complete change of perfusate in the recording chamber within approximately 2-3 minute. All the experiments were conducted at 32 °C.

In this work, all experiments were done using patch clamp recording technique. In this electrophysiological method, an electrolyte filled glass electrode used for recording forms an electrically tight (resistance in the order of gigaohms) seal with the target membrane. The "patch" refers to the circular tightly sealed membrane region in the tip of the electrode. Electrodes for recordings consisted of an AgCl-coated Ag wire inside a glass micropipette filled with electrolyte solution. The micropipettes were fabricated from a borosilicate glass capillary (outer/inner diameter 1.5/0.86 mm) with a Narishige PC-10 dual-stage puller. The intracellular electrolyte solution varied depending on the application (see below). Electrodes had a resistance of 3 – 6 MΩ.

The recording electrode can be targeted to the target neuron with either "blind" or visually guided method. Both were used in this study. In both methods, recording electrode is advanced within the tissue while maintaining a positive pressure in the electrode to maintain the tip clean for optimal seal formation. In the blind method, target neuron is located based on the sudden increase of resistance in the tip of the electrode upon contact to cellular membrane. It lends itself well for regions with high density of neuron somata and in this work it was utilized

for most CA3 pyramidal neurons and all dentate granule cells because the somata of these neurons are tightly packed in easily identifiable layers. In the visually guided method, target neurons are visually identified under infrared illumination combined to differential interference contrast or Dodt gradient optics and the electrode is targeted on them under high resolution microscope. This method was used for all interneurons and some CA3 pyramidal neurons. Interneurons were selected for recordings based on their localization in the CA3 *s. lucidum*, multipolar or bipolar shape of the cell soma, and perpendicular orientation of the soma to the pyramidal layer.

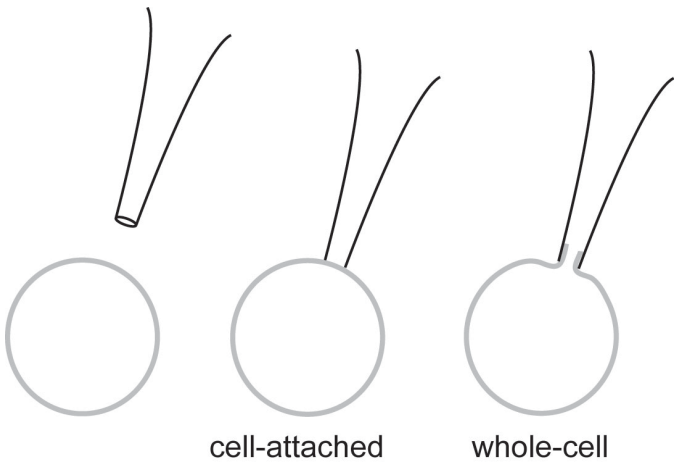

Figure 4 Patch-clamp recording configurations
Schematic representation of patch clamp recording configurations.

Left A recording electrode is approaching a target cell. *Mlddle,* Once the tip of the recording electrode makes contact with the membrane of the target cell, it forms by a not yet satisfactorily explained molecular interaction of the membrane and the glass surface an electrically tight contact, "a seal".

Right the membrane enclosed by tip of the electrode may be ruptured by application of negative pressure in the electrode to achieve electrical continuity with the intracellular compartment of the cell and electrode filling solution.

The patch recordings are divided into two main configurations: in the on-cell (or cell attached) configurations the cell membrane within the patch remains intact, in whole-cell configurations it is ruptured to gain electrical continuity between the electrolyte solution in the electrode and the interior of the cell (see figure 4). In the studies included in this thesis, on-cell patch recordings were used to monitor spontaneous firing of action potentials of neurons. The amplifier (see below) was set to voltage clamp circuit in the track-mode, which dynamically adjusts the

offset potential so that the current in the electrode averages to zero thus minimizing the influence of the recording artifacts on cell activity (Perkins 2006). Despite introducing some distortion to the recorded signal it allows reliable temporal detection of electrical events such as action potentials with amplitudes clearly exceeding background noise. Whole-cell configuration was used to record intracellular currents or membrane potential with voltage clamp and current-clamp, respectively. In voltage clamp the membrane voltage is controlled at set value by injecting transmembrane current. Voltage clamp allows the measurement of currents flowing through the cell membrane and is well suited for monitoring synaptic currents. In current-clamp a known current is applied and the changes in membrane potential are measured. This type of experiment mimics the physiological state of the cell and allows monitoring of voltage-sensitive ion channel activity in the membrane.

Spontaneous action currents were recorded in cell-attached configuration with aCSF-filled electrodes, and the amplifier was set to "track" mode (Perkins 2006). In other recordings, the electrodes were filled with solutions the composition which depended on the application (Table 3 on page 28). In all cases, except on-cell recordings, magnesium salt of adenosine triphosphate (ATP-Mg) and sodium salt of guanosine triphosphate (GTP-Na) were included in the solution to support intracellular metabolism, either EGTA or BAPTA to buffer intracellular calcium, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to buffer intracellular pH. The pH of the electrode filling solutions was adjusted to 7.20–7.30 with cesium or potassium hydroxide (CsOH and KOH, respectively, both at 1 M concentration) depending on the main cation in the solution. The osmolarities were 275–285 mOsm/l. In some voltage clamp recordings, chloride salt of the membrane impermeable sodium channel blocker N-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium (QX314) was included in the electrode filling solution. In Studies I and III, all measured potentials were corrected for calculated liquid junction potentials (LJPs; 6-14 mV) that occur between electrolyte solutions of different composition (Barry 1994). LJPs were not corrected in Study II.

The electrodes were connected to an amplifier (model Axopatch 200B or Multiclamp 700A from Molecular Devices, www.moleculardevices.com) via headstage resistor (CV 203BU) for amplification and low-pass filtering (at 1 −5 kHz) of the recorded signal. The signal was digitized with Digidata 1322A digitizer (Molecular Devices) and recorded with an acquisition software (Clampex from Molecular Devices or the MiniAnalysis software from Synaptosoft) at sampling frequency ca. 2.5 times the low-pass filter frequency. The bathing solution was grounded by a AgCl-coated Ag wire.

Pharmacological agents that were used in the Studies I-III are listed in table 4 on page 29. In some experiments, the concentration of extracellular glutamate was reduced utilising an enzymatic "scavenger", a combination of pyruvate with an enzyme glutamic puryvic transaminase (GPT) catalyzing a transamination reaction converting glutamate and pyruvate to α-ketoglutarate and alanine. The elevated concentration of pyruvate drives the metabolization of endogenous extracellular glutamate to α-ketoglutarate (Overstreet *et al.* 1997; Min *et al.* 1998).

Table 3 Electrode filling solutions

composition (in mM)	**used for**	**used in**
Cs-methanesulfonate 90 CsCl 30 HEPES 10 EGTA 10 ATP-Mg 4 GTP-Na 0,3	Voltage clamp recordings of synaptic currents from rat CA3 pyramidal neurons to observe network acitivity patterns.	Study I
Cs-methanesulfonate 115 HEPES 10 EGTA or BAPTA 10 ATP-Mg 4 GTP-Na 0.3	Voltage clamp recordings of glutamatergic synaptic currents from rat CA3 pyramidal neurons and *s. lucidum* interneurons.	Study I
K-gluconate 110 KCl 20 HEPES 10 EGTA 0.1 ATP-Mg 4 GTP-Na 0.3	Current-clamp recordings of firing from rat CA3 pyramidal neurons and *s. lucidum* interneurons.	Study I
K-gluconate 130 NaCl 8 HEPES 10 EGTA 0.4 Mg-ATP 4 Na-GTP 0.3	Current-clamp recordings of firing of murine *s. lucidum* interneurons.	Study II
K-gluconate 130 KCl 10 HEPES 10 EGTA 0.2 Mg-ATP 4 Na-GTP 0.3	Voltage clamp recordings of medium afterhyperpolarizing current (ImAHP) from murine *s. lucidum* interneurons.	Study II
K-gluconate 135 HEPES 10 EGTA 5 Mg-ATP 4 Na-GTP 0.5 KCl 2 $Ca(OH)_2$ 2	Voltage clamp recordings of synaptic currents from rat CA3 pyramidal neurons and murine *s. lucidum* interneurons. The low chloride concentration allows simultaneous recording and discrimination of glutamatergic and GABAergic currents when transmembrane voltage is clamped around -60 mV.	Study II and III

Table 4 List of pharmacological tools used in this study. All compounds were from Tocris or Sigma, except for LY382884, which was generously provided by Eli Lilly.

Drug	Action	Concentration	Used in Study
ACET	GluK1 antagonist	200 nM	III
ATPA	GluK1 agonist	1 μM	II
CGP 55845	$GABA_B$ receptor antagonist	1 μM	I
D-AP5	NMDA receptor antagonist	50 μM	I
DCG IV	mGluR-II receptor antagonist	1 μM	I
domoate	KA receptor agonist	5 nM	I
GYKI53655	AMPA receptor antagonist	50 μM	I
kainate	agonist of KA and AMPA receptors	25-100 nM	I
LY382884	GluK1 antagonist	10 μM	I,II
NBQX	AMPA/KA receptor antagonist	50 μM	I
picrotoxin	$GABA_A$ receptor antagonist	100 μM	I and II
TTX	A blocker of voltate-gated sodium channels	1 μM	I, II

3.3 Data analysis

Spontaneous events were detected with either Clampfit (Molecular Devices) or MiniAnalysis (Synaptosoft). Detected events were verified visually. Current-clamp recordings were analyzed in Igor Pro (WaveMetrics; www. wavemetrics.com) with the Neuromatic module by Jason Rothman (www.neuromatic.thinkrandom.com).

All average data are reported as mean ± SEM, together with the number of recordings (n). Effects of drugs are quantified as percentage ratio over baseline conditions. Statistical significance of the difference between means was determined using unpaired or paired Student's two-tailed t-test. ANOVA following Tukey's honestly significant difference (HSD) comparison was used for assessing the significance of difference between multiple means. P values < 0.05 were considered statistically significant.

4 Results

4.1 Activation of high-affinity kainate receptors promotes network burst generation via enhancing spontaneous spiking of immature CA3 pyramidal neurons (I)

The Study I aimed at characterizing the consequences of selective activation of the population of KA receptors with high agonist affinity in CA3 region of neonatal (P3-5) rat hippocampus. In order to specifically activate high-affinity KA receptors, KA was bath-applied at concentration of 50 nM, which is close to the dissociation constant of native hippocampal KA receptors (Straub *et al.* 2011).

Spontaneous activity of individual neurons was assessed by recording neuronal firing in on-cell configuration. In these recordings, $GABA_A$, NMDA and AMPA receptors were blocked with picrotoxin (100 μM), D-AP5 (50 μM), and GYKI53655 (50 μM), respectively (Fig. 5 on p. 33). Both CA3 pyramidal neurons and interneurons in *stratum lucidum* displayed spontaneous firing. Activation of high-affinity KA receptors with 50 nM KA reversibly increased firing of CA3 pyramidal neurons by 440 ± 170% but had no effect on firing of the interneurons. Application of an unselective AMPA/KA receptor blocker NBQX decreased spike frequency in CA3 pyramidal neurons by 77±10% suggesting that high-affinity KA receptors are endogenously activated. Separate current clamp recordings of CA3 pyramidal cells and interneurons in whole-cell configuration revealed that 50 nM KA neither caused any detectable membrane currents or changes in input resistance nor affected the response to injection of depolarizing current. The results indicate that activation of high-affinity KA receptors in the immature hippocampus specifically enhances the activity of CA3 pyramidal neurons.

Local pyramidal neurons provide the major glutamatergic input to neurons of CA3 region and increase in their activity supposedly enhances glutamatergic drive in the CA3 network. Glutamatergic input to neurons in CA3 was assessed by recording spontaneous excitatory postsynaptic currents (sEPSCs) in whole-cell voltage clamp configuration under block of $GABA_A$ and NMDA receptors with picrotoxin (100 μM) and D-AP5 (50 μM), respectively. KA at 50 nM increased the occurrence of sEPSCs in pyramidal neurons by 1380 ± 840% and interneurons by 490 ± 90%. In pyramidal neurons, no changes in current properties were observed but in interneurons the amplitude of currents increased reversibly by 53 ± 20% with no changes in rise or decay times. Supposedly selective suppression of mossy fibre transmission by blocking mGluR-II receptors with (2S,2'R,3'R)-2-(2',3'-di-

carboxycyclopropyl)glycine (DCG IV) (Kamiya *et al.* 1996; Kasyanov *et al.* 2004; Marchal and Mulle 2004; Safiulina *et al.* 2006) had not effect on sEPSC occurrence nor influenced the effects of KA. This indicates that mossy fibers do not substantially contribute to spontaneous unitary transmission on CA3 and that increased glutamatergic input after high-affinity KA receptor activation most probably originates from recurrent A/C fibers. Thus, activation of high-affinity KA receptors in immature CA3 pyramidal neurons leads to increased glutamatergic drive at CA3 region.

Apart from neuronal firing, the synaptic drive is dictated by the strength of activated synapses. Presynaptic KA receptors of immature CA3 pyramidal neurons regulate transmitter release from glutamatergic terminals at both CA3 (Lauri *et al.* 2005) and CA1 (Lauri *et al.* 2006). Spontaneous glutamate release was assessed by recording action potential independent (*i.e.* miniature) glutamatergic EPSCs (mEPSCs) in whole-cell voltage clamp configuration under block of voltage-gated sodium channels with tetrodotoxin (TTX; 1 μM) in addition to block of $GABA_A$ and NMDA receptors with picrotoxin and D-AP5. Under these conditions, mEPSCs result from spontanenous release of glutamate from presynaptic terminals. mEPSCs were unaffected by 50 nM KA in both pyramidal neurons and interneurons, suggesting that either the presynaptic KA receptors that regulate glutamate release at synapses onto immature CA3 pyramidal neurons do not have high affinity for KA or that they are tonically activated to such an extent by extracellular glutamate that they cannot be further activated by external agonist (Lauri *et al.* 2005). Either way, the results indicate that enhancement of glutamatergic drive in CA3 by high-affinity KA receptor activation is not due to enhanced glutamate release but solely to increased firing of CA3 pyramidal neurons.

Glutamatergic drive is a crucial factor for the generation of network bursts in immature hippocampus (Bolea *et al.* 1999; Sipilä *et al.* 2005; Sipilä *et al.* 2006). To assess the consequences of activation of high-affinity KA receptors to network bursts, spontaneous synaptic input to CA3 pyramidal neurons was recorded in whole cell voltage clamp recording configuration with pharmacologically intact synaptic transmission. In these conditions, the characteristic network activity with spontaneous network bursts were observed on a background of unitary synaptic activity. Application of KA at 50 nM increased the frequency of bursts by 700 ± 240% and the regularity of bursts by 54 ± 8% as measured by a decrease in the coefficient of variation of interburst interval. In contrast, burst size (amplitude as well as area) showed inverse relation to frequency although burst duration was not affected indicating a reduction in the size of the neuronal population participating in a given burst (*i.e.* synchrony). KA at 25 nM and domoate at 5 nM had similar

but less pronounced effect on network bursts. Block of GluK1-containing KA receptors with specific antagonist LY382884 suppressed GDP occurrence as reported by (Lauri *et al.* 2005) but had no influence on the effect of KA at 50 nM. The effect of 50 nM KA on interictal-like bursts recorded in the presence of $GABA_A$ receptor blocker picrotoxin (100 μM) was similar as on network bursts in the presence of intact GABAergic tranmission. As a whole, these results suggest that activation of high-affinity KA receptors lacking GluK1 enhance the spontaneous generation of network bursts in CA3 independently of fast GABAergic transmission.

Unlike concentrations at or below 50 nM, KA at 100 nM replaced the natural pattern of network activity with intense unsynchronous activity as shown before with KA at 250 nM or higher by Khalilov *et al.* (1999b). Importantly, whereas KA at 50 nM induced no measurable inward currents in either CA3 pyramidal neurons nor interneurons a clear depolarizing current was observed with KA at 100 nM in both neuron types in accordance with (Khalilov *et al.* 1999). Thus, the consequences of KA receptor activation in the immature hippocampus are qualitatively different when the agonist concentration is high enough to activate depolarizing currents in CA3 pyramidal neurons.

To conclude, findings in Study I indicate that activation of high-affinity KA receptors in immature hippocampus specifically leads to increased spiking of CA3 pyramidal neurons, which enhances excitatory synaptic drive in the CA3 region and therefore promotes the spontaneous generation of network bursts. Importantly, activation of high-affinity KA receptors increases both occurrence and regularity of spontaneous network bursts without inducing hypersynchronous network activity as with higher KA concentrations.

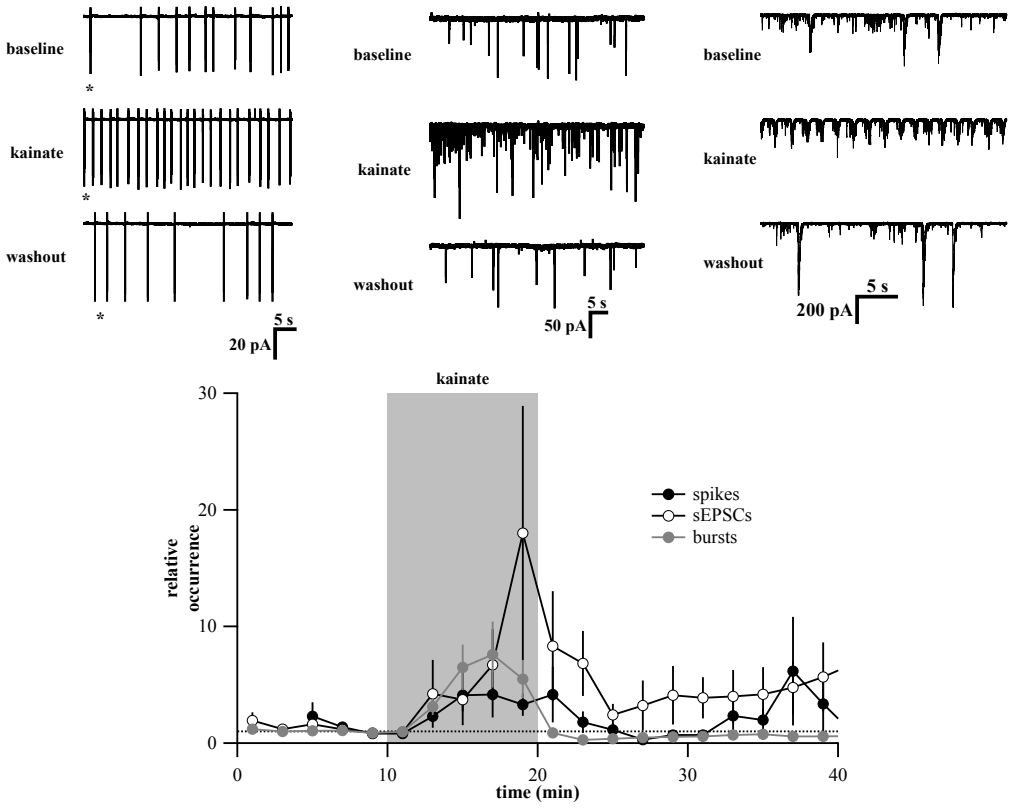

Figure 5 Enhanced spiking of pyramidal neurons by activation of high-affinity KA receptors promotes network bursting via enhancement of glutamatergic drive at neonatal CA3.

Top left Example traces from an on-cell recording of an immature CA3 pyramidal neuron in the presence of AMPA, NMDA, and GABAA receptor blockers. The neuron fires spontanously and firing is increased in the presence of KA at 50 nM. ***Top middle*** Example traces from a whole-cell voltage clamp recording of an immature CA3 pyramidal neuron in the presence of picrotoxin (100 μM) and D-AP5 (50 μM) illustrating unitary glutamatergic activity and its increase in the presence of KA (50 nM). ***Top right*** Example traces from a whole-cell voltage clamp recording of an immature CA3 pyramidal neurons with pharmacologically intact synaptic transmission illustrating spontaneous network bursts on a background of unitary glutamatergic activity and the increase in burst occurrence in the presence of KA (50 nM). ***Bottom*** Pooled data of the occurrence of spikes, sEPSCs and network bursts.

4.2 Subunit GluK1-containing kainate receptors regulate network activity via metabotropically controlling afterhyperpolarization in immature interneurons (II)

Although GABAergic transmission is regarded crucial for hippocampal development (Ben-Ari *et al.* 2004; Bitzenhofer and Hanganu-Opatz 2014) and GABAergic currents dominate the synaptic input to immature neurons (Lauri *et al.* 2005) very little is known about the firing properties of immature interneurons. Recordings in on-cell configuration revealed that interneurons in the immature CA3 display sustained firing and that their firing was reduced by 82 ± 3% when GluK1 antagonist LY382884 was applied. In current clamp recordings in whole-cell configuration interneurons responded to intracellular injection of depolarizing current with a non-accommodating train of action potentials. In the presence of LY382884, interneurons fired ca. 30 to 70% less intensely, depending on magnitude of current injected. Additionally, an AHP that was not evident in control conditions followed spike train in the presence of LY382884. AHP had a duration of ca. 200 ms, which corresponds with the AHP of medium duration (mAHP) (Aoki and Baraban 2000; Savić *et al.* 2001) (see figure 6 on page 36) . These results suggest that endogenously activated GluK1-containing KA receptors control the excitability of immature CA3 interneurons.

The current underlying the AHP was assessed directly in voltage clamp recordings in whole-cell configuration. Intrasomatic injections of depolarizing current evoked an outward current that was underlying mAHP and had charateristics of I_{mAHP} previously described in juvenile CA1 interneurons (Aoki and Baraban 2000) and immature CA3 interneurons (Savić *et al.* 2001). I_{mAHP} was regulated by the activity of GluK1-containing KA receptors as it was enhanced by 28 ± 5% upon application of LY382884 whereas GluK1 agonist ATPA suppressed it by 41 ± 13%. The I_{AHP} was suppressed by 31 ± 7% by apamin, a specific blocker of the small conductance calcium-activated potassium channels (SK channels). Accordingly, apamin occluded the suppressing effect of ATPA and LY382884 enhanced the suppressing effect of apamin on I_{mAHP} by 81 ± 7%. Scavenging of extracellular glutamate with glutamate pyruvate transaminase and pyruvate (2 mM) increased I_{mAHP} and occluded the effect of LY382884 on I_{mAHP}. No tonic inward current was observed in interneurons upon either application of LY382884 nor scavenging of glutamate. ATPA, however, activated a depolarizing current revealing a presence of ionotropically active KA receptors. These results suggest that tonically activated

GluK1-containing KA receptors suppress I_{AHP} mediated that is mediated by apamin-sensitive potassium channels in immature CA3 interneurons.

The functions of KA receptors in the presynaptic glutamatergic terminals of immature hippocampus are developmentally downregulated (Lauri *et al.* 2006). To see whether also I_{mAHP} modulation is a feature restricted to neonatal period, similar recordings as above were conducted from interneurons in hippocampal slices from juvenile mice. These neurons displayed a slower firing rate in on-cell recording as well as less intense spike response to depolarization and more prominent AHP in whole-cell recordings compared to neonatal neurons. Also, neither spiking nor I_{mAHP} was affected by LY382884 or ATPA. Like in immature interneurons, ATPA induced a depolarizing current in juvenile interneurons indicating the presence of functional KA receptors. In light of these findings, the lower firing rate of juvenile interneurons seems to be due to developmental uncoupling of GluK1-containing KA receptors from apamin sensitive potassium channels.

Interneuronal firing is an important factor for the generation of network bursts by providing GABAergic excitatory drive and synchronization of neuronal populations (Bonifazi *et al.* 2009). Promotion of interneuronal firing by suppression of apamin sensitive I_{mAHP} should lead to increased GABAergic drive in the network. Because inhibition of GluK1-containing KA receptors modulate glutamate release in addition to interneuronal I_{mAHP} (Lauri *et al.* 2005; Lauri *et al.* 2006), direct assessment of network effects of I_{mAHP} modulation by presently available KA receptor modulating pharmacological tools is not possible. Therefore the consequences of selective inhibition of I_{mAHP} for the network activity in the immature hippocampus was investigated by bath application of apamin. Apamin increased the frequency of spontaneous network bursts recorded in whole-cell voltage clamp from CA3 pyramidal neurons in hippocampal slices from P3-P5 mice. This was accompanied by an increased firing of interneurons revealed in on-cell recordings. These observations show that regulation of apamin-sensitive I_{mAHP} is physiologically relevant means for controlling the activity in the network of immature hippocampus and suggest that by controlling the activity of interneurons GluK1-containing KA receptors are able to modulate the activity of whole network of immature CA3.

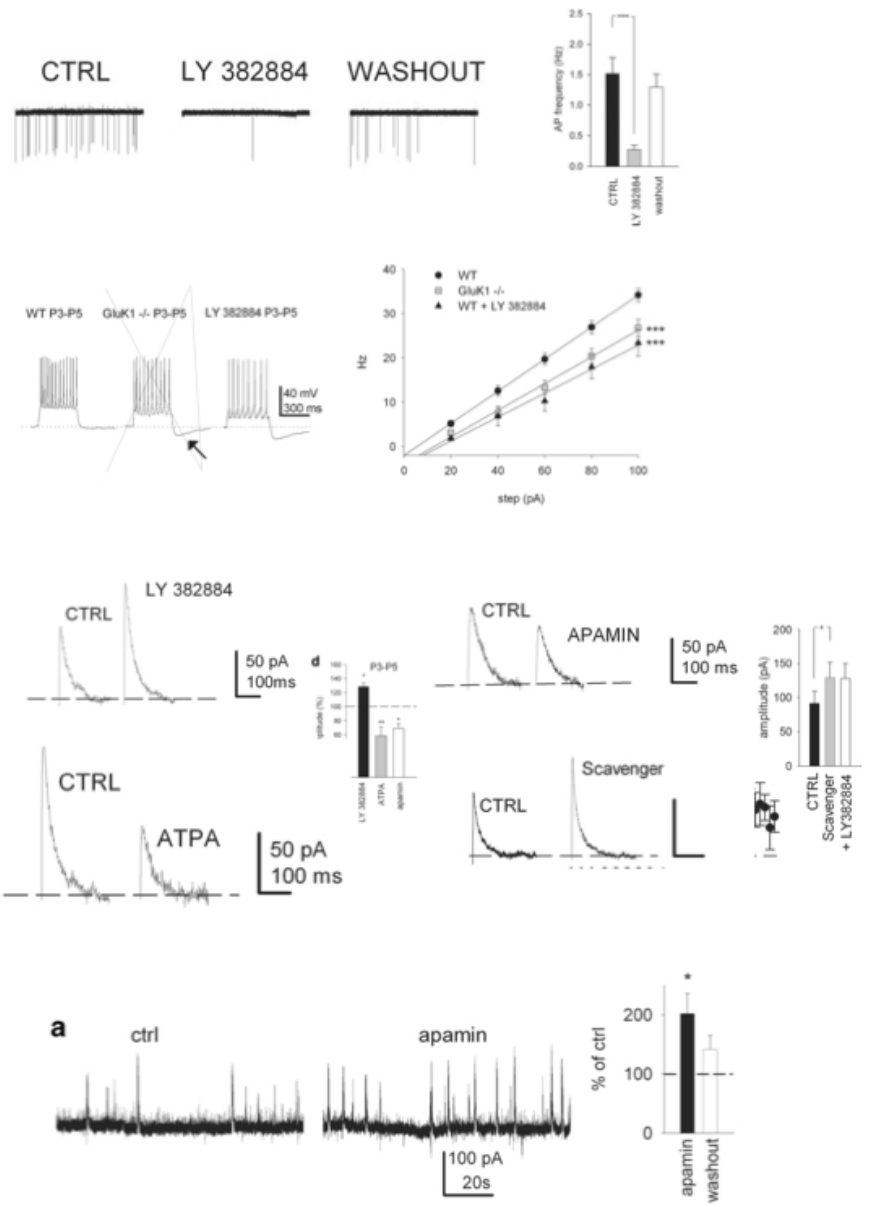

Figure 6 GluK1-containing KA receptors in neonatal interneurons suppress afterhyperpolarizing current of medium duration (I_{mAHP})

1st row: GluK1-containing KA receptors modulate interneuronal firing. ***left*** Example traces of on-cell recordings illustrating the less frequent spiking of interneurons after block of GluK1-containing KA receptors. ***right*** Average frequency of interneuronal firing**.**

2nd row: ***left*** example traces of current-clamp whole cell recording illustrating that in the presence of LY382884 interneuronse responded to depolarizing pulse

with less intense firing and that an apparent AHP follows spike train. ***right*** Average firing rates of interneurons in response to depolarizing current of varying magnitude

3rd row: example traces and pooled data of I_{AHP} evoked by injection of depolarizing current in whole-cell voltage clamp recordings of interneurons.

4th row: example traces of whole-cell voltage clamp recordings illustrating the increased occurrence of network bursts upon block of ImAHP with apamin.

4.3 Block of GluK1-containing kainate receptors suppresses network effect of ethanol in a developmental manner (III)

KA receptors are inhibited by ethanol in the mature hippocampus (Weiner *et al.* 1999; Carta *et al.* 2003) and ethanol increases network bursting in the neonatal hippocampus (Galindo *et al.* 2005). In Study III, the aim was to assess whether GluK1-containing KA receptors are involved in the network effects of ethanol in the immature hippocampus. Network bursts in acute hippocampal slice preparations were monitored by recording synaptic currents from CA3 pyramidal neurons using whole cell voltage clamp. Application of ethanol at 50 mM revealed that network effects of ethanol were dependent on the developmental stage of the network as burst frequency was increased by 35 ± 15% at P3-5, in accordance with (Galindo *et al.* 2005), but decreased by 29 ± 8% and 36 ± 6% at P1 and P10, respectively (see figure 7 on page 38). ACET, a selective blocker of GluK1-containing KA receptors, occluded the effect of ethanol on bursts at P3-5 but had no apparent influence on the effect at P1 and P10 indicating that GluK1-containing KA receptors contribute to effects of ethanol only at P3-5.

The effect of ethanol in the immature hippocampus has been suggested to be due to increased frequency of GABA-mPSCs in interneurons in the CA3 region (Galindo *et al.* 2005). To assess whether GluK1-containing KA receptor activation contributes to this effect, GABA-mPSCs were recorded from CA3 pyramidal neurons and *s. lucidum* interneurons at P3-5. Ethanol at 50 mM increased frequency of GABA-mPSCs in interneurons by 30±7% but had no effect on it in pyramidal neurons in accordance with Galindo *et al.* (2005). Co-applied ACET had no influence in this effect indicating that GluK1 activation plays no role in the effect of ethanol on GABA-mPSC frequency. However, ACET, coapplied with ethanol, caused a partially reversible decrease in the GABA-mPSC amplitude of (9.4±3.0% decrease from baseline, 6.3±% decrease from EtOH) in interneurons but not pyramidal neurons indicating that ACET reduces the postsynaptic influence of GABAergic input to interneurons.

GluK1-containing KA receptors modulate network bursting by regulating glutamate release at immature CA3 (Lauri *et al.* 2005). Ethanol had no effect on Glu-mPSCs in CA3 pyramidal neurons at P3-5 suggesting that it has no effect on presynaptic KA receptor population in these synapses.

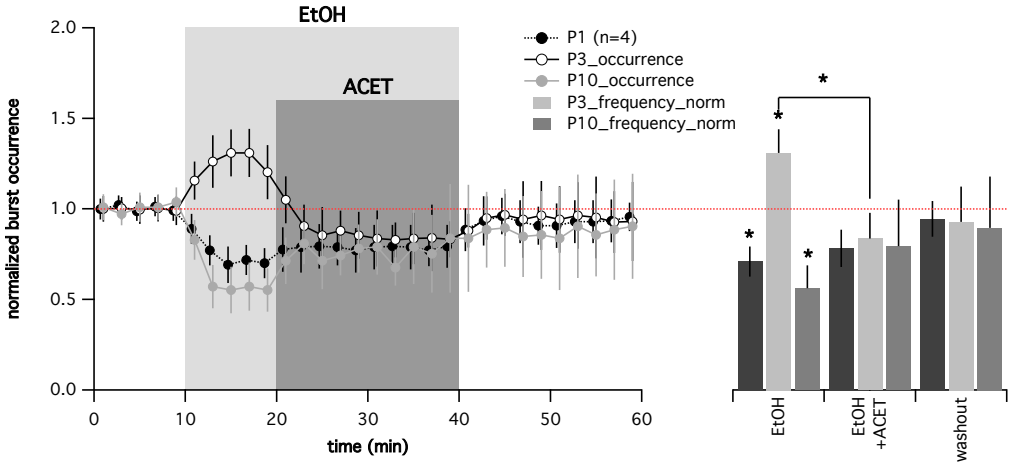

Figure 7 Network effects of ethanol are partially suppressed under pharmacological block of GluK1-containing KA recepors
Pooled data of the occurrence of spontaneous network bursts in hippocampal slices from P1 (top), P3-5 (middle) and P10 (bottom) rats.

5 Discussion

In the studies included in this thesis KA receptors were shown to act as efficient modulators of action potential firing activity of neurons in the CA3 region of immature hippocampus. In Study I it was showed that activation of high-affinity KA receptors enhances spontaneous firing of immature pyramidal neurons. Study II revealed that subunit GluK1-containing KA receptors control the firing of immature interneurons. With these and previously described actions in immature hippocampus (summarized in figure 8 on page 40), KA receptors possess a capacity for powerful modulation of neurotransmission and propensity for spontaneous burst generation in the developing network. This is clearly evidenced by the dramatic effects of KA receptor modulation on network burst occurrence. High-affinity KA receptors may act as a physiological mechanism to control bursting activity in the immature network and thereby have a strong influence on the function and maturation of the network of immature hippocampus. Modulation of KA receptor function by drugs of abuse such as ethanol or clinically used pharmacological agents in the early stages of brain development thus has important physiological and pathophysiological implications.

The activity of pyramidal neurons in the CA3 region is a critical determinant of the glutamatergic drive in the immature hippocampus as they provide the majority of glutamatergic input in the scarcity of mossy fiber transmission and perforant path innervation, which occur later in development (Skutella *et al.* 1999; Marchal and Mulle 2004). In Study I, high-affinity KA receptors specifically modulated the firing of pyramidal neurons in immature CA3. Importantly, these receptors appear to be activated endogenously as NBQX suppressed firing of CA3 pyramidal neurons under block of AMPA and NMDA receptors as well as $GABA_A$ receptors. The endogenous activation is most probably due to the presence of ambient extracellular glutamate as is the case with tonic activation of presynaptic KA receptors at immature CA3 as well as CA1 (Lauri *et al.* 2005; Lauri *et al.* 2006). It also agrees well with the high affinity of these receptors for KA, as KA and glutamate affinity of KA receptor subunits is correlated (Jane *et al.* 2009). Notably, the activation of high-affinity KA receptors may be more pronounced under physiological conditions since firing of pyramidal neurons was assessed under block of fast synaptic transmission and the concentration of ambient glutamate could be assumed to be higher in acute slices when synaptic transmission is intact. Taking into account that the *in vitro* slices are under constant superfusion with saline, the level of ambient glutamate in extracellular space could be even higher *in vivo*.

The exact mechanism by which high-affinity KA receptors control firing of CA3 pyramidal neurons is unknown. The lack of effect of KA on somatic excitabil-

ity argues against somatic ionotropic effect and suggests that the activated receptors are located at some distance from the soma. The action potentials in CA3 pyramidal neurons are normally initiated in the initial axonal segment around 40 µm from the soma (Meeks and Mennerick 2007) but also ectopic spikes at more distal axonal regions have been observed (Stasheff *et al.* 1993; Bucher and Goaillard 2011). In axons of mature and immature hippocampal interneurons activation of KA receptors is able to induce antidromic spiking through depolarization of the axonal membrane (Semyanov and Kullmann 2001; Maingret *et al.* 2005). At mossy fibers KA reduces the stimulation threshold for generation of antidromic spikes (Kamiya and Ozawa 2000; Schmitz *et al.* 2000). Thus, a possible explanation for promoted spiking of CA3 pyramidal neurons could be an increased axonal excitability and enhanced generation of spikes initiated at either normal or an ectopic site. Whether the mechanism relies on ionotropic or metabotropic signaling remains to be determined.

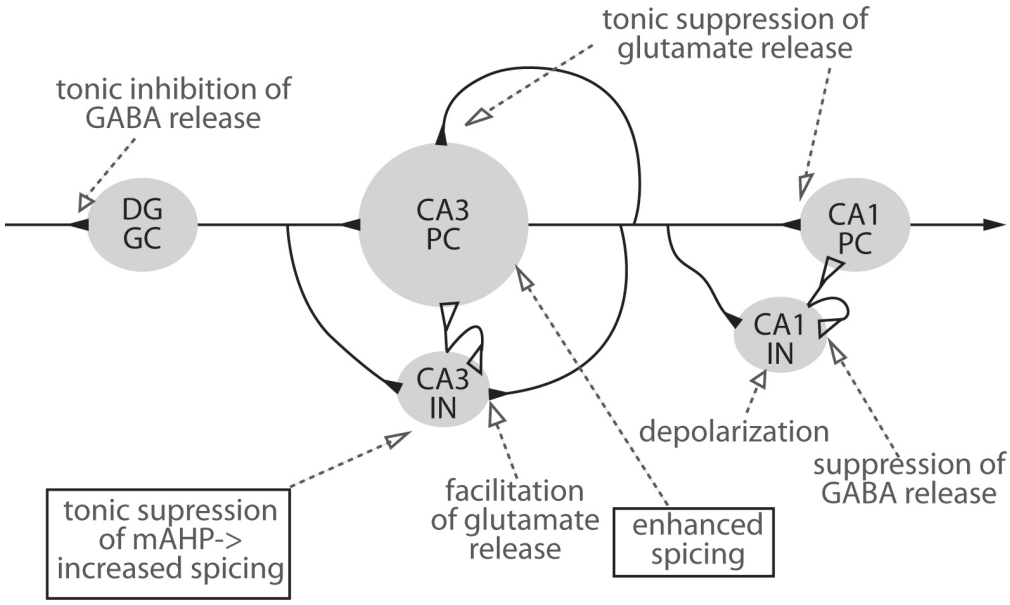

Figure 8 Updated summary of kainate receptor functions in immature hippocampus

KA receptors are highly expressed in interneurons of the immature hippocampus but few studies have addressed their physiological role. Study II revealed that subunit GluK1-containing KA receptors control the firing of immature interneurons in murine CA3. Tonical activation of these receptors by ambient glutamate suppressed mAHP and allowed interneurons to fire at higher frequency than in juvenile or adult hippocampus. I_{mAHP} was first demonstrated in interneu-

rons of *s. lacunosum-moleculare* (L-M) of rat CA1 (Aoki and Baraban 2000). In these neurons, I_{mAHP} can be observed from P3 and is developmentally upregulated in a similar manner as showed for mouse CA3 interneurons in Study II. The Study II now extends these findings by showing that this regulation is due to KAR activity. The KA receptors in interneurons mediated their effects on I_{mAHP} metabotropically as the effect was dependent on intracellular G protein signaling but they also mediated a depolarizing current when activated by ATPA. This implies that either same receptors acted both ionotropically and metabotropically or there are two receptor populations with different modes of action. The metabotropic regulation of I_{mAHP} by KA receptors was downregulated in juvenile animals in a similar manner as metabotropic suppression of glutamate release from synapses at CA3 and CA1 (Lauri *et al.* 2005; Lauri *et al.* 2006). The coupling between KA receptors and mAHP was downregulated by the 2nd PNW. It is not known whether this uncoupling is activity dependent akin to the tonic activation of presynaptic KA receptors in glutamatergic presynaptic terminals at CA1 (Lauri *et al.* 2006; Sallert *et al.* 2009).

Native KA receptors are believed to be heteromers but the exact subunit composition of KA receptors in immature hippocampus, as in most other cases, remains uncertain. Subunits GluK1, GluK2, GluK4 and GluK5 are expressed in CA3 pyramidal neuron layer in the early development (Ritter *et al.* 2002) and GluK1, GluK4 and GluK5 are targeted to axonal compartments in cultured immature CA3 pyramidal neurons (Vesikansa *et al.* 2012). One (or both) of the high-affinity KA receptors subunits GluK4 and GluK5 most probably participates in the high-affinity KA receptors in the immature CA3 pyramidal neurons. However, these receptors appear to not contain GluK1 as LY382884 had no influence on network effect of KA (Study I). However, the effects of GluK1 antagonists were not directly tested on CA3 pyramidal neuron firing. In mature interneurons of CA3, KA receptors appear to be principally composed of subunits GluK1, GluK2 and GluK5 (Paternain *et al.* 2000; Mulle *et al.* 2000; Christensen *et al.* 2004). Interneuronal spiking was similar in GluK5-/- as in WT mice in Study II suggesting that GluK5 is not a component of these receptors.

Previous studies have established presynaptic KA receptors as important regulators of function of immature synapses (reviewed by Lauri and Taira 2012). In Study I it was observed that low doses of KA had no effect on action potential independent glutamate release onto CA3 pyramidal neurons. However, it is influenced by both pharmacological manipulation of GluK1-containing KA receptors as well as manipulation of the level of extracellular glutamate (Lauri *et al.* 2005). It is possible that the KA receptors in the presynaptic terminals in question have lower affinity for KA or that the effect of KA is occluded by tonic activation of ambi-

ent glutamate as has been shown in the area CA1 (Lauri *et al.* 2006). Release of GABA is suppressed from presynaptic terminals of immature CA1 interneurons (Maingret *et al.* 2005) and KA receptor mediated modulation of GABA release has also been demonstrated in CA1 of mature hippocampus (Rodríguez-Moreno *et al.* 1997; Jiang *et al.* 2001; Lourenço *et al.* 2010). Spontaneous GABAergic transmission in CA3 region is not affected by manipulation of GluK1-containing KA receptors in the immature hippocampus (Lauri *et al.* 2005) (Study III). KA receptor mediated modulation of GABA release in mature CA3 has not been reported and whether KA receptors are present in presynaptic terminals of CA3 interneurons remains unknown. Interneurons are very heterogenous and the properties of interneuron subpopulations may differ also with regards to the KA receptors they are expressing.

The network bursts in immature hippocampus are generated once a certain threshold level of excitatory synaptic traffic is exceeded (Menendez de la Prida and Sanchez-Andres 2000). Burst initiation depends to a great deal on activity of CA3 pyramidal neurons, apparently because a subpopulation of them preferably fires action potentials in bursts (Sipilä *et al.* 2005; Sipilä *et al.* 2006). Thus, the increased CA3 pyramidal neuron firing can explain the promotion of network burst generation by low concentrations of KA and domoate obsrved in Study I. It is worth to stress that on Study I KA at concentration 25-50 nM did not induce pathological activity but merely enhanced the spontaneous generation of natural activity pattern. However, application of KA at 100 nM replaced the natural activity pattern with intense asynchronous activity in accordance with the previously reported epileptogenic infuence of KA receptor agonist application. A simple and most probably sufficient explanation for this qualitative difference in network effect is that KA at 50 nM or lower concentration evoked no depolarizing current but at 100 nM it did so in both pyramidal neurons and interneurons, thereby producing overactivity in the network. In agreement with this, it has previously been shown that KA (at 300 nM) induces no depolarizing current in rat CA3 pyramidal neurons at P0 but the susceptibility to KA induced depolarization gradually increases on the first PNW (Khalilov *et al.* 1999).

Although not absolutely required for burst generation (Bolea *et al.* 1999; Sipilä *et al.* 2005), interneuronal activity is a central determinant of the activity of the whole hippocampal network as depolarizing GABAergic input contributes synergistically to synaptic excitation of both interneurons and pyramidal neurons (Khazipov *et al.* 1997) and synchronises neuronal activity (Bonifazi *et al.* 2009). The consequences of modulation of interneuronal firing by KA receptors for network activity are difficult to directly assess pharmacologically as bath application of

drugs also affects receptors at presynaptic terminals (Lauri *et al.* 2005). However, in Study II it was observed that suppression of I_{mAHP} by bath-applied apamin increased the frequency of both network bursts and interneuronal firing indicating that I_{mAHP} in interneurons limits the overall activity in the network. This suggests that suppression of mAHP in interneurons by KA receptors promotes network bursts by enhancing firing of interneurons. Some of the network effects of apamin may be due to suppression of an uncharacterized SK-channels in other neuron types. I_{mAHP} has been demonstrated in juvenile (P13-16) murine CA3 pyramidal neurons (Fisahn *et al.* 2005) but their function in the neonatal stage has not been assessed.

In both pyramidal neurons and interneurons of immature CA3, KA receptors promoting firing were activated endogenously. Thus, the excitatory tone in the immature CA3 is seemingly set by KA receptors. In line with these findings, many of the KA receptors in the developing hippocampus are tonically activated by ambient extracellular glutamate. Despite the determination of the exact concentration of extracellular glutamate is technically challenging, the ambient glutamate level has been estimated to be in the range of 0.5 to 5 μM in the resting/anesthetized mammalian brain (Featherstone and Shippy 2008) and submicromolar in acute hippocampal slice (Herman and Jahr 2007). Albeit glutamate concentration in neonatal slices has not been measured, the above value lies well within the affinity ranges reported for native glutamate receptors. In fact, it is reasonable to assume that ambient glutamate levels are higher in the early development because expression of glutamate transporters is upregulated during development and extracellular diffusion is less limited in immature brain due to relatively high ratio of extracellular and intracellular volumes (Danbolt 2001; Syková 2004). As it is believed that concentration of glutamate may vary depending on the neuronal activity (Featherstone and Shippy 2008), KA receptors could act as sensors of general glutamatergic activity level in the immature hippocampus. In CA3, a rise in extracellular glutamate concentration would increase firing of both pyramidal neurons (Study I) and interneurons (Study II), suppress glutamatergic synaptic input to pyramidal neurons while increasing it to interneurons (Lauri *et al.* 2005), and suppress release of GABA from mossy fiber synapses onto pyramidal neurons (Caiati *et al.* 2010). Additionally, glutamate release from synapses that CA 3 pyramidal neurons form on CA1 pyramidal neurons would be suppressed. This would lead to enhanced synaptic traffic with a balance shifted to favor activation of interneurons and to more pronounced filtering of asynchronous activity in glutamatergic synapses. Notably, elevated glutamate level would influence other targets in addition to KA receptors. Especially NMDA receptors are very sensitive to glutamate and experimental ele-

vation of ambient glutamate by block of glutamate transporter results in slow network oscillation in acute slices of immature hippocampus by activation of exrasynaptic NMDA receptors preferentially in interneurons (Cattani *et al.* 2007).

The strong influence of nanomolar KA on network activity in the developing hippocampus may well explain the changes in neurobehavioral development and hippocampal morphology produced by subconvulsive doses of KA receptor agonists (Doucette *et al.* 2003; Doucette *et al.* 2004; Burt *et al.* 2008). Notably, the size of the neuron population participating in a given burst was inversely related to burst frequency. The mechanisms underlying this inverse relationship between burst frequency and size of neuronal population participating in bursts are unclear but may be hypothesized to be due to increase in some neurons that are in a functionally refractory state due, for example, to inactivation of voltage gated ion channels or activation of mechanisms such as metabotropic transmitter signalling suppressing the excitability of neurons. Whatever the reason is, decreased synchronization during network bursts could lead to disturbed development of the synaptic connections as synchronous activity promotes the maturation of glutamatergic connections while asynchronous activity promotes their elimination (Hanse *et al.* 2009). Additionally, externally applied KA receptor agonists may interfere with the several presynaptic KA receptors and influence the maturation of presynaptic functions (Lauri *et al.* 2005; Maingret *et al.* 2005; Lauri *et al.* 2006; Caiati *et al.* 2010).

The results of Study III revealed that at least some of the effects of ethanol on early network activity could be prevented by inhibition of GluK1-containing KA receptors. The actual mechanisms, however, remain unclear. Ethanol inhibits KA receptors containing GluK1 (Valenzuela and Cardoso 1999) yet in the immature hippocampus ethanol appears to not influence the functions known to be modulated by GluK1 KA receptors. Firstly, ethanol has no effect on glutamatergic input to CA3 pyramidal neurons (Study III) although LY382884 enhances it (Lauri *et al.* 2005). Secondly, ethanol has no effect on firing of interneurons in response to depolarizing current injection (Galindo *et al.* 2005) although LY382884 decreases it (Study II). Thirdly, ethanol does not influence release from glutamatergic synapses onto interneuron at immature CA3 (Galindo *et al.* 2005) but LY382884 does (Lauri *et al.* 2005). In light of this, it is somewhat surprising that ACET completely blocks the effect of ethanol on network bursts. However, in Study III it was verified that ethanol does enhance release from GABAergic synapses onto interneurons but not pyramidal neurons at immature CA3 as reported by Galindo *et al.* (2005) although ACET did not affect release in either case but it slightly decreased amplitude of GABAergic currents in interneurons. This may be due to prevention of net change in GABAergic input to interneurons by suppressing the postsynaptic effect of indi-

vidual currents. However, as the partially redundant mechanisms generating network bursts involve all neuron types and transmitter systems, there is always the possibility that ethanol and ACET mutually mitigate their effects on the network level by acting on completely different targets. Clearly, more detailed investigation is required to settle these issues.

6 Conclusions

KA receptors remain the least understood member of the iGlu receptor family. The findings in this work add to the growing amount of data indicating that KA receptors are expressed at key locations to modulate spontaneous network activity of the immature hippocampus and strengthen the view that KA receptors act as modulators of network functions in general. Maturation of hippocampal synapses and connections depends on electrical activity (Zhang and Poo 2001; Lauri *et al.* 2003; Colin-Le Brun *et al.* 2004; Spitzer 2006; Swann *et al.* 2007; Huupponen *et al.* 2013) and changes in network activity influence the modulation and balance of developing synapses (Kasyanov *et al.* 2004; Huupponen *et al.* 2007). Additionally, alterations in glutamatergic transmission may contribute to the aetiology of a number of neuropathologies (Eubanks *et al.* 1993; Sander *et al.* 1995; Sander *et al.* 1997; Mulle *et al.* 1998; MacDonald *et al.* 1999). By regulating the activity of both glutamatergic and GABAergic neurons and function of their synapses, KA receptors provide an efficient means for fine-tuning the hippocampal network. As such, KA receptors also predispose the network to influences of external agents that modulate their function. Indeed, because KA receptors primarily act as modulators of neuronal networks but are not absolutely required for the function of synaptic transmission as the viability of KA receptor subunit knockout shows, they are considered to be tempting targets for pharmacological interventions (Jane *et al.* 2009). The findings that distinct KA receptor populations control various physiological functions in developing hippocampus may provide basis for development of pharmacological tools for treatment of activity disorders of immature hippocampus. Better understanding of KA receptor functions should help in evaluating the contribution of these receptords to deleterious effects that drugs of abuse have in the developing brain. Understanding of the mechanisms underlying the functional maturation of neuronal networks in the early development may also have direct implications for mature brain. In recent years, it has been demonstrated that neurogenesis is not restricted to early development but also occurs in the mature brain. Interestingly, it occurs in the dentate gyrus where granule cells are generated long into the postnatal life. Very little is known of the incorporation of newly generated neurons into the already functioning hippocampal network but there is evidence that this process depends on the local network activity (Overstreet-Wadiche *et al.* 2006; Ge *et al.* 2007; Piatti *et al.* 2011).

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