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Activity-dependent regulation of GABA release at immature mossy fibers-CA3 synapses: role of the *Prion* protein

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TABLE OF CONTENTS

Declaration

The original work presented in this thesis was carried out at the International School for Advanced Studies, Trieste, between November 2008 and November 2012. I performed and analyzed the majority of the electrophysiological experiments and contributed to papers writing.
In situ hybridization experiments have been performed by Elodie Richard and Dany Verrier in Dr. Giovanni Marsicano's laboratory in Bordeaux.

N. 1:

Caiati MD*, Sivakumaran S*, Cherubini E.
Early in postnatal development, tonic activation of kainate receptors by ambient glutamate reduces GABA release from mossy fiber terminals. *The Journal of Neuroscience 2010 Feb 3;30(5):1750-9.*
* equally contributed

N. 2:

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Developmental regulation of CB1-mediated spike-time dependent depression at immature mossy fiber-CA3 synapses. *Sci Rep. 2012;2:285.*

N. 3:

Caiati MD, Safiulina VF, Fattorini G, Sivakumaran S, Giuseppe Legname and Enrico Cherubini
Prp^C controls via PKA the direction of synaptic plasticity in the immature hippocampus. *Under revision in The Journal of Neuroscience*

The following publications have been included in the present thesis in the *Annex*.

Safiulina VF, **Caiati MD**, Sivakumaran S, Bisson G, Migliore M and Enrico Cherubini.

Control of GABA release at single mossy fiber-CA3 connections in the developing hippocampus.
Frontiers in Synaptic Neuroscience, 2010 Feb 22;2:1 doi: 10.3389/neuro.19.001.

R Tyzio, C Allene, R Nardou, MA Picardo, S Yamamoto, Sivakumaran S, **Caiati MD**, S Rheims, M Minlebaev, P Ferré, R Khazipov, JL Romette, J Lorquin, G. Chazal, R Cossart, I Khalilov, A Nehlig, E Cherubini, Y Ben-Ari.
Ketone body metabolic substrate beta-hydroxybutyrate does not alter excitatory GABA actions on neonatal cortical neurons.
The Journal of Neuroscience 2011 Jan 5, 31(1):34-45.

Cherubini E, **Caiati MD**, Sivakumaran S.

In the Developing Hippocampus Kainate Receptors Control the Release of GABA from Mossy Fiber Terminals via a Metabotropic Type of Action.
Adv Exp Med Biol. 2011;717:11-26.

Caiati MD
Is GABA co-released with glutamate from immature mossy fiber terminals?
In press in *The Journal of Neuroscience*.

ABBREVIATIONS

ACSF	Artificial CerebroSpinal Fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP	Adenosine 5'-triphosphate
BAPTA	1,2-bis(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF	Brain-derived neurotrophic factor
CA1/4	Cornu Ammonis regions 1/4
cAMP	3'-5'-cyclic Adenosine MonoPhospate
CCK	Cholecystokinin
CNQX	6-Cyano-7-NitroQuinoXaline-2,3-dione
CNS	Central Nervous System
D-AP5	D-2-amino-5-phosphonovalerate
DCG-IV	(2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine
DG	Dentate Gyrus
DMSO	DiMethylSulphOxide
DNQX	6,7-DiNitroQuinoXaline-2,3-dione
EC	Entorhinal Cortex
EGTA	Ethylene Glycol-bis(2-aminoethylether)-N-N-N'-N'-Tetraacetic Acid
EPSC	Excitatory PostSynaptic Current
EPSP	Excitatory PostSynaptic Potential
ERK	Extracellular signal-Regulated Kinases

GABA	γ-aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GAT	γ-aminobutyric acid transporter
GDP	Giant Depolarizing Potential
GYKI	52466 4-(8-methyl-9H-1,3-dioxolo[4,5-h][2,3] benzodiazepin-5-yl)-benzenamine Hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid
KAR	Kainate Receptor
KCC2	K+/Cl- co-transporter isoform 2
L-AP4	L(+)-2-amino-4-phosphonobutyric acid
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MAPK	Mitogen-activated protein (MAP) kinases
MF	Mossy Fiber
mGluR	metabotropic glutamate receptor
nAChR	nicotinic acetylcholine receptor
NKCC1	Na-K-Cl cotransporter isoform 1
NMDA	N-Methyl-D-Aspartate
O-LM	Oriens–Lacunosum Moleculare
O-OR	Oriens–Oriens and Radiatum
P-LM	Pyramidale–Lacunosum Moleculare
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A

PPR	Paired-Pulse Ratio
PTX	Picrotoxin
R–LM	Radiatum–Lacunosum Moleculare
STD-LTD	spike-timing-dependent-long-term depression
STD-LTP	spike-timing-dependent-long-term potentiation
TTX	Tetrodotoxin
VGAT	Vesicular GABA Transporter

"Study the science of art. Study the art of science.
Develop your senses- especially learn how to see.
Realize that everything connects to everything else."
Leonardo Da Vinci

ABSTRACT

In adulthood, mossy fibers (MFs), the axons of granule cells of the dentate gyrus (DG), release glutamate onto CA3 principal cells and interneurons. In contrast, during the first week of postnatal life MFs release γ-aminobutyric acid (GABA), which, at this early developmental stage exerts a depolarizing and excitatory action on targeted cells. The depolarizing action of GABA opens voltage-dependent calcium channels and NMDA receptors leading to calcium entry and activation of intracellular signaling pathways involved in several developmental processes, thus contributing to the refinement of neuronal connections and to the establishment of adult neuronal circuits. The release of GABA has been shown to be down regulated by several neurotransmitter receptors which would limit the enhanced excitability caused by the excitatory action of GABA. It is worth noting that the immature hippocampus exhibits spontaneous correlated activity, the so called giant depolarizing potentials or GDPs that act as coincident detector signals for enhancing synaptic activity, thus contributing to several developmental processes including synaptogenesis. GDPs render the immature hippocampus more prone to seizures.

Here, I explored the molecular mechanisms underlying synaptic transmission and activity-dependent synaptic plasticity processes at immature GABAergic MF-CA3 synapses in wild-type rodents and in mice lacking the prion protein (*Prnp*$^{0/0}$ mice).

In the first paper, I studied the functional role of kainate receptors (KARs) in regulating GABA release from MF terminals. Presynaptic KARs regulate synaptic transmission in several brain areas and play a central role in modulating glutamate release at adult MF-CA3 synapses. I found that functional presynaptic GluK1 receptors are present on MF terminals where they down regulate GABA release. Thus, application of DNQX or UBP 302, a selective antagonist for GluK1 receptors, strongly increased the amplitude of MF-$GABA_A$-mediated postsynaptic currents (GPSCs). This effect was associated with a decrease in failure rate and increase in PPR, indicating a presynaptic type of action.

GluK1 receptors were found to be tonically activated by glutamate present in the extracellular space, since decreasing the extracellular concentration of glutamate with a glutamate scavenger system prevented their activation and mimicked the effects of KAR antagonists. The depressant effect of GluK1 on GABA release was dependent on pertussis toxin (PTx)-sensitive G protein-coupled kainate receptors since it was prevented when hippocampal slices were incubated in the presence of a solution containing PTx. This effect was presynaptic since application of UBP 302 to cells patched with an intracellular solution containing GDPβS still potentiated synaptic responses. In addition, the depressant effect of GluK1 on GABA release was prevented by U73122, which selectively inhibits phospholipase C, downstream to G protein activation. Interestingly, U73122, enhanced the probability of GABA release, thus unveiling the ionotropic type of action of kainate receptors. In line with this, we found that GluK1 receptors enhanced MF excitability by directly depolarizing MF terminals via calcium-permeable cation channels. We also explored the possible involvement of GluK1 in spike time-dependent (STD) plasticity and we found that GluK1 dynamically regulate the direction of STD-plasticity, since the pharmacological block of this receptor shifted spike-time dependent potentiation into depression.
The mechanisms underlying STD-LTD at immature MF-CA3 synapses have been investigated in detail in the second paper.

STD-plasticity is a Hebbian form of learning which consists in bi-directional modifications of synaptic strength according to the temporal order of pre and postsynaptic spiking. Interestingly, we found that at immature mossy fibers (MF)-CA3 synapses, STD-LTD occurs regardless of the temporal order of stimulation (pre *versus* post or *viceversa*). However, as already mentioned, while STD-LTD induced by positive pairing (pre before post) could be shifted into STD-LTP after blocking presynaptic GluK1 receptors, STD-LTD induced by negative pairing (post before pre) relied on the activation of CB1 receptors. At P3 but not at P21, endocannabinoids released by the postsynaptic cell during spiking-induced membrane depolarization retrogradely activated CB1 receptors, probably expressed on MF terminals and persistently depressed GABA release in the rat hippocampus. Thus, bath application of selective CB1 receptor antagonists prevented STD-LTD.

Pharmacological tools allow identifying anandamide as the endogenous ligand responsible of activity-dependent depressant effect. To further assess whether STD-LTD is dependent on the activation of CB1 receptors, similar experiments were performed on WT-littermates and *CB1*-KO mice. While in WT mice the pairing protocol produced a persistent depression of MF-GPSCs as in rats, in *CB1*-KO mice failed to induce LTD. Consistent with these data, in situ hybridization experiments revealed detectable levels of CB1 mRNA in the granule cell layer of P3 but not of P21 mice. These experiments strongly suggest that at immature MF-CA3 synapses STD-LTD is mediated by CB1 receptors, probably transiently expressed, during a critical time window, on MF terminals.

In the third paper, I studied synaptic transmission and activity dependent synaptic plasticity at immature MF-CA3 synapses in mice devoid of the prion protein ($Prnp^{0/0}$). The prion protein (PrP^{C}) is a conserved glycoprotein widely expressed in the brain and involved in several neuronal processes including neurotransmission. If converted to a conformationally altered form, PrP^{Sc} can cause neurodegenerative diseases, such as Creutzfeldt-Jakob disease in humans. Previous studies aimed at characterizing $Prnp^{0/0}$ mice have revealed only mild behavioral changes, including an impaired spatial learning, accompanied by electrophysiological and biochemical alterations. Interestingly, PrP^{C} is developmentally regulated and in the hippocampus its expression parallels the maturation of MF. Here, we tested the hypothesis that at immature (P3-P7) MF-CA3 synapses, PrP^{C} interferes with synaptic plasticity processes. To this aim, the rising phase of Giant Depolarizing Potentials (GDPs), a hallmark of developmental networks, was used to stimulate granule cells in the dentate gyrus in such a way that GDPs were coincident with afferent inputs. In WT animals, the pairing procedure induced a persistent increase in amplitude of MF-GPSCs. In contrast, in $Prnp^{0/0}$ mice, the same protocol produced a long-term depression (LTD). LTP was postsynaptic in origin and required the activation of cAMP-dependent PKA signaling while LTD was presynaptic and was reliant on G protein-coupled GluK1 receptor and protein lipase C downstream to G protein activation.

In addition, at emerging CA3-CA1 synapses of PrP^C-deficient mice, stimulation of Schaffer collateral failed to induce LTP, known to be PKA-dependent. Finally, we also found that LTD in *Prnp*$^{0/0}$ mice was mediated by GluK1 receptors, since UBP 302 blocked its induction. These data suggest that in the immature hippocampus PrP^C controls the direction of synaptic plasticity.

INTRODUCTION

1. The hippocampus

The hippocampus is a well characterized brain structure located deeply within the medial temporal lobe.

Its name derives from the Greek words hippos and kampos, meaning 'horse' and 'sea monster', respectively, since its shape resembles a seahorse. It is also named "Cornu Ammonis" (CA) because it is reminiscent of the horns of a ram.

The structures that line the edge of the cortex make up the so-called limbic system (*limbus* = *border*): these include the hippocampus, the cingulated cortex, the olfactory cortex, and the amygdala.

The term hippocampal formation generally applies to the dentate gyrus, the CA1-CA3 fields and the subiculum (Fig. 1). Information flowing through the hippocampus proceeds from the dentate gyrus (DG) to the CA3, the CA1 regions and the subiculum. The CA2 region represents only a very small portion of the hippocampus and is less studied since it is difficult to define its boundaries. A particular feature of the CA2 region is its high resistance to epileptic damage (Mercer et al., 2007). The CA3 region is critical for the initiation of hippocampal interictal-like activity due to the profuse recurrent excitatory connections between CA3 pyramidal cells (Miles and Wong, 1983). In area CA3, Lorente de No (1934) has identified 3 subareas by anatomical position CA3a, CA3b and CA3c, corresponding to the bend of CA3, the ventral portion between the bend and the lateral end of the DG and the portion encapsulated by the blades of the DG, respectively. CA3a pyramidal cells are more excitable and fire in bursts more frequently than CA3b cells and this may explain why the CA3a region is involved in the initiation of ensemble activity (Wittner and Miles, 2007). Hence, at least in guinea pigs, CA3a cells have more complex dendritic arbors than CA3b cells, especially in zones targeted by recurrent synapses (Wittner and Miles, 2007).

The entorhinal cortex (EC), the greatest source of hippocampal input and target of hippocampal output, is strongly and reciprocally connected with many other parts of the cerebral cortex, and thereby serves as the main "interface" between the hippocampus and other parts of the brain. The superficial layers of the EC project to the dentate gyrus through the so-called “perforant path”. The flow of information is largely unidirectional, with signals propagating from the EC to the dentate gyrus, hence to the CA3 field, to the CA1 field, to the subiculum, then out of the hippocampus back to the deep layers of EC.

In CA1-CA3 regions, pyramidal cell somata are arranged in stratum pyramidale. Other regions include the stratum oriens where the basal dendrites of pyramidal cells can be found, the stratum radiatum and stratum lacunosum moleculare, where the apical dendrites are radially oriented.

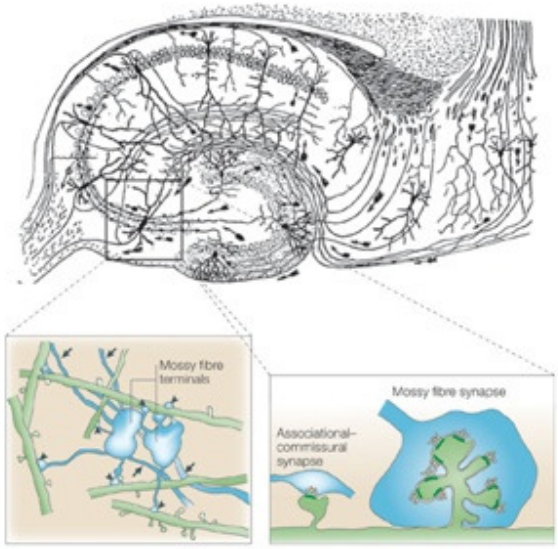

Figure 1: Classical drawing of the hippocampal formation by Ramón y Cajal. The left-hand panel shows a graphic representation of two large mossy fibre terminals, each with several filopodial extensions. The terminals of the filopodial extensions contact GABA (γ-aminobutyric acid)-containing neurons (arrowheads). Arrows point to the main axon of dentate gyrus granule cells. The right-hand panel compares a large mossy fibre synapse with the associational–commissural synapse, which are in close proximity on the dendrites of CA3 pyramidal cells (From Nicoll and Schmitz, 2005).

2. The hippocampal mossy fibers pathway

The mossy fibers (MFs) are the axons of dentate gyrus granule cells. First described by Golgi (1886) and Sala (1891) were named "mossy' by Ramon y Cajal (1911), because of their particular appearance at the light microscopic level, which reminds, as the MFs in the cerebellum, the shape of moss on trees (Fig. 1).

MFs not only project to CA3 pyramidal neurons, but also make synaptic contacts with basket cells in the dentate gyrus and with pyramidal-like neurons, the mossy cells, in the hilus (Johnston and Amaral, 1978). In addition, MFs make synaptic contacts with GABAergic interneurons present in the hilus and in the CA3 area and these represent the majority of all MF connections (Acsady et al. 1998). The ratio of synaptic contacts onto interneurons is approximately fourfold higher than that onto principal cells. Each granule cell contacts approximately 15 pyramidal neurons, while each pyramidal cell receives up to 50 MF inputs from granule cells (Claiborne et al., 1986). MFs give rise to large en passant swellings (up to 5 µm in diameter) and terminal expansions on CA3 principal neurons or mossy cells, seen as giant boutons at the electron microscopic level. The presynaptic varicosities adapt very well to the specialized postsynaptic elements present on proximal dendrites of CA3 principal cells, called thorny excrescences. The MF synaptic complex contains multiple active zones (up to 50) associated with postsynaptic densities. Acsady et al. (1998) demonstrated that MF connections terminate into interneurons with small boutons or filopodial extensions (Fig. 1). Differences in morphology between MF terminals at principal cells and interneurons may account for the distinct functional properties of these synapses which appear to be regulated in a target-specific way (Nicoll and Schmitz, 2005). However, all synapses made by the MF pathway (on both excitatory and inhibitory neurons) are relatively strong when compared to other excitatory cortical synapses.

2.1 Synaptic physiology

The MF synapses have been originally proposed as a model for studying synaptic transmission in the CNS (Brown and Johnston, 1983). In particular, in contrast to other excitatory synapses, they are located close to the soma of pyramidal cells making them ideally suited for accurately measuring synaptic currents in voltage clamp conditions (Brown and Johnston, 1983; Henze *et* al., 2000). Unfortunately, there are several complicating factors associated with the electrophysiological study of this pathway. First, the sparse connectivity between granule cells (GCs) and CA3 pyramidal cells hampers the possibility to perform pair recordings from these cells. In addition, the high complexity of the local circuit makes it difficult to study *pure* MF synaptic responses using bulk electrical stimulation of granule cells in the DG, or stimulation of the hilus, or of the stratum lucidum (the usual methods of MF stimulation) (reviewed in Henze *et* al., 2000). The existence of these contaminating factors has led to a set of criteria to optimize the probability of studying a "pure" MF response. MF responses should have a relatively fast rising time (~3 ms), without inflections and variable latencies due to polysynaptic recruitment of interconnected CA3 pyramidal cells or anti-orthodromic activation of MF hilar collaterals. In some cases polisynaptic activity can be minimized by using a low concentration of AMPA receptor antagonists or high divalent cations (Henze *et* al., 2000). The selective inhibition of MF responses by group II and III mGluR agonists has been successfully used to discriminate between monosynaptic responses evoked by MF and responses due to antidromic activity of CA3 axons (Salin *et* al. 1996; Henze *et* al., 2000).

An alternative approach to study MF transmission consists in recording single fiber responses (Allen and Stevens, 1994; Jonas et al., 1993). With this technique, a small stimulating electrode is placed in the granule cell layer and the stimulation intensity is decreased until only a single axon is activated. This is achieved when the mean amplitude of the postsynaptic currents and failure probability remain constant over a range of stimulus intensities near threshold for detecting a response. Small movements of the stimulating electrode 20–30 μm away from the initial location lead to the loss of the evoked response.

In addition, in monosynaptic responses the latency and the shape of synaptic currents should remain constant in case of repeated stimuli and/or reduced extracellular Ca^{2+} /Mg^{2+} concentration ratio.

2.1.1 Adult MFs are glutamatergic

In adulthood, the primary excitatory neurotransmitter released from MF terminals is glutamate. This acts on AMPA, NMDA and kainate receptors (KARs). Activation of KARs by a single stimulus or a brief train of stimuli to the MFs generate slow (tens of milliseconds) EPSCs (Castillo *et* al., 1997; Vignes and Collingridge, 1997; Fig. 2), which account for less than 10% of the total amplitude of MF-EPSCs (Pinheiro *et* al., 2007).

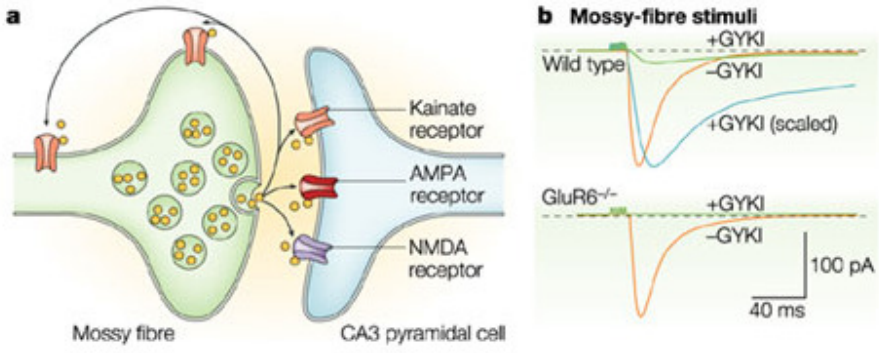

Figure 2: Schematic diagram showing the pre- and postsynaptic arrangement for kainate receptors at the mossy fibre–CA3 contact. At these synapses, synaptically released glutamate activates kainate receptors located at both presynaptic and postsynaptic sites. b. Under selective antagonism of the postsynaptic AMPA receptor-mediated current by GYKI 53655, the activation of mossy fibres by a short train of impulses reveals a smaller synaptic component produced by activation of kainate receptors. This kainate receptor-mediated excitatory postsynaptic current (EPSC) have much slower activation and deactivation kinetics than those mediated by AMPA receptors. In GluR6 knockout mice (GluR6 $^{-/-}$), the slower synaptic response is absent (Modified from Lerma, 2003).

The different kinetics between AMPA- and KAR-mediated responses may interfere with synaptic integration and temporal coding (Frerking and Ohliger-Frerking 2002). According to a recent paper by Pinheiro *et* al. (2012), the sustained KAR-mediated synaptic depolarization would contribute to heterosynaptic facilitation when additional inputs (i.e. the associative/commissural pathway) are concomitantly activated.

In addition to their ionotropic function, postsynaptic KARs inhibit a Ca^{2+}-activated K^+ current (I_{sAHP}) *via* a metabotropic signaling pathway, leading to increased excitability of CA3 pyramidal cells (Ruiz et al. 2005).

2.1.2 In particular conditions, adult MFs can co-release glutamate and GABA

In particular conditions, in addition to glutamate, MFs can release GABA. Sandler and Smith (1991) were the first to describe the presence of GABA in glutamatergic MF terminals in both humans and monkeys. Further evidence in favor of a co-release of GABA and glutamate has been recently provided by Zander et al., (2010) who, using post-embedding immunogold double labeling, have demonstrated the coexistence of VGAT and VGLUT (vesicular transporters for GABA and glutamate, respectively) in MF terminals. Gutierrez and Heinemann (2001) have convincingly shown that, in kindled rats, stimulation of granule cells in the dentate gyrus can evoke in addition to a glutamatergic response a monosynaptic bicuculline-sensitive GABAergic inhibitory postsynaptic current (IPSPs). Interestingly, high-frequency MF stimulation led to IPSPs summation and consequent reduction in the probability of generating an action potential. Other studies have shown that the expression of GAD 67, GAD 65, and VGAT can be transiently up-regulated in an activity-dependent way (Gutierrez, 2002; Ramırez and Gutierrez, 2001). Moreover, hippocampal pyramidal neurons are able to express not only glutamate receptors but also 'mistargeted' $GABA_A$ receptors which, in particular conditions may become functional (Rao et al., 2000).

Together all these evidences strongly suggest that, in particular conditions, MFs can use GABA as a neurotransmitter since they posses all the machinery for synthesizing, storing, releasing, and sensing GABA.
The release of GABA from MF terminals during seizures might counteract the enhanced excitability associated with the epileptic activity (Jaffe and Gutierrez, 2007).

2.1.3 Presynaptic modulation of glutamate release

MFs are endowed with a number of receptors whose activation regulates glutamate release, including metabotropic glutamate receptors (Kamiya *et* al., 1996), KARs (Castillo *et* al., 1997; Vignes and Collingridge, 1997), adenosine receptors (Thompson *et* al., 1992), peptides (Weisskopf *et* al., 1993), $GABA_A$ (Ruiz *et* al., 2003; Jang *et* al., 2006) and $GABA_B$ receptors (Min *et* al., 1998; Vogt and Nicoll, 1999).

Glutamate receptors

MFs express specific subtypes of metabotropic glutamate receptors (mGluRs), which strongly depress neurotransmitter release (Nicoll and Schmitz, 2005). Molecular cloning has revealed a family of eight genes encoding for mGluRs. These are divided into three groups: group I (mGluR1 and 5); group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8). MF terminals express group II and III (Lanthorn *et* al., 1984; Shigemoto *et* al., 1997). Interestingly, while type II are localized at the preterminal level (Yokoi *et* al., 1996), type III at terminal level. Therefore, in order to be activated group II mGluRs need synaptic spillover of glutamate following high frequency stimulation of MF (Toth *et* al., 2000; Scanziani *et* al., 1997). Synaptically released glutamate can diffuse out of the synapses and activate type II mGLuRs present at extrasynaptic sites and also on neighboring mossy fiber terminals (Vogt and Nicoll, 1999). In conclusions, presynaptic mGluRs act as autoreceptors to limit the amount of glutamate release.

Granule cells express kainate receptors encoded by four different genes (GluK1, GluK2, GluK4 and GluK5; Wisden and Seeburg, 1993). KARs play a central role in modulating MF-CA3 synaptic transmission (Contractor *et* al., 2001; Lauri et al., 2001; Schmitz et al., 2001; Sachidhanandam et al., 2009).

An intriguing aspect of KARs function is that they control glutamate release in a bi-directional fashion. While a high concentration of kainate (>100 nM) inhibits glutamate release, a lower concentration (<50 nM) facilitates glutamate release (Rodriguez-Moreno *et* al., 2004; Lauri *et* al., 2001-2003a; Contractor *et* al., 2000; Schmitz *et* al., 2001; Breustedt and Schmitz, 2004; Kamiya et al., 2002).

Synaptically released glutamate mimics the effects of bath application of low doses of kainate and contributes to the pronounced frequency-dependent facilitation characteristic of mossy fiber synapses (Schmitz *et* al., 2001; Lauri *et* al., 2001; Contractor *et* al., 2001). This may occur *via* depolarization of presynaptic boutons or axon terminals (Lauri *et* al., 2001; Kamiya *et* al., 2002; Nicoll and Schmitz, 2005).

Synaptic facilitation has been found to be significantly reduced in GluK2-deficient mice, but unaffected in mice lacking the GluK1 subunit (Contractor et al., 2001; Breustedt and Schmitz 2004). On the other hand, the reduction of glutamate release induced by high concentrations of kainate may involve a G protein and a metabotropic type of action (see Rodriguez-Moreno and Sihra, 2011; Fig. 3).

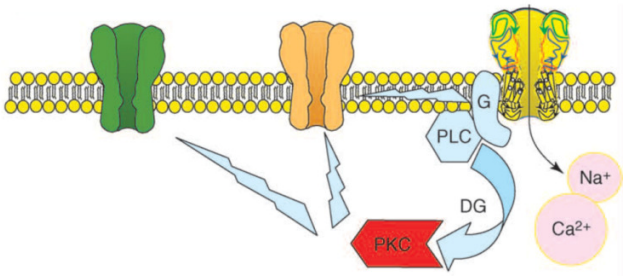

Figure 3: Dual signaling by KARs. KARs could open *via* a canonical pathway cationic ion channels and depolarize the membrane.

However, they can also activate a non-canonical signaling pathway involving the activation of a pertussis toxin-sensitive G protein (G) and phospholipase C (PLC). This in turn leads to the activation of PKC, probably through an increase in diacylglycerol (DG). This second-messenger system may inhibit Ca2+ channels and modulate transmitter release (Modified from Lerma, 2006).

$GABA_A$ and $GABA_B$ receptors

The presence of ionotropic $GABA_A$ receptors on adult MF terminals has been unambiguously demonstrated by Alle and Geiger (2007) who, using patch-clamp recordings from single MF boutons, detected $GABA_A$-mediated responses following local application of GABA from a puff pipette. Activation of these receptors by ambient GABA or GABA released from neighboring interneurons leads to a reduction of MF's excitability (Ruiz *et* al., 2003). Interestingly, in mechanically dissociated rat hippocampal CA3 neurons with adherent presynaptic nerve terminals, exogenously application of muscimol has been found to significantly increase the frequency of spontaneous excitatory postsynaptic currents, an effect that was prevented by selective $GABA_A$ receptor antagonists (Jang *et* al., 2006).
$GABA_B$ receptor activation has been shown to inhibit glutamate release from MF terminals in both acute hippocampal slices (Henze *et* al., 2000) and organotypic slice cultures (Thompson *et* al., 1992)

Adenosine Receptors

Pharmacological block, genetic deletion of A1 receptors or exposure to adenosine-degrading enzymes, enhances synaptic transmission at MF-CA3 synapses and reduces short-term synaptic potentiation (Moore *et* al., 2003). Therefore, adenosine acting on A1 receptors contributes to the low initial release probability of these synapses.

Neuropeptides

The MF pathway contains neuropeptides stored in large dense-core vesicles from which they can be released. The giant MF boutons contain several peptides including dynorphin, enkephalin (McGinty *et* al., 1983), cholecystokinin (CCK; Gall, 1984) neuropeptide Y (NPY; Sperk *et* al., 1992) and neurokinin-B (Schwarzer and Sperk, 1995).

3. Synaptic plasticity

MF synapses are highly plastic. Several forms of homosynaptic short- and long-term synaptic plasticity have been reported at MF-CA3 synapses, including paired-pulse facilitation, frequency-dependent facilitation, post-tetanic potentiation, long-term potentiation (LTP) and long-term depression (Salin *et* al., 1996; Toth *et* al., 2000; reviewed in Nicoll and Schmitz, 2005).

3.1 Short-term plasticity

Short-term plasticity differs significantly from that observed in the majority of CNS synapses: MF-CA3 synapses exhibit very strong paired-pulse facilitation (usually with a ratio >3; Salin *et* al., 1996) and frequency-dependent facilitation. The strong paired-pulse facilitation largely depends on the basal release probability which, in spite of the elevated number of release sites, is very low (Chicurel and Harris, 1992), leading in some cases to synapse silencing. In contrast, MF-interneurons synapses usually exhibit paired-pulse inhibition due to a larger release probability and a small number of release sites (Lawrence et al., 2004).

Impressive is also the selective ability of MF synapses to undergo frequency-dependent facilitation. By increasing the frequency of stimulation from low (i.e. 0.05 Hz) to moderate (1 Hz) rates causes manifold increase in synaptic strength (Nicoll and Schmitz, 2005).

In striking contrast to MF synapses, neighboring associational-commissural (A/C), and Shaffer collateral-CA1 synapses, show little facilitation (Salin *et* al., 1996; Dobrunz and Stevens, 1999). Pronounced short-term frequency-dependent facilitation is particularly prominent at large MF boutons targeting CA3 pyramidal neurons, whereas mossy fibre terminals synapsing into GABAergic interneurons show much less facilitation or even depression (Toth *et* al., 2000). Frequency-dependent synaptic depression in interneurons leads to a reduced feed-forward inhibition and further facilitation of CA3 pyramidal neurons (Mori *et* al., 2004). The cellular mechanisms underlying short-term plasticity are incompletely understood. As already mentioned, at MF-CA3 synapses, frequency-dependent facilitation involves presynaptic KARs (Contractor *et* al., 2001; Lauri *et* al., 2001; Schmitz *et* al., 2001, see above). A simple explanation for this phenomenon is that KARs activated by glutamate released from MF terminals depolarize presynaptic boutons or axons (Lauri *et* al., 2001; Kamiya *et* al., 2002; Nicoll and Schmitz, 2005). Indeed, the axonal depolarization induced by elevating the extracellular K^+ concentration can mimic synaptic facilitation associated with presynaptic KARs activation (Schmitz *et* al., 2001). Repetitive spiking would lead to the inactivation of presynaptic K^+ channels, to action potentials broadening, increase in calcium entry and enhanced transmitter release (Geiger and Jonas, 2000). Calcium entry may in turn enhance Ca^{2+} release from internal Ca^{2+} stores *via* a calcium-induced calcium release mechanism (Lauri et al., 2003; Scott and Rusakov, 2006; Shimizu et al., 2008). A similar mechanism occurs at single MF boutons (Scott *et* al., 2008; Fig. 4).

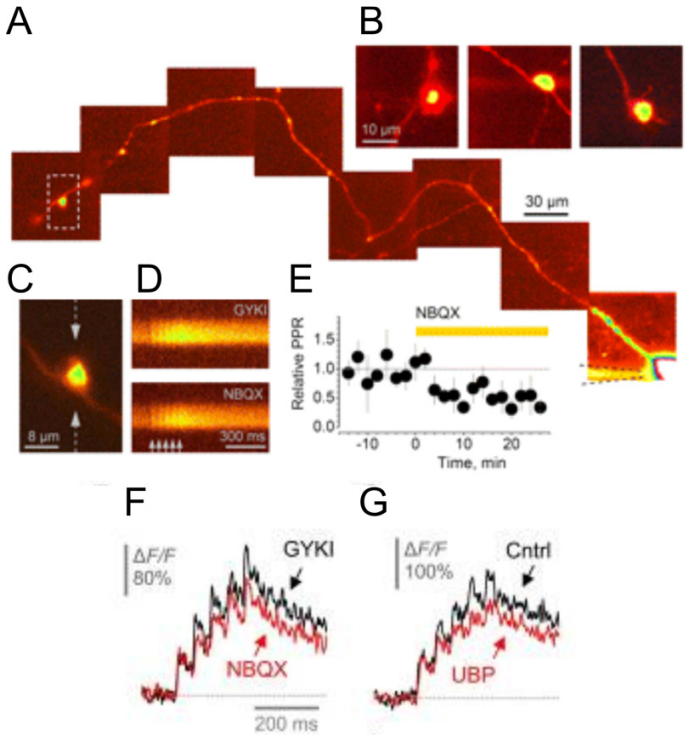

Figure 4: Kainate receptors regulate short-term plasticity by modulating presynaptic Ca^{2+} entry at individual MF–CA3 pyramidal cell synapses. (A) a granule cell axon traced from the soma (Alexa Fluor 594 emission, collage); the CA3 giant bouton of interest is marked by a dashed frame and enlarged in *C*. (B) examples of giant MF boutons traced in area CA3, 300–700 µm from cell bodies. (C–D) in the giant bouton shown in C (also A), five action potentials at 20 Hz (arrows) evoke clear Ca^{2+}-dependent fluorescence signals (line-scans in *D*, Fluo-4 emission; 10-sweep average), in the presence of 20 µm GYKI53655 (GYKI), and after application of NBQX, as indicated. Line scan positioning is shown in *C* by dotted arrows. (E) The average time course for the effect of KAR blockade by NBQX (in the presence of GYKI, n = 10) on the paired-pulse ratio of action-potential-evoked Ca^{2+} responses, PPR = $(\Delta F/F)_2/(\Delta F/F)_1$. Error bars, SEM. (F–G), blocking KARs with NBQX (F) or with 10 µm UBP302 (*G*) has little effect on the resting Ca^{2+} fluorescence but decreases PPR. Traces, characteristic line scans (10-sweep average), as indicated (Modified by Scott et al., 2008).

MF synapses also undergo post-tetanic potentiation (PTP), a form of short-term plasticity that decays with a time constant of about 3 min (Henze et al., 2000).

3.2 Long-term plasticity

MF-CA3 synapses exhibit long-term synaptic plasticity that differs in many aspects from that observed in other brain regions. In particular, high frequency stimulation of MF induces in CA3 principal cells a form of long-term potentiation (LTP) that is independent of NMDA receptor (NMDAR) activation and is presynaptically in origin (reviewed in Nicoll and Schmitz, 2005). The signaling pathways underlying LTP induction are still debated, with evidence in favor and against the involvement of postsynaptic mechanisms including mobilization of calcium from internal stores after mGluR activation or calcium entry through voltage-gated calcium channels (Yeckel *et* al., 1999; Mellor and Nicoll, 2001; reviewed in Nicoll and Schmitz, 2005). Because the expression of LTP occurs presynaptically, any postsynaptic induction mechanism must involve a retrograde messenger (Contractor *et* al., 2002; Armstrong *et* al., 2006). Presynaptic expression of MF-LTP is known to engage cAMP and its downstream signaling pathway (Weisskopf *et* al., 1994), which leads to an increase in release probability (Nicoll and Schmitz, 2005).

Recently, it has been found that mossy fiber synapses display a form of LTP selective for NMDARs which acts as a metaplastic switch making mossy fiber synapses competent for generating NMDAR-dependent LTP of AMPA-mediated EPSCs (Rebola *et* al., 2011).

Another form of synaptic plasticity extensively studied at excitatory glutamatergic synapses is spike-time-dependent plasticity (STDP). This is a particular form of Hebbian type of learning which consists in bidirectional modifications of synaptic strength according to the temporal order of pre and postsynaptic spiking (Dan and Poo, 2006; Fig. 5).

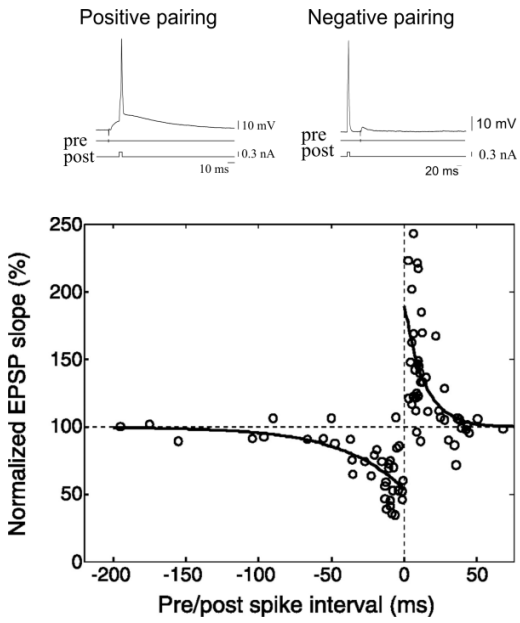

Figure 5: Synaptic modification induced by repetitively pairing pre- and postsynaptic spikes in layer 2/3 of visual cortical slices from the rat. Each symbol represents result from one experiment. Curves are single exponential, least-squares fits of the data. *Insets* depict the sequence of spiking in the pre- and postsynaptic neurons in cases of positive and negative pairing, respectively. EPSP, excitatory postsynaptic (Modified from Dan and Poo, 2006).

Thus, positively correlated pre and postsynaptic spiking (pre before post) within a critical window leads to LTP, whereas a negative correlation (post before pre) leads to long-term depression (LTD). At juvenile MF-CA3 synapses STD-LTP or LTD needs the involvement of postsynaptic NMDA receptors (Astori *et* al., 2010). According to these authors, timing-dependent LTP could be induced when MF stimulation is followed by a burst of three postsynaptic action potentials (at 50 Hz). This protocol closely mimics the firing rate of granule cells in freely moving animals (Jung and McNaughton, 1993; Henze *et* al*,* 2002). Timing-dependent LTD, instead, can be induced when a single AP precedes synaptic activation by 30 ms.

In vivo, both single APs and AP bursts occur at a low frequency in granule cells as well as in CA3 neurons (McNaughton *et* al. 1983; Jung and McNaughton, 1993; Hahn *et* al. 2007). Therefore, Astori *et* al. propose that the near-coincidence of single spikes and spike bursts might be a relevant mechanism for adapting synaptic strength *in vivo*.

Besides spike-time-dependent LTD, evidence exists that stimuli similar to those used to induce NMDAR-dependent LTD at Schaffer collateral-CA1 synapses (for example, 1 Hz for 15 min) promote LTD at MF-CA3 synapses (Kobayashi *et* al., 1996). This form of LTD, which requires the activation of presynaptic mGluR2 (Kobayashi *et* al., 1996) and reduction of adenylyl cyclase activity, leads to a decrease in the activity of the cAMP-dependent protein kinase (PKA) and a reversal of the presynaptic processes responsible for mossy fiber LTP (Tzounopoulos et al., 1998).

4. Functional role of MF

The hippocampus has long been known to be involved in high cognitive functions such as memory formation (i.e., episodic memory; see Squire, 1992) and spatial navigation (O'Keefe and Dostrovsky, 1971). It allows the formation of distinct memories of similar episodes by generating distinct representations of the temporal and spatial relationships comprising the events (pattern separation). This ability is critical because although many episodes we experience daily have similarities, it is often important to memorize distinct features of a particular episode. The hippocampus is also involved in the recall of previously acquired memories by reactivating their full representations using partial information as recall cues (pattern completion). For pattern completion, synaptic transmission and plasticity in the recurrent associative network in the CA3 region have been proposed to play a crucial role on a theoretical basis (Marr, 1971; McNaughton and Morris, 1987; O'Reilly and McClelland, 1994; Treves and Rolls, 1994; Hasselmo et al., 1995).

Conversely, synaptic transmission and plasticity in the feed-forward pathway from the entorhinal cortex-DG-CA3 have been implicated in pattern separation (Marr, 1971; McNaughton and Morris, 1987; O'Reilly and McClelland, 1994; Treves and Rolls, 1994; Leutgeb *et* al., 2007; Bakker *et* al., 2008).

Importantly, CA3 pyramidal cells receive a prominent excitatory input *via* MF to their proximal apical dendrites (Ramon y Cajal, 1911; Claiborne *et* al., 1986), which makes possible a strong spike coupling between the DG and the CA3 region (Henze *et* al., 2002), unusual in excitatory cortical circuits. Moreover, unlike most pyramidal cells, which exhibit spontaneous firing, background activity in hippocampal CA3 principal cells and granule cells is very low (Barnes *et* al., 1990; Quirk *et* al., 1992; Jung and McNaughton, 1993). This allows pyramidal cells to accurately modulate their firing pattern according to environmentally relevant stimuli. Apparently, indeed, the hippocampus and the other cortical regions ''speak'' different neuronal languages. In computational terms, in comparison to other cortical areas, the hippocampus uses a sparse code. Therefore, an interface is needed between the neocortex and the hippocampus. At the gate of the hippocampal formation, the DG possesses a set of unusual properties which enable it to implement the task of code transformation between cortical afferents and the hippocampus. Specifically, the DG translates noisy signals of upstream cortical areas to the sparse and specific code of hippocampal formation, which is indispensable for the formation of multiple memory items.

As argued on theoretical grounds by Treves and Rolls (1992), the storage of new memory traces, ideally accomplished through the CA3 recurrent network, requires a type of input characterized by a small number of individually strong synapses per cell. The MF pathway definitely meets these requirements. Available behavioral data from rodents with lesions of dentate granule cells or MF are consistent with a crucial role of DG in providing input to area CA3 during the acquisition of spatial information (Gilbert *et* al., 2001; Lassalle *et* al., 2000; Lee and Kesner, 2004).

It seems therefore likely that memory formation in the hippocampus occurs in a two-steps process: sorting of entorhinal signals by granule cells, followed by association of the sparse code in the CA3 network.

According to this hypothesis, the output of the small population of granule cells that become active in a given environmental context (e.g., during a couple of theta cycles as the rat passes through their place fields) activates GABAergic interneurons which exert fast and strong feedback inhibition on the somata and dendrites of non-coding granule cells, shunting their entorhinal inputs and precluding them from firing. This effect is facilitated by the lack of interaction among basket cells and other interneurons in the hilus (Acsady *et* al., 2000). Moreover, synaptic transmission at MF-interneuron synapses is characterized by very fast kinetics (Geiger et al., 1997). This effect has been attributed to the high synchrony of transmitter release and the rapid time course of AMPA receptor deactivation. The fast postsynaptic response allows a rapid activation of feedback inhibition that can be used to suppress the activity of granule cells in a competitive manner. As a result, synaptic plasticity only takes place at the perforant path input of the coding (granule) cells and this would further strengthen the competitive process, resulting in the typical sharp and focused place fields of the granule cells (Jung and McNaughton, 1993) which are in sharp contrast to the grid-like entorhinal signals (Hafting *et* al., 2005). The cortical signal will be then transmitted to the associational station within the CA3 region for memory storage.

Giant mossy terminals display all the morphological features of a classic sparse ‘‘detonator’’. As already mentioned, presynaptic varicosities present on MF terminals adapt very well to the specialized postsynaptic elements present on proximal dendrites of CA3 principal cells, called thorny excrescences. The MF synaptic complex contains multiple active zones (up to 50) associated with postsynaptic densities (Chicurel and Harris, 1992; Acsady *et* al., 1998). The short electrotonic distance of MF terminals from the soma facilitates the probability of driving postsynaptic cells to fire (Mori *et* al., 2004; Henze *et* al., 2002). At the same time, the number of large mossy terminals originating from a single granule cell and impinging within the area CA3 is very low (average: 12.3; Acsady *et* al., 1998). Thus, the sparse code generated in DG granule cells is transferred very effectively only to a restricted set of postsynaptic pyramidal cells.

In addition, by activating GABAergic interneurons, granule cells provide an unusually strong feed-forward inhibition to the CA3 area.

Since even a single basket cell is able to block action potential generation in a large number of pyramidal cells (Miles *et* al., 1996), feed-forward inhibition will effectively reduce the number of active CA3 pyramidal cells during MF activation. In this way only a small number of CA3 pyramidal cells receiving MF signals will be active, and following the Hebbian rule, only the recurrent synapses between the activated CA3 cells will be potentiated. The ensemble of potentiated synapses would represent a memory trace.

In summary, the mossy fiber pathway maximally utilizes the possibility to differentially regulate its postsynaptic partners. Selective innervations of pyramidal cells and interneurons by distinct presynaptic terminals create a favorable condition to differentially regulate short-term and long-term plasticity at MF-pyramidal neurons and MF-interneurons synapses. Moreover, feed-forward inhibition makes the system highly dynamic, determining exactly which firing patterns of granule cells will induce permanent modification of the auto-associational network of the CA3 region, required for memory storage.

5. Development of the hippocampus

Both the neocortex and the archicortex, including its main derivative, the hippocampus, originate from the dorso-medial telencephalic pallium. In the early stages of development, the undifferentiated germinal cells forming the so-called neuroepithelium are localized in the ventricular zone. Pluripotent progenitors arise from the neuroepithelial cells and lead to the generation of neuronal and glial precursors. The first postmitotic cells migrate in a radial manner, out of the neuroepithelium to form the preplate (or primordial plexiform layer), which represents the first recognizable cortical layer. In the rat, the preplate is present at day 12-15 after conception. The cortical plate expands and forms six cortical layers which compose the adult rat neocortex. In the developing hippocampus, the marginal zone is subdivided into two segregated sublayers: the inner marginal zone (IMZ) and the outer marginal zone (OMZ). The developing hippocampal cortical plate (HP) does not become a multi-laminar cortex but remains a cell dense pyramidal cell layer (PY). The stratum lacunosum moleculare (SLM) originate from the OMZ, whereas the stratum radiatum (SR) derives from the IMZ and the subplate becomes the stratum oriens (SO).

The hippocampal pyramidal neurons are generated between the embryonic day 16 (E16) and 20 (E20), in the ventricular zone of the pallium (Bayer, 1980a; Bayer, 1980b). From here, they migrate towards the hippocampal plate, using the radial glial fibres. The migration features an inside-out sequence with the newly arrived neurons settling in the upper level of the already migrated cells. The pyramidal cell layer becomes recognizable in areas CA1 and CA3 around E20. Once the migration is completed, the radial glial cells undergo changes in their morphology and become astrocytes. At about the same time, the granule cells in the DG are also generated, but most of the DG neurogenesis occurs later. Approximately 85% of the granule cells are generated, indeed, during the first postnatal month (Bayer, 1980a; Bayer, 1980b). They originate mainly in the ventricular zone and in the hilus, with only a small proportion being generated within the DG itself. Granule cells migrate along glia fibres. GABAergic cells in the hippocampus are generated before pyramidal neurons between E14 and E15. Between E16 and E17 they migrate tangentially from the ventricular zone of the subpallial telencephalon to their final destination. This migration process is supposed to be calcium dependent and regulated by NMDA, GABA receptors and voltage-dependent calcium channels (Spitzer, 2002; Komuro and Rakic, 1998). GABA depolarizes neuronal progenitor cells thus favoring calcium entry *via* voltage-dependent calcium channels. Calcium signaling in turn activates several metabolic pathways necessary for cell survival and differentiation including DNA synthesis (LoTurco et al., 1995). At this early developmental stage, neurotransmitters can influence neurogenesis by acting at distance from their release sites (growth cones) in a paracrine way (Demarque *et* al., 2002). Thus, "ambient" GABA and glutamate, present in the extracellular space, may influence neuronal signaling by binding mainly to extrasynaptic receptors. The release of neurotransmitters may occur in a non-vesicular fashion and does not require vesicular release proteins. Later in development, after the formation of conventional synapses, this system is replaced by a more focal form of neuronal communication. Besides neurotransmitters, other factors such as extracellular matrix proteins, neurotrophins and adhesion molecules contribute to regulate neurogenesis (Owens and Kriegstein, 2002). It is worth noting that in granule cells, neurogenesis persists in adulthood (van Praag *et* al.,2002). Also in this case, before being innervated by the pre-existing functional circuitry (Ge *et* al., 2006a), newborn granule cells are tonically activated by ambient GABA.

Studies on rats (Tyzio *et* al., 1999; Hennou *et* al., 2002) and non-human primates (Khazipov *et* al., 2001) have shown that in both interneurons and pyramidal cells, functional GABAergic synapses are formed before glutamatergic ones. Interestingly, the GABA - glutamate sequence is retained in newborn granule cells during adult neurogenesis (Ge *et* al., 2006a).

5.1 GABAergic signaling early in postnatal development

GABA is the main inhibitory transmitter in the adult brain. GABA binds and activates two different classes of receptors: $GABA_A$ and $GABA_B$. $GABA_A$ receptors are ligand-gated ion channels, permeable mainly to Cl^- and $HCO3^-$ (Cherubini and Conti, 2001). The opening of $GABA_A$ receptor channels causes a net flux of chloride inside the cells (while $HCO3^-$ flux is directed outwards) with consequent membrane hyperpolarization and reduction of cell firing.

The $GABA_B$ receptors do not contain an integral ion channel and are coupled with cationic channels (usually inwardly rectifying potassium channels) *via* G_i and G_o proteins (reviewed in Bettler *et* al., 2004; Couve *et* al., 2000). Activation of $GABA_B$ receptors causes a membrane hyperpolarization and a reduction of cell firing (Luscher *et* al., 1997). As already mentioned, $GABA_A$ and $GABA_B$ receptors are localized also on GABAergic or glutamatergic terminals where they contribute to regulate the release of GABA and glutamate, respectively (Amico *et* al., 1995; Mintz and Bean, 1993; Poncer, 1997).

Interestingly, in the immediate postnatal period, when glutamatergic synapses are still poorly developed (Hosokawa *et* al., 1994; Tyzio *et* al., 1999;), GABA *via* $GABA_A$ receptors depolarizes and excites targeted cells through an outwardly-directed flux of chloride (Ben-Ari *et* al., 1989; Fig. 6).

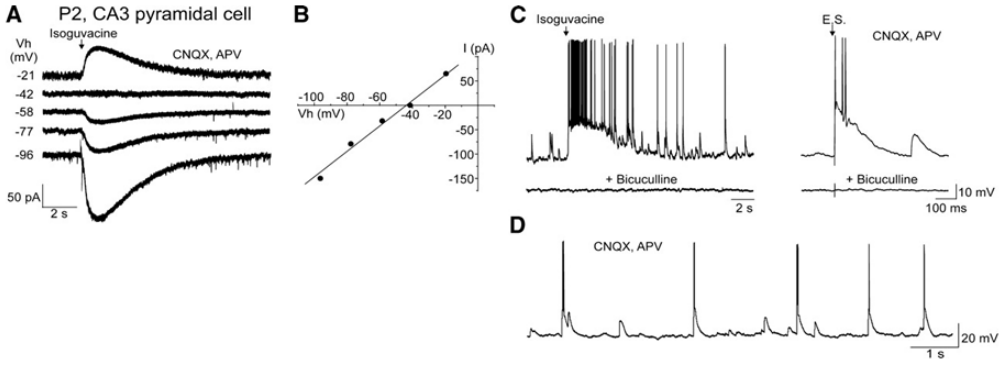

Figure 6: $GABA_A$-mediated postsynaptic responses in a P2 CA3 pyramidal cell with gramicidin perforated patch. (A) responses evoked by pressure activation of the $GABA_A$ agonist isoguvacine in voltage-clamp mode at different holding potentials. (B) dependence of the peak of the isoguvacine-induced responses on the membrane potential. Note that the responses reverse at −44 mV. (C) current-clamp recordings of the same neuron. Brief application of isoguvacine (left) and electrical stimulation (E.S.) of the slice in the presence of the ionotropic glutamate receptors antagonists CNQX and d-APV (right) evoke a membrane depolarization and action potentials. The responses are blocked by bicuculline (20 µM, traces below). (D) in the presence of the glutamate receptors antagonists CNQX and D-APV, spontaneous $GABA_A$-mediated postsynaptic potentials often result in action potentials generation. In C and D, the membrane potential was held at −80 mV (Modified by Ben-Ari et al., 2007).

Later on, towards the end of the second postnatal week, GABA action shifts from the depolarizing to the hyperpolarizing direction as in adulthood. In the developing hippocampus, the depolarizing action of GABA has been shown to be dependent on the elevated intracellular chloride concentration (Ben-Ari *et* al., 1989). The intracellular chloride concentration is under control of two main Cl^- co-transporters, the NKCC1 and KCC2 that enhance and lower $[Cl^-]_i$, respectively (Blaesse *et* al., 2009). Due to the low expression of the KCC2 extruder at birth, chloride accumulates inside the neuron *via* NKCC1.

Starting from the end of the first postnatal week, the progressive up-regulation of the expression of KCC2 is responsible for the shift of GABA from depolarizing to hyperpolarizing (Rivera *et* al., 1999). KCC2, indeed, extrudes K^+ and Cl^- using the electrochemical gradient for K^+ (Khirug *et* al., 2005; Luhmann and Prince, 1991).

Several studies strongly suggest that the depolarizing action of GABA is essential for the morphological maturation of neonatal cortical neurons *in vivo* (Cancedda *et* al., 2007; Wang and Kriegstein, 2008; Dzhala *et* al., 2005; Sipila *et* al., 2006).

Importantly, in the immature hippocampus, the depolarizing action of GABA enables the induction of synchronized activity, the so called giant depolarizing potentials (GDPs), which consist in recurrent membrane depolarization with superimposed action potentials, separated by quiescent intervals (Ben-Ari *et* al., 1989; Fig. 7).

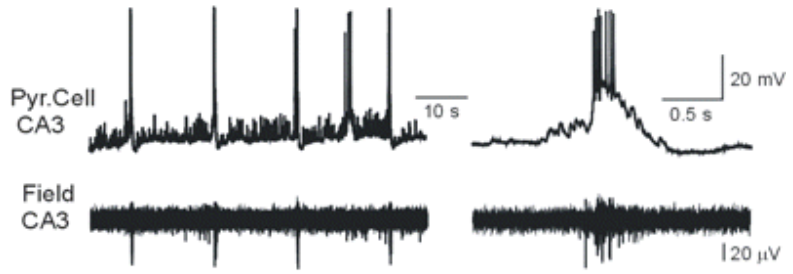

Figure 7: Giant depolarizing potentials in the neonatal rat hippocampus. A: gramicidin perforated patch recordings from a CA3 pyramidal cell in P6 rat hippocampus. Concomitant field potential recordings are shown on the trace below. Note that GDPs in CA3 pyramidal cell coincide with the field potential population burst. One GDP and the corresponding extracellular field potentials are shown on expanded time scale in the right panel (Modified from Ben-Ari, 2007).

GDPs can be considered a primordial form of synchrony between neurons, which usually precedes more organized forms of activity such as theta and gamma rhythms (Buzsaki and Draguhn, 2004).

Correlated network activity constitutes a hallmark of developmental networks, well preserved during evolution, that has been observed not only in the hippocampus but in almost every brain structure examined, including the retina (Feller *et* al., 1997), the neocortex (Owens *et* al.,1996; Dammerman *et* al., 2000; Maric *et* al., 2001), the hypothalamus (Chen *et* al., 1996), the cerebellum (Yuste and Katz, 1991; Eilers *et* al., 2001) and the spinal cord (Wang *et* al.,1994; O' Donovan, 1999).

The depolarizing action of GABA during GDPs results in calcium influx through the activation of voltage-dependent calcium channels and N-methyl-D-aspartate (NMDA) receptors (Leinekugel *et* al., 1997; Garaschuk *et* al., 1998). Calcium entry leads to the activation of intracellular signaling pathways known to contribute to several developmental processes such as neuronal migration, differentiation and synaptogenesis (Owens and Kriegstein, 2002).

How GDPs are generated is still a matter of debate. A tonic $GABA_A$-mediated conductance, well developed at birth (Marchionni *et* al., 2007), would bring the membrane to the voltage window where voltage-dependent bursts are generated (Sipila *et* al., 2005).

The presence of intrinsically bursting neurons, which, by virtue of their spontaneous discharge and large spike output, can drive other neurons to fire in synchrony may contribute to trigger GDPs (Menendez de la Prida and Sanchez-Andres, 2000; Sanabria *et* al., 2001; Sipila *et* al., 2005; Safiulina *et* al. 2008).

5.2 GABA is the main neurotransmitter released from immature MF terminals

Immediately after birth, GABA appears to be the only neurotransmitter released from MF terminals (Fig. 8). Minimal stimulation of granule cells in the dentate gyrus evokes in CA3 principal cells monosynaptic currents that completely fulfill the criteria for MF identification. These can be identified on the basis of their sensitivity to group III mGluR agonist L-AP4 and on the basis of their strong paired pulse facilitation and short term frequency-dependent facilitation (Salin *et* al., 1996).

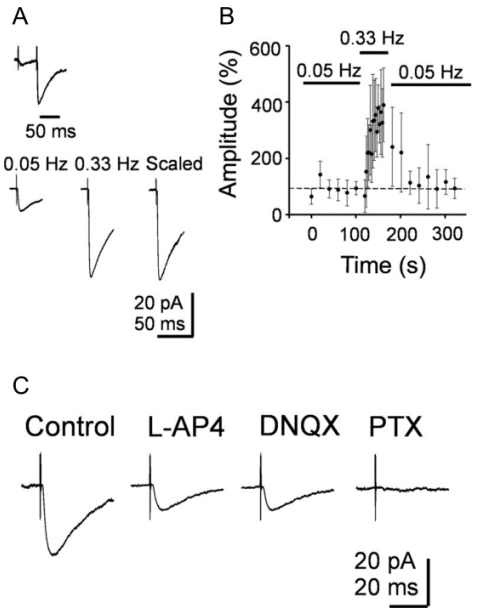

Figure 8: Minimal stimulation of granule cells in the dentate gyrus evokes $GABA_A$-mediated monosynaptic responses in CA3 pyramidal cells. (A) Paired stimuli delivered at 50 ms interval to granule cells in the dentate gyrus evoked at -70 mV synaptic responses exhibiting strong paired-pulse facilitation (average of 50 individual responses). Bottom, mean amplitude of synaptic responses (average of 15 individual traces) evoked by stimulation of granule cells in the dentate gyrus at 0.05 and 0.33 Hz. On the right, the two responses are normalized and superimposed. (B) The mean amplitude of synaptic currents evoked in three pyramidal cells at 0.05 and 0.33 Hz (bars) is plotted against time. Note the slow build-up of facilitation of synaptic responses at 0.33 Hz that completely reversed to control values after returning to 0.05 Hz stimulation. Error bars represent SEM. (C) Synaptic responses from another pyramidal cell before and during application of L-AP4 (10 μm) and L-AP4 plus DNQX (20 μm). Addition of picrotoxin (PTX; 100 μm) completely abolished synaptic currents. Each response is the average of 20 individual traces (including failures). Note that $GABA_A$-mediated synaptic currents were depressed by L-AP4 but were unaffected by DNQX (Modified from Safiulina et al., 2006).

The probability of transmitter release is so low that MF stimulation often fails to induce synaptic responses in principal cells. However, a second stimulus occurring with a delay of 50 ms from the first causes large amplitude synaptic currents, indicating that immature MF-CA3 synapses are "presynaptically" silent (Kasyanov *et* al., 2004).

A moderate shift in MF stimulation frequency (from 0.05 to 0.3 Hz) is able to transiently induce a strong synaptic facilitation (Safiulina *et* al., 2010; Fig. 8). The magnitude of synaptic facilitation largely depends on the age of the animals. While at P2-P3 is rather modest (the amplitude of synaptic responses reaches 200% of controls; Safiulina *et* al., 2010), at P5-P6 it is more consistent (the amplitude of synaptic responses reaches 400% of controls). MF-interneuron synapses, instead, show both paired pulse facilitation and paired pulse depression. MF-induced synaptic currents are readily blocked by picrotoxin, bicuculline, or gabazine, suggesting that they are mediated by GABA acting on $GABA_A$ receptors (Fig. 8). As expected for $GABA_A$-mediated postsynaptic potentials or currents, MF-GPSCs can be potentiated by flurazepam, an allosteric modulator of $GABA_A$ receptors, or by selectively blocking the GABA transporter GAT-1 (Safiulina et al., 2006). Depolarizing the cell to positive potentials (40 mV) reveals bicuculline-sensitive outward currents with latency, onset, and deactivation kinetics similar to those obtained at -70 mV, further indicating that they are mediated by $GABA_A$ receptors. Additional evidence in favor of GABA as a neurotransmitter at MF-CA3 synapses is given by the experiments in which chemical application of glutamate into the dendrites of granule cells in stratum moleculare (in the presence of the AMPA/kainate receptor antagonists to prevent the recruitment of GABAergic interneurons) depolarizes *via* activation of NMDA receptors granule cells and induces in CA3 principal cells barrages of L-AP4-sensitive currents completely abolished by picrotoxin. Moving the pressure pipette a few micrometers away towards the hilus, to activate hilar interneurons, causes a barrage of synaptic currents insensitive to L-AP4 but blocked by picrotoxin, implying that they are mediated by the release of GABA from GABAergic interneurons (Safiulina *et* al., 2006). Perforated patch experiments, to preserve the anionic conditions of the recorded cells, have revealed that GABA released from MF terminals exerts a depolarizing action on targeted cells (Sivakumaran *et* al., 2009). Thus, MF-GPSPs reversed at a potential more positive that the resting membrane potential (on average GPSPs reversed -47.6 ± 3.3 mV while the resting membrane potential was -56 ± 2.2 mV).

Interestingly, the E_{GPSP} value is very close to the threshold for action potential generation (-49.3 ± 2.4mV) suggesting that GABA may also be excitatory.

However, experiments performed in the cell-attached mode (to keep the intracellular milieu intact) revealed that, in spite its depolarizing action, GABA released from MF terminals is only rarely able to trigger action potentials in pyramidal cells, suggesting that its shunting effect may sometimes prevail over the excitatory one (Safiulina *et* al., 2010).

Later on, towards the end of the first postnatal week, MFs release both GABA and glutamate. Therefore, at this postnatal age, single-fiber-evoked synaptic currents fluctuate between outward, biphasic, and inward (Safiulina *et* al., 2006). Although with respect to GPSCs, mixed GABAergic and glutamatergic currents occur with a shorter latency, they involve MF synapses as they are sensitive to L-AP4. Accordingly, in juvenile animals, stimulation of granule cells in the dentate gyrus induces monosynaptic GABAergic and glutamatergic responses in CA3 principal cells (Gutierrez *et* al., 2003; Walker *et* al., 2001), and immunogold experiments have demonstrated that AMPA and $GABA_A$ receptors are co-localized on the same synapse in close apposition to MF terminals (Bergersen *et* al., 2003).

Further support in favor of a mixed GABAergic and glutamatergic phenotype of MF terminals in juvenile animals has been recently provided by Beltran and Gutierrez (2012).

These authors have demonstrated that selective stimulation of single, identified MF boutons attached to the apical dendrites of dissociated pyramidal cells of developing rats, produces synaptic currents mediated by either glutamate receptors or by both glutamate and $GABA_A$ receptors. By contrast, stimulation of MF boutons of adult rats produces exclusively glutamate receptor-mediated responses. The appearance of glutamatergic responses towards the end of the first postnatal week has been also reported by Marchal and Mulle, (2004). GABA has been found to be stored only in a particular subset of glutamatergic terminals (hippocampal and cerebellar MFs) particularly enriched with synaptic vesicles (Zander *et* al., 2010), and immunocytochemical experiments performed from newborn rats have clearly demonstrated the co-localization of the vesicular GABA and glutamate transporter VGAT and VGLUT, respectively (Safiulina *et* al., 2006, Zander *et* al., 2010).

The GABAergic nature of immature MF-CA3 synapses has been questioned by Uchigashima *et* al. (2007) on the assumption that the stimulation protocol, generally used to activate MF, may induce synchronous release of GABA from adjacent GABAergic terminals leading to misinterpretation of the results. While caution should be taken in interpreting results obtained from neonatal animals, it should be stressed that, unlike putative MF responses that occur with very low probability and exhibit short-term frequency-dependent facilitation, those generated by GABA released from GABAergic interneurons (such as those reported by Uchigashima et al., 2007) display only minimal facilitation upon repeated stimuli or even depression. In addition, unlike MF-evoked responses, synaptic currents generated by GABAergic interneurons are insensitive to L-AP4 (Walker *et* al. 2003; Safiulina *et* al. 2006; Gutierrez, 2005).

A more recent study on organotypic hippocampal slices from GAD 67-GFP transgenic mice failed to report unitary $GABA_A$-mediated synaptic currents in pair recordings from interconnected granule cells and CA3 principal cells, in spite the expression of GAD 67 in a sub-population of immature granule cells (Cabezas *et* al. 2012). Therefore, the results of Cabezas et al. (2012) do not support the conclusion that postsynaptic $GABA_A$ receptors are activated by GABA release from MF terminals. According to the authors, however, repetitive firing of GAD 67-positive granule cells transiently reduced the probability of evoking antidromic spikes, an effect that was blocked by selective $GABA_B$ receptor antagonists suggesting that GABA, released with glutamate from presynaptic MF terminals, may activate presynaptic $GABA_B$ receptors to reduce cell excitability.

It is important to stress that these observations were made in organotypic slice cultures obtained from P10-P20 old animals, whereas all previous studies were performed in acute slices immediately after birth. Therefore, it is difficult to compare experiments performed from different preparations at different developmental stages.

The possibility that the same fiber can release different neurotransmitters has been well documented in several brain structures including the retina (O'Malley and Masland, 1989) and the spinal cord (Jonas *et* al., 1998). In the developing auditory system, glutamate has been reported to be released from inhibitory terminals (Gillespie *et* al., 2005).

5.3 Presynaptic modulation of GABA release by $GABA_A$ and $GABA_B$ autoreceptors

Early in postnatal development, MF are endowed with several neurotransmitter receptors including $GABA_A$ (Nakamura *et* al., 2007), $GABA_B$ receptors (Safiulina and Cherubini, 2009), mGluRs (Scanziani *et* al., 1997), nAChRs, mAChRs (Maggi *et* al., 2004), adenosine and purinergic P2Y receptors (Zhang *et* al., 2003; Safiulina *et* al., 2005), known to down regulate GABA release.

As already mentioned, activation of group III mGluR depresses GABA release (Gutierrez *et* al., 2003; Safiulina *et* al., 2006; Walker *et* al., 2001) probably by down-regulating the exocytotic machinery (Kamiya and Ozawa, 1999). This unique property of MF synapses is commonly used to determine whether a given response is of MF origin.

While in 3-4 weeks-old guinea pigs, activation of presynaptic MF $GABA_A$ receptors by ambient GABA or GABA spillover from neighboring interneurons leads to a reduction of MF's excitability (Ruiz *et* al., 2003), in juvenile rats it leads to a depolarization of MF terminals by the outward flux of chloride and to an increase in cell firing (Nakamura *et* al., 2007). Whether a similar effect occurs also during the first week of postnatal life remains to be elucidated.

Postsynaptic $GABA_B$ receptors are developmentally regulated and, immediately after birth, are poorly expressed. In contrast, presynaptic $GABA_B$ receptors are already present and functional (Gaiarsa *et* al., 1995; Lei and McBain, 2003). Presynaptic $GABA_B$ receptors reduce transmitter release mainly by inhibiting P/Q and N types of voltage-dependent calcium channels (Poncer *et* al., 1997). Application of a selective $GABA_B$ receptor antagonist enhances the probability of GABA release from immature MF terminals switching on 'presynaptically' silent synapses. This suggests that the constitutive activation of $GABA_B$ receptors by spillover of GABA from MF terminals or from neighboring GABAergic interneurons contributes to synapse silencing (Safiulina and Cherubini, 2009). In contrast, the $GABA_B$ receptor agonist baclofen induces a powerful depression of MF-GPSCs, an effect that is associated with a significant increase in transmitter failures and in the paired-pulse ratio.

Interestingly, the IC_{50} value for baclofen (400 nM) was found to be at least one order of magnitude lower than that obtained at GABAergic synapses in CA3 stratum radiatum interneurons (Lei and McBain, 2003). This may be related to differences in $GABA_B$ receptors expression, distribution, and/or affinity among different cell types, and suggests a target-specific modulation of GABAergic signaling by $GABA_B$ autoreceptors.

Furthermore, by increasing the concentration of GABA in the extracellular space by repetitively stimulating MF at 3-10 Hz or by blocking GABA uptake with NO711, a selective blocker of the GABA transporter GAT-1, typically results in synaptic depression that can be rescued by CGP55845 (Safiulina and Cherubini, 2009). This further indicates the involvement of $GABA_B$ auto receptors in controlling the release of GABA from immature MF terminals.

5.4 Activity-dependent changes of synaptic efficacy

Activity-dependent changes in synaptic strength such as long-term potentiation (LTP) or long-term depression (LTD) are critical for information storage in the brain and for the development of neuronal circuits. One interesting question is whether, early in postnatal development, MF-GPSCs undergo activity-dependent modifications of synaptic efficacy. Immature neurons are characterized by an elevated number of 'silent' synapses (Durand *et* al., 1996). These are synapses that do not conduct at rest because the neurotransmitter is not released when the presynaptic terminal is invaded by an action potential (presynaptically silent) or because they are unable to detect the release of the neurotransmitter due to the lack of the respective receptors on the subsynaptic membrane (postsynaptically silent). Silent synapses can be converted into active ones by activity-dependent processes and this represents the most common mechanism for LTP induction, not only during development but also in the mature brain (Voronin and Cherubini, 2004).

5.4.1 Calcium transients associated with GDPs act as coincident detectors for enhancing synaptic strength

A pairing procedure was developed to test the hypothesis that, early in postnatal development, correlated network activity such as GDPs may act as coincident detector signals for enhancing, in an associative type of manner, synaptic efficacy at poorly developed MF-CA3 synapses (Kasyanov *et* al., 2004; Fig. 9).

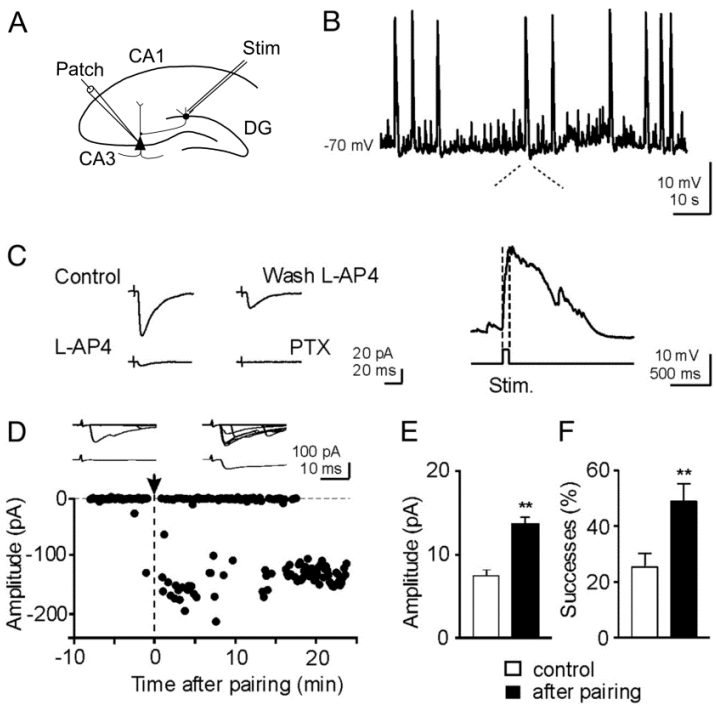

Figure 9: Pairing GDPs with MF stimulation persistently enhances synaptic efficacy at MF-CA3 synapses. (A) Diagram of the hippocampus showing a CA3 pyramidal cell with the MF input (Stim). (B) GDPs recorded from a CA3 cell in current clamp mode from the hippocampus of a P3 old rat. Below the trace, a single GDP is shown on an expanded time scale. Note the absence of spikes riding on the top of GDP due to block of the sodium channel with QX 314.

The rising phase of GDPs (between the dotted lines) was used to trigger synaptic stimulation (Stim). (C) Typical MF response recorded in control conditions (Control), after adding 10 μM of L-AP4 to the bathing solution, after washing out L-AP4 and after adding 100 μM picrotoxin (PTX). Each response is the average of 30 trials (including failures). (D) Amplitudes of synaptic responses (dots) evoked by stimulation of MF before and after pairing (arrow at time 0) are plotted against time. The traces above the graph represent 7 individual responses evoked before (Left) and 15 min after pairing (Right); lower traces represent the average of 48 individual responses (successes and failures) obtained before (Left) and 15 min after pairing (Right). (E and F) Mean EPSC amplitude (E) and mean percentage of successes (F) for 20 P1-P6 CA3 pyramidal neurons examined before (white column) and 15 min after pairing (black column). (Modified from Kasyanov et al., 2004).

Pairing consisted in triggering MF stimulation with the rising phases of GDPs in such a way that calcium transients associated with GDPs occurred simultaneously with MF-GPSPs. After a control period of 5-10 min in which GPSCs were evoked by stimulation of granule cells in the dentate gyrus at 0.05 Hz, the patch was switched from voltage-clamp to current-clamp mode and MF responses were evoked by spontaneously occurring GDPs (pairing typically lasted 5 min). After this period, the patch was switched back to voltage-clamp mode and synaptic currents were recorded as in control. In the case of presynaptically silent synapses, the pairing protocol caused the appearance of responses to the first stimulus and increased the number of successes to the second one. In the case of non silent low-probability synapses, the pairing procedure produced a strong and persistent potentiation of MF responses associated with a significant increase in the number of successes and, in double-pulse experiments with a significant reduction in the paired-pulse ratio and a significant increase in the inverse squared value of the coefficient of variation. This suggests that an increased probability of transmitter release accounts for the persistent increase in synaptic efficacy. In the absence of pairing, no significant changes in synaptic efficacy occurred.

Moreover, when the interval between GDPs and MF stimulation was progressively increased, the potentiation declined and reached the control level when presynaptic signals were activated 2-3 s after GDPs (Kasyanov *et* al., 2004).

Pairing-induced synaptic potentiation was prevented by chelating calcium in the postsynaptic cell with BAPTA or when nifedipine was added to the extracellular medium. In contrast, the NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-APV) failed to prevent pairing-induced potentiation, indicating that calcium rise through voltage-dependent calcium channel is the common trigger for activity-dependent changes in synaptic efficacy at GABAergic MF–CA3 synapses. In conclusions, during development, coincident detection signals provided by GDPs are crucial for enhancing synaptic transmission at emerging MF-CA3 connections. How this contributes to the establishment of appropriate synaptic connections remains to be established.

5.4.2 Spike-time-dependent plasticity (STDP)

In a recent study (Sivakumaran *et* al., 2009) it was found that pairing postsynaptic spikes with unitary MF GPSPs consistently up- or down-regulates synaptic strength according to the temporal order of stimulation. STDP was induced in current-clamp mode by pairing 10 postsynaptic spikes (at 0.1 Hz) with afferent stimulation, varying the relative timing of pre and postsynaptic activity. A strong potentiation of MF-GPSCs was obtained when antidromic spikes coincided with the peak of MF-evoked synaptic potentials (15 ms after the onset of MF stimulation; Fig.10).

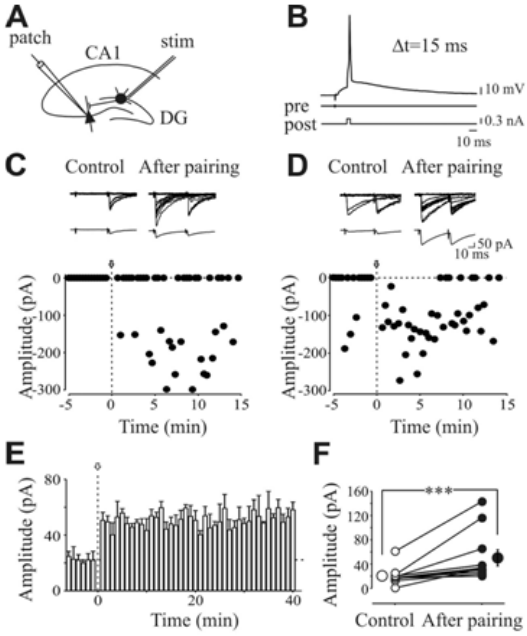

Figure 10: Spike-timing dependent LTP induced by pairing MF GPSPs with postsynaptic spiking. (A) Schematic representation of the experimental design. (B) Spike-timing protocol. GPSP preceded the postsynaptic spike by 15 ms (Δt). (C, D) Peak amplitude of MF GPSCs in presynaptically silent (C) and low probability neurons (D) evoked before and after pairing (arrows at time 0) as a function of time. Insets above the graphs represent individual (top traces) and averaged GPSCs (bottom traces) evoked before and after pairing. (E) Summary plot of mean GPSCs amplitude recorded before and after pairing versus time (n = 12). (F) Amplitude measured in individual cells before (o) and 40 min after pairing (•). Bigger symbols represent averaged values. ***$p < 0.001$ (Modified from Sivakumaran et al., 2009).

The increased GPSC amplitude and successes rate persisted without decrement for at least 40 to 60 min. These effects were associated with a significant reduction in PPR and a significant increase in the inverse squared value of the coefficient of variation of responses amplitude, suggesting a presynaptic site of expression of STD-LTP. In addition, when pairing was induced in current-clamp conditions using gramicidin-perforated patch to preserve the intracellular chloride concentration, LTP was associated with a persistent increase in GPSP slope and in the probability of cell firing.

Interestingly, in silent neurons the pairing protocol enhanced the probability of GABA release, thus making MF-CA3 connections active. As for GDPs, STD-LTP required a rise of calcium in the postsynaptic cell *via* voltage-dependent calcium channel for its induction.

These data clearly show that, in spite its postsynaptic induction mechanism (LTP could be prevented by chelating calcium in the postsynaptic cell with BAPTA), the expression of STD-LTP is presynaptic, as demonstrated by the increased probability of GABA release. Therefore, the postsynaptic cell should provide a trans-cellular retrograde signal to the presynaptic neuron. One attractive candidate is the brain-derived neurotrophic factor (BDNF), which can be released in a calcium-dependent way by depolarization of the postsynaptic cell (Kuczewski *et* al., 2008) and has been shown to be the retrograde messenger required for increasing the probability of GABA and glutamate release at GABAergic and glutamatergic synapses, respectively (Gubellini *et* al., 2005; Mohajerani *et* al., 2007). In line with this hypothesis, BDNF was found to be the signaling molecule responsible for increasing *via* TrkB receptors GABA release from MF terminals.

These data clearly show that during postnatal development, pairing back propagating action potentials with MF-GPSPs persistently enhances synaptic efficacy and brings CA3 principal cells to fire, thus providing a reliable way to convey information from granule cells to the CA3 associative network at a time when glutamatergic synapses are still poorly developed.

6. Adult neurogenesis

Neurogenesis, the process by which functional neurons are generated from precursors, was traditionally thought to occur only during embryonic and perinatal stages of mammals development (Ming and Song, 2005). Altman's pioneering studies decades ago provided the first anatomical evidence that newly generated granule cells are present in the dentate gyrus in the postnatal rat hippocampus (Altman and Das, 1965). Active adult neurogenesis is spatially restricted under normal conditions to two specific ''neurogenic'' brain regions, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Gage, 2000).

In the SGZ, proliferating radial and non-radial precursors give rise to intermediate progenitors, which in turn generate neuroblasts (Fig.11).

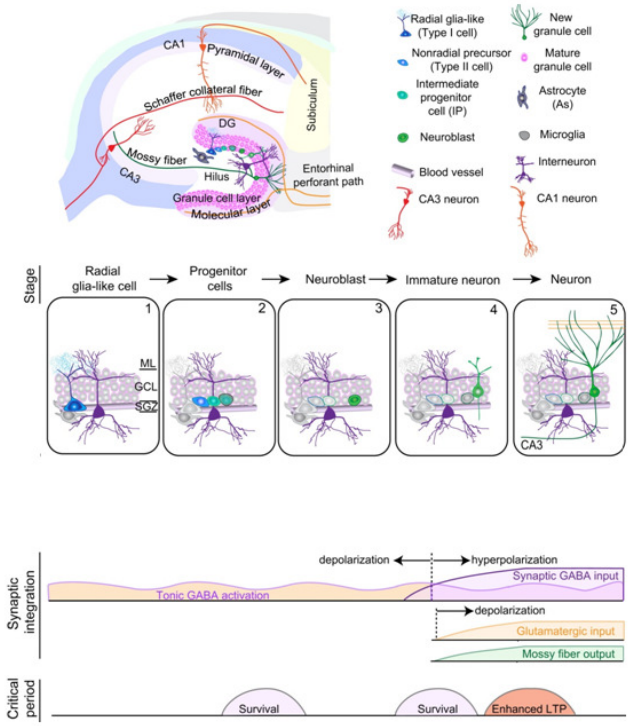

Figure 11: Generation of new granular neurons in the dentate gyrus of the hippocampus from neural stem cells in the subgranular zone (SGZ). Adult neurogenesis in the dentate gyrus of the hippocampus undergoes five developmental stages. Stage 1. Proliferation: Stem cells with their cell bodies located within the subgranular zone in the dentate gyrus have radial processes that project through the granular cell layer and short tangential processes that extend along the border of the granule cell layer and hilus. These stem cells give rise to transient amplifying cells. Stage 2. Differentiation: transient amplifying cells differentiate into immature neurons. Proliferating progenitors in the SGZ are tightly associated with astrocytes and vascular structures. Stage 3. Migration: Immature neurons migrate a short distance into the granule cell layer. Stage 4. Axon/dendrite targeting: Immature neurons extend their axonal projections along mossy fiber pathways to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction toward the molecular layer. Stage 5.

Synaptic integration: New granule neurons receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus regions. The specific properties of each stage are summarized below, mainly on the basis of studies in adult mice. DG, dentate gyrus region; ML, molecular cell layer; GL, granular cell layer. (Modified from Ming and Song, 2005).

These migrate into the inner granule cell layer and differentiate into dentate granule cells. Within days, newborn neurons extend dendrites toward the molecular layer and project axons through the hilus toward the CA3 (Zhao *et* al., 2006). New neurons undergo progressive synaptic integration into the existing circuitry (reviewed by Ge *et* al., 2008; Fig.11). They are initially tonically activated by ambient GABA released from local interneurons (Bhattacharyya et al., 2008; Ge *et* al., 2006). Although newborn granule cells have functional glutamate receptors, they receive GABAergic synaptic inputs before the formation of glutamatergic ones and are depolarized by GABA due to high expression levels of the chloride importer NKCC1 (Ge *et* al., 2008). Furthermore, evoked and spontaneous $GABA_A$-mediated synaptic currents exhibit immature characteristics, including slow rise and decay phases and depolarized reversal potentials. In comparison with neighboring mature granule cells, synaptic currents are relatively insensitive to the $GABA_A$ receptor modulator zolpidem, indicating lack of $GABA_A$ receptors containing the α1subunits (Esposito et al., 2005; Ge *et* al., 2006a; Overstreet-Wadiche *et* al., 2006b). Finally new born granule cells make synaptic contacts with hilar interneurons and CA3 principal cells (Faulkner *et* al., 2008; Toni *et* al., 2008). Compared to mature granule cells, newborn neurons display elevated intrinsic excitability partially dependent on a reduced $GABA_A$-mediated inhibition (Ge *et* al., 2008; Schmidt-Hieber *et* al., 2004; Marín-Burgin *et* al., 2012). With maturation, adult-born neurons exhibit similar electrophysiological characteristics of mature neurons, including firing behavior and kinetic properties of GABAergic and glutamatergic responses (reviewed by Mongiat and Schinder, 2011).

Interestingly, major milestones of neuronal development are highly conserved among embryonic, early postnatal, and adult neurogenesis. One notable difference is a significantly slower “tempo” of neuronal maturation in adult compared to embryonic development (Overstreet-Wadiche *et* al., 2006a; Zhao *et* al., 2006).

The physiological significance of this prolonged development remains unknown. However, it appears crucial since an acceleration of the maturation "tempo" leads sometimes to aberrant integration of newborn neurons in the adult hippocampus (Duan *et* al., 2007; Overstreet-Wadiche *et* al., 2006; Parent *et* al., 1997).

Newborn neurons also exhibit enhanced synaptic plasticity of their glutamatergic inputs during specific critical developmental periods (Ge *et* al., 2007; Nissant *et* al., 2009; Schmidt-Hieber *et* al., 2004). Such enhanced plasticity renders adult born neurons competitive in comparison with pre-existing ones for selective formation and stabilization of local synaptic connections (Tashiro *et* al., 2006; Toni *et* al., 2007).

7. Involvement of DG granule cells in neuropsychiatric disorders

The hippocampus and the DG are the targets of many different forms of neuropsychiatric disorders, including epilepsy, neurodegenerative diseases, schizophrenia and depression (Danzer et al., 2012; Kobayashi, 2009). A critical factor is represented by the impairment of DG granule cells gating functions, which control the amount of excitation reaching the CA3 associational network. This is particularly relevant in epilepsy and in Alzheimer's disease, where changes in the excitatory/inhibitory balance account for network hyper-excitability and memory loss, respectively. Moreover, the strategic location of the DG within the limbic system and its reciprocal connections with brain regions crucially involved in emotion, explain the involvement of this structure in disorders such as schizophrenia and depression.

Interestingly, many of these diseases are known to be associated with alterations of adult neurogenesis in the DG (Ming and Song, 2005). Here, I will focus only on the involvement of DG adult neurogenesis in neurodegenerative diseases and epilepsy.

Neurodegenerative diseases comprise a wide range of diseases that share the common characteristic of progressive loss of structure and function of neurons and glial cells in the brain and spinal cord (Glass *et* al., 2010).

Neurodegenerative diseases are based on slowly progressive processes which affect mainly neurons in selective brain areas controlling cognitive functions [the hippocampus and cerebral cortex; (Selkoe, 2002)] and their communication through the synapses (In addition, these disorders are associated with an altered and/or reduce neurogenesis). Genes involved in neurodegenerative diseases [a-synuclein, presenilin (PSEN)1, cellular prion protein (PrP^C), tau, huntingtin] are also physiologically involved in modulating brain plasticity in the embryonic brain (Winner *et* al., 2011**).** Interestingly, proteins encoded by these genes are highly expressed at synapses. Therefore, it is intriguing that the mechanisms of neurodegenerative diseases are closely linked to brain plasticity. Whether an impaired adult neurogenesis contributes to deficits observed in neurodegenerative diseases is still unclear. However, impaired olfaction and hippocampal related cognitive and emotional functions are common findings in many different neurodegenerative disorders.

Interestingly, in Parkinson's and Huntington's diseases, specific alterations in neurogenic areas parallel premotor symptoms seen at early stages, such as depression, anxiety and olfactory dysfunction (Simuni and Sethi, 2008). The expression of human PSEN variants, linked to the early-onset of the familial form of Alzheimer's disease, in microglia localized in the adult SGZ, impairs proliferation and neuronal fate commitment (Choi *et* al., 2008). Deletion of PSEN1 in forebrain excitatory neurons affects enrichment-induced hippocampal neurogenesis (Feng *et* al., 2001). PSEN mutants also exhibit impaired self-renewal and differentiation of adult SVZ precursors involving notch signaling (Veeraraghavalu *et* al., 2010). It is worth mentioning that PrP^C, a protein extensively studied for its central role in mammalian neurodegenerative disorders such as Creutzfeldt–Jakob disease and the transmissible spongiform encephalopathies (Prusiner, 1998; Aguzzi, and Polymenidou, 2004)] exerts a strong positive influence on both developmental and adult mammalian neurogenesis, increasing cellular proliferation *in vivo*, in both the SVZ and the DG (Steele *et* al., 2006).

All together, these findings raise the intriguing possibility that aberrant postnatal neurogenesis may contribute to the juvenile and adult onset of many neurodegenerative disorders (reviewed by Christian *et* al., 2010).

Epilepsy is a multifarious and debilitating disease affecting 1–2% of the population. Epilepsy is defined clinically by the occurrence of two or more unprovoked seizures. Seizures can originate from different regions of the brain, depending on the type of epileptic syndrome.

Temporal lobe epilepsy (TLE) represents a common and difficult to treat form of the disease. Interestingly, a large body of literature in the epilepsy field has focused on the DG of the hippocampus (Dudek and Sutula, 2007). While there are many mechanisms in the brain for regulating the balance between excitation and inhibition, it appears that the ability of the DG to control excitation is unusually pronounced and robust (Hsu, 2007). Disruption of the DG gating function, therefore, may have disproportionate effects on the excitatory/inhibitory balance in the brain (Heinemann *et* al., 1992; Behr *et* al., 1998; Gloveli *et* al., 1998; Pathak *et* al., 2007; Shao and Dudek, 2011).

The question follows: what causes this failure? Although loss of inhibitory interneurons (Dudek and Sutula, 2007) likely plays a role, three main "defects" of the DG have been identified as responsible for the formation of recurrent excitatory circuits among granule cells, in the epileptic brain. The first, termed mossy fibers sprouting, has been described long ago (Tauck and Nadler, 1985; Nadler, 2003). Mossy fiber sprouting occurs when granule cell axons sprout into the dentate inner molecular layer and form excitatory synaptic connections with neighboring granule cells, likely promoting hyper-excitability within the hippocampus (for review see Sutula and Dudek, 2007). Mossy fiber sprouting has been found in both animals and humans with TLE, and is considered to be a hallmark of the disease. The second alteration consists in a defect of migration: granule cells migrate from the granule cell layer to ectopic locations within the hilus. Displacement of these cells into the hilus, a primary target region of granule cell axons, likely accounts for the extensive innervation of ectopic granule cells by neighboring granule cells in rodents (Scharfman *et* al., 2000-2003; Pierce *et* al., 2005). Ectopic granule cells have also been identified in tissue from humans with epilepsy (Parent *et* al., 2006).

The third anomaly arises when granule cells develop basal dendrites projecting into the hilus (Buckmaster and Dudek, 1999). In rodents, mature granule cells typically possess only apical dendrites, which project to the molecular layer where they are innervated by afferents from entorhinal cortex.

By contrast, granule cells with basal dendrites receive significant recurrent input from neighboring granule cells, as evidenced by both anatomical and physiological studies (Ribak *et* al., 2000; Austin and Buckmaster, 2004; Shapiro and Ribak, 2006). Interestingly, the majority of these abnormal granule cells are newly-generated neurons (Danzer, 2012). Newborn granule cells exhibit critical periods during which they are vulnerable to developing specific abnormalities. Conversely, granule cells already mature at the time of an epileptogenic insult are only minimally affected.

Studies of ectopic dentate granule cells, which appear to be almost exclusively newborn (Walter *et* al., 2007; Kron *et* al., 2010), have revealed that many of these neurons fire with bursts (Scharfman *et* al., 2000). Bursting is not typical of normal granule cells, and this property has been suggested to be pro-epileptogenic. Ectopic granule cells also exhibit higher ratios of excitatory to inhibitory inputs than normal granule cells (Zhan *et* al., 2010). By contrast, in epileptic animals, newborn granule cells correctly located in the granule cell layer have been found to be less excitable than age-matched controls, raising the possibility that some newborn cells integrate to perform a homeostatic role (Jakubs *et* al., 2006).

While at early stages of the disease epilepsy is associated with an increased neurogenesis and aberrant integration of newborn cells, at late stages, is accompanied with a reduced neurogenesis. Thus, the hippocampus of animals with chronic seizures (five months after KA administration), exhibit a dramatically reduced neurogenesis (Hattiangady *et* al., 2004). The extensive cell loss, gliosis and shrinkage observed in this condition may lead to disruption of the neurogenic niche, in which progenitor cells are lost entirely or cell proliferation, survival or differentiation are disrupted. Hattiangady and Shetty (2010) found that while neurogenesis is profoundly reduced or absent in damaged rodent hippocampi, cell birth continued, indicating the progenitor cells are still present. The progeny of these progenitor cells, however, differentiates into glia rather than into neurons.

In summary, good evidence exists from animal models to indicate that the DG has an important role in maintaining the excitatory/inhibitory balance in the brain.

AIM OF THE STUDY

Aim of my thesis was to investigate the molecular mechanisms underlying synaptic transmission and activity-dependent synaptic plasticity processes at immature MF-CA3 synapses in wild-type rodents and in mice lacking the prion protein (*Prnp*$^{0/0}$ mice).

Unlike adult MF which are glutamatergic, immature MF release GABA which exerts a depolarizing and excitatory action on targeted neurons (Safiulina *et* al., 2006, 2010). Several neurotransmitter receptors, including $GABA_B$ receptors (Safiulina and Cherubini, 2009), mGluRs (Scanziani et al., 1997), nAChRs, mAChRs (Maggi et al., 2004), adenosine and purinergic P2Y receptors (Zhang et al., 2003; Safiulina et al., 2005) are known to down-regulate GABA release from MF terminals. Their action is crucial for maintaining the right balance between excitation and inhibition at this early developmental stage. Interestingly, several studies have revealed a strong developmentally regulated correlation between the expression of kainate receptors (KARs) and hippocampal spontaneous activity, suggesting that these receptors might play a critical role during postnatal development (Bahn et al., 1994; Ritter et al., 2002; Ben-Ari, 1989). In addition, in the neonatal hippocampus, KARs have been shown to regulate glutamatergic transmission (Segerstrale et al., 2010; Lauri *et* al., 2003, 2006). Hence, the first aim of my work was to investigate whether KARs are expressed and functional on immature GABAergic MF terminals and whether and through which mechanisms they can eventually regulate GABA release. I found that endogenous activation of presynaptic GluK1 receptors by "ambient" glutamate, severely depresses MF-evoked synaptic currents in CA3 principal cells, an effect that appears to be mediated *via* a metabotropic type of action. Furthermore, I found that GluK1 receptors dynamically regulate the direction of spike time-dependent plasticity occurring by pairing MF stimulation with postsynaptic spiking.

The results of this study are illustrated and discussed in paper N. 1.

Researches over the last decade have shown that endocannabinoid/CB1 receptor signaling exerts powerful inhibitory effects on both glutamatergic and GABAergic synaptic transmission in many brain regions, including the hippocampus (Kano *et* al., 2009). Typically, endocannabinoids are formed in activated neurons, released from the postsynaptic membrane, diffuse to and stimulate CB1 receptors on presynaptic terminals to modulate transmitter release (Kreitzer and Regehr, 2002; Freund *et* al., 2003; Szabo and Schlicker, 2005; Lovinger, 2010). Interestingly, CB1 receptors are developmentally regulated: in the cortex they are highly expressed at early developmental stages and then slowly decline with age (Ellgren *et* al., 2008; Heng *et* al., 2011), suggesting their involvement in the formation of cortical networks (Berghuis et al., 2007; Harkany *et* al., 2007- 2008; Mulder *et* al., 2008). According to both immunohistochemistry and electrophysiological data, adult MF lack CB1 receptors (Katona *et* al., 2006; Hofmann et al., 2008). We speculated that, similarly to prefrontal cortex (Ellgren *et* al., 2008; Heng *et* al., 2011), early in postnatal development, CB1 receptors, may be transiently expressed on MF terminals where they can regulate GABA release. I used pharmacological and genetic manipulations, to examine whether CB1 receptors are expressed and functional on GABAergic MF terminals during the first week of postnatal life (between P3 and P6). I found that presynaptic CB1 receptors are present on MF terminals already at P3. Activation of these receptors by endocannabinoids released during membrane depolarization from CA3 principal cells, causes spike-time dependent LTD. This can be induced by pairing postsynaptic spikes with MF stimulation. Thus, STD-LTD can be prevented by CB1 receptor antagonists and is absent in CB1-KO mice. Consistent with these data, in situ hybridization experiments, performed in Bordeaux, in Dr. Marsicano lab. have revealed detectable level of CB1 mRNA in the granule cell layer at P3 but not at P21.

The results of this study are illustrated and discussed in paper N. 2.

Finally, during the last year of my PhD course, I studied synaptic transmission and activity-dependent synaptic plasticity processes occurring at immature MF-CA3 synapses in the hippocampus of *Prnp*$^{0/0}$ mice.

The prion protein, PrP^{C}, is a conserved glycoprotein, highly expressed in the brain, which can be converted into its abnormally folded and aggregated isoform PrP^{Sc}, known to cause neurodegenerative disorders in mammals, including Creutzfeldt-Jakob disease in humans. Its prevalent localization at pre- and post-synaptic sites suggests a role for this protein in synaptic transmission and plasticity (Collinge *et* al., 1994; Fournier *et* al., 1995; Mironov *et* al., 2003). Interestingly, PrP^{C} is developmentally regulated (Manson et al., 1992; Sales et al., 2002; Benvegnu et al., 2010), and its high expression in the immature brain may account for the shorter incubation time of the disease in younger animals (McKinley et al. 1989). Moreover, the high levels of the protein in late pre- and early post-natal life are crucial for regulating neurogenesis and cell proliferation (Steele et al. 2006). In the hippocampus, developmental changes in PrP^{C} immunoreactivity parallel those of mossy fiber terminals (MFs), the axons of granule cells in the dentate gyrus (Sales et al., 2002) that reach full maturation around P15-P20 (Amaral and Dent, 1981). All these evidences led us to hypothesize that PrP^{C} might play a crucial role at MF-CA3 synapse at this early stage of postnatal development.

The immature hippocampus is characterized by correlated network activity such as the giant depolarizing potentials or GDPs which are generated by the synergistic action of glutamate and GABA, both of which depolarizing and excitatory (Ben-Ari et al., 1989; Ben-Ari et al. 2007). Calcium transients associated with GDPs are thought to be instrumental for enhancing synaptic efficacy at emerging glutamatergic (Mohajerani et al., 2007) and GABAergic (Kasyanov et al., 2004) synapses. Therefore, I used a pairing procedure to correlate GDPs with MF stimulation. This procedure caused long-term potentiation (LTP) in wild-type animals and long-term depression (LTD) in $Prnp^{0/0}$ mice.

LTP was postsynaptic in origin and required the activation of cAMP-dependent PKA signaling while LTD was presynaptic and was reliant on G protein-coupled GluK1 receptor and protein lipase C downstream to G protein activation. In addition, at emerging CA3-CA1 synapses of PrP^{C}-deficient mice, stimulation of Schaffer collateral failed to induce LTP, known to be PKA-dependent.

The results of this study are illustrated and discussed in paper N. 3.

METHODS

All experimental procedures and materials used are described in detail in the original papers included in the next section (Results).

Animals

All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609EEC) and were approved by the local authority veterinary service. All efforts were made to minimize animal suffering and to reduce the number of animals used. The FVB *Prnp*$^{0/0}$ mice were obtained from George A. Carlson, McLaughlin Research Institute, Great Falls, Montana, USA, and were bred by backcrossing with the original *Prnp*$^{0/0}$ mice at least 20 time.

Slice preparation

Experiments were performed on hippocampal slices from P2-P6 and P19-P25 Wistar rats, from *CB1*-KO mice and WT littermate of the same postnatal age and from P3 to P7 FVB/N wild-type and FVB *Prnp*$^{0/0}$ mice. Briefly, animals were decapitated after being anesthetized with an i.p. injection of 2 g/kg urethane. The brain was quickly removed from the skull and placed in ice-cold artificial cerebrospinal fluid. Transverse hippocampal slices (400 μm thick) were cut with a vibratome and stored at room temperature in a holding bath containing the same solution. After a recovery period of at least 1 h, an individual slice was transferred to the recording chamber, where it was continuously superfused with oxygenated artificial cerebrospinal fluid at a rate of 2 to 3 ml/min at 33-34°C.

Electrophysiological recordings

Electrophysiological experiments were performed mainly from CA3 pyramidal cells using the whole-cell configuration of the patch-clamp technique in current or voltage-clamp mode. Bipolar twisted NiCr-insulated electrodes localized into stratum granulosum of the dentate gyrus were used to evoke synaptic responses in CA3 pyramidal cells. We used minimal stimulation in order to activate only one or few presynaptic fibers. According to the technique described by Jonas et al., (1993) and Allen and Stevens (1994) the stimulation intensity was decreased until only one single axon was activated. This was achieved when the mean amplitude of the postsynaptic currents and failure probability remained constant over a range of stimulus intensities near the threshold for detecting a response. Further enhancing the stimulus intensity led to an abrupt increase in the mean peak amplitude of synaptic currents. This "all-or-none" behavior suggests that only a single granule cell was stimulated. When the stimulation intensity was turned down, the probability of failures in synaptic transmission was near 1. The latency and shape of individual synaptic responses remained constant over repeated stimuli. In most cases paired stimuli were applied at 50 ms interval.

Patch electrodes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, D). They had a resistance of 4-6 MΩ when filled with an intracellular solution containing (in mM): 140 KCl, 1 MgCl2, 10 HEPES, 4 MgATP, 0.5 EGTA, pH 7.3. In some cases, guanosine 5'-[β-thio]diphosphate (GDPβS) (Sigma-Aldrich) at the concentration of 0.3 mM was included into the patch pipette to block postsynaptic G-protein-coupled receptors. In the experiments in which GDP-pairing protocol was performed, the intracellular solution used contain (in mM): K-gluconate 115, KCl 20, disodium phosphocreatine 10, HEPES 10, MgATP 4, GTP 0.3, QX 314 5 (pH 7.3). In some experiments recordings were performed with patch pipettes containing the calcium chelator 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'- tetraacetic acid (BAPTA, 20 mM; purchased from Sigma, Milan, Italy).

Experiments aimed at assessing synaptic plasticity at glutamatergic CA3-CA1 synapses were performed from CA1 pyramidal cells using patch pipettes filled with a solution containing (in mM): Cs-MeSO4 120, KCl 20, HEPES 10, EGTA 0.5, Na-GTP 0.3 and Mg-ATP 4 (pH 7.2; osmolality 275-280 mOsm). Spontaneous miniature GABAergic currents (mGPSCs) were recorded from CA3 principal cells in the presence of DNQX (20 mM) and TTX (1 mM).

Recordings were made with a patch clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA). The stability of the patch was checked by repeatedly monitoring (every 5 min) the input and series resistance. Cells exhibiting more than 15% changes in series resistance were excluded from the analysis.

During the first week of postnatal life, MF-CA3 responses are mainly GABAergic since they are insensitive to the AMPA receptor antagonist GYKI 52466 (Caiati *et* al., 2010) but they are readily and reversibly blocked by bicuculline (20 μM), picrotoxin (100 mM) or gabazine (2mM; see Walker et al., 2001 and Safiulina *et* al., 2006). MF inputs were identified on the basis of their sensitivity to group III metabotropic glutamate receptor agonist 2-amino-4-phosphonobutyric acid (L-AP4, Gutierrez et al., 2003; Kasyanov et al., 2004), their paired pulse facilitation and their short-term frequency-dependent facilitation (Safiulina et al., 2006). GABAA-mediated synaptic potentials or currents (GPSPs or GPSCs) were evoked at 0.05 Hz from a holding potential of –60 mV.

The detailed description of the stimulation protocols used in the experiments aimed at assessing synaptic plasticity is reported in the original papers included in the next section (Results).

Data Analysis

Data were acquired and digitized with an A/D converter (Digidata 1200, MolecularDevices) and stored on a computer hard disk. Acquisition and analysis of evoked responses were performed with Clampfit 9 (Molecular Devices). Data were sampled at 20 kHz and filtered with a cut off frequency of 2 kHz.

Mean GPSCs amplitude was obtained by averaging successes and failures. The paired pulse ratio (PPR) was calculated as the mean amplitude of the synaptic response evoked by the second stimulus over that evoked by the first one. The coefficient of variation (CV) of response amplitude was determined as the ratio between the standard deviation and the mean.

Statistical Analysis

Statistical analysis was carried out using Student's paired *t*-test to compare conditions that allowed recordings from the same neuron before and after drug treatment. The unpaired *t*-test was employed to compare two independent groups and one-way ANOVA was used to compare several independent groups. When the ANOVA resulted in a significant general group effect, Dunnett's *post hoc* test was used to compare different groups versus the control group for single or multiple comparisons. A p value < 0.05 was considered as statistically significant.

Fluorescent in situ hybridization (ISH).

The brains of P3 and P21 null CB1 mutant mice and wild-type littermates were isolated, quickly frozen on dry ice and stored at -80º C until sectioning in a cryostat (14 µm, Microm HM 500 M, Microm Microtech, France). ADIG-labelled riboprobe against mouse CB1 receptor was used (Marsicano and Lutz, 1999) and procedures were as previously described using the TSA amplification System (Bellocchio *et* al., 2010). Slides were analyzed by epifluorescence microscopy at 20X (Leica) and photographed using a Coolsnap HQ2 camera (Roper). In order to detect low but specific levels of labelling, the acquisition parameters were chosen using CB1-KO sections in order to exclude any background signal and exactly the same parameters were immediately used to acquire the images from wild-type P3 and P21 sections. Images of CB1mRNA labelling did not undergo any post-acquisition processing.

Images of CB1 mRNA FISH were acquired in epifluorescence and the levels of expression were evaluated using the program Image J on 4–8 sections corresponding to dorsal DG of wild-type and CB1-KO P3 and P21 mice (2 mice per group). The levels of fluorescent signal were measured by pixel intensity in black and white images after background subtraction for each section (same parameters for all samples).

Six regions of interest were randomly chosen for each section corresponding to the granule cell layer of the dentate gyrus, with the only limit to avoid brightly stained cells, presumably corresponding to GABAergic interneurons(Marsicano and Lutz, 1999). The average values of each section represented one data point. Values were also obtained in a similar way from P3 and P21 *CB1*-KO mice.

RESULTS

Development/Plasticity/Repair

In the Developing Rat Hippocampus, Endogenous Activation of Presynaptic Kainate Receptors Reduces GABA Release from Mossy Fiber Terminals

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Presynaptic kainate receptors regulate synaptic transmission in several brain areas but are not known to have this action at immature mossy fiber (MF) terminals, which during the first week of postnatal life release GABA, which exerts into targeted cells a depolarizing and excitatory action. Here, we report that, during the first week of postnatal life, endogenous activation of GluK1 receptors by glutamate present in the extracellular space severely depresses MF-mediated GABAergic currents [$GABA_A$-mediated postsynaptic currents (GPSCs)]. Activation of GluK1 receptors was prevented by treating the slices with enzymatic glutamate scavengers that enhanced the clearance of glutamate from the extracellular space. The depressant effect of GluK1 on MF-GPSCs was mediated by a metabotropic process sensitive to pertussis toxin. In the presence of U73122 (1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), a selective inhibitor of phospholipase C, along the transduction pathway downstream to G-protein, GluK1 activation increased the probability of GABA release, thus unveiling the ionotropic action of this receptor. In line with this type of action, we found that GluK1 enhanced MF excitability by directly depolarizing MF terminals via calcium-permeable cation channels. Furthermore, GluK1 dynamically regulated the direction of spike time-dependent plasticity occurring by pairing MF stimulation with postsynaptic spiking and switched spike time-dependent potentiation into depression. The GluK1-induced depression of MF-GPSCs would prevent excessive activation of the CA3 associative network by the excitatory action of GABA and the emergence of seizures in the immature brain.

Introduction

Kainate receptors (KARs) are highly expressed in the CNS, where they are involved in different physiological functions (Jane et al., 2009). Five different receptor subtypes have been cloned, GluK1-5, which coassemble in various combinations to form functional receptors with distinct pharmacological and physiological properties (Collingridge et al., 2009). Although at some excitatory connections KARs carry at least in part current charges of synaptic responses (Castillo et al., 1997; Vignes and Collingridge, 1997; Mulle et al., 1998; Cossart et al., 2002), at presynaptic sites they exert a powerful control of transmitter release (Lerma, 2003). In addition, evidence has been provided that, in several CNS regions, KARs control cell excitability through a metabotropic type of action, which involves the activation of G-protein and intracellular signal cascades (Rodríguez-Moreno and Lerma, 1998; Rozas et al., 2003; Melyan et al., 2004; Lerma, 2006; Rodríguez-Moreno and Sihra, 2007). The hippocampus is endowed with KARs with preponderant presynaptic localization (Represa et al., 1987; Ben-Ari and Cossart, 2000). In particular, activation of KARs localized on glutamatergic mossy fibers (MFs), the axons of dentate gyrus granule cells, enhances glutamate release particularly during frequency-dependent facilitation, a form of short-term plasticity characteristic of MF–CA3 synapses (Contractor et al., 2001; Lauri et al., 2001a; Schmitz et al., 2001) (but see Kwon and Castillo, 2008). This effect has been attributed to kainate-induced depolarization of presynaptic boutons or axon terminals (Lauri et al., 2001b; Kamiya et al., 2002; Nicoll and Schmitz, 2005) and the release of calcium from local stores (Scott et al., 2008). Interestingly, KARs are present also on presynaptic GABAergic terminals, where they exert either a facilitatory (Mulle et al., 2000; Cossart et al., 2001) or a depressant (Clarke et al., 1997; Rodríguez-Moreno et al., 1997; Rodríguez-Moreno and Lerma, 1998; Maingret et al., 2005) effect on GABA release. Activation of these receptors relies on the source of glutamate, on the spatiotemporal feature of glutamate release in the extracellular space, and their affinity for glutamate.

Several lines of evidence suggest a role of KARs in neuronal development. Signaling via presynaptic GluK1 containing KARs has been shown to be critical for regulating the number of functional glutamatergic synapses (Vesikansa et al., 2007) and the balance between GABAergic and glutamatergic transmission, which control correlated network activity (Lauri et al., 2005). Interestingly, during the first week of postnatal life, the main neurotransmitter released from MF terminals is GABA (Safiulina et al., 2006), which exerts a depolarizing and excitatory action on targeted cells (Sivakumaran et al., 2009). MF-evoked glutamatergic currents comprising a kainate component start appearing after postnatal day 6 (P6) (Marchal and Mulle, 2004).

Here, we addressed whether, immediately after birth, MF-induced $GABA_A$-mediated postsynaptic currents (GPSCs) in CA3

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principal cells are controlled by presynaptic KARs. We report that, at this developmental stage, MF-GPSCs are downregulated by G-protein-coupled GluK1 receptors, which are endogenously activated by glutamate present in the extracellular medium. We further demonstrated that, at these synapses, GluK1 receptors dynamically regulate the direction of spike time-dependent plasticity (STDP).

Materials and Methods

Slice preparation. Experiments were performed on hippocampal slices from P2 to P5 Wistar rats as previously described (Gasparini et al., 2000). All experiments were performed in accordance with the European Community Council Directive of 24 November 1986 (86/609EEC) and were approved by local authority veterinary service. Briefly, animals were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 g/kg). The brain was quickly removed from the skull and placed in ice-cold artificial CSF (ACSF) containing the following (in mM): 130 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 27 $NaHCO_3$, 1.3 $MgCl_2$, 2 $CaCl_2$, 25 glucose, saturated with 95% O_2 and 5% CO_2, pH 7.3–7.4. Transverse hippocampal slices (400 μm thick) were cut with a vibratome and stored at room temperature (20–24°C) in a holding bath containing the same solution as above. After a recovery period of at least 1 h, an individual slice was transferred to the recording chamber, in which it was continuously superfused with oxygenated ACSF at a rate of 2–3 ml/min at 33–35°C.

Electrophysiological recordings. Electrophysiological experiments were performed from CA3 pyramidal cells using the whole-cell configuration of the patch-clamp technique in current- or voltage-clamp mode. Neurons were visualized using an upright microscope (Olympus BX51WI) equipped with differential interference contrast optics and infrared video camera. Patch electrodes were pulled from borosilicate glass capillaries (Hilgenberg). They had a resistance of 4–6 MΩ when filled with an intracellular solution containing the following (in mM): 140 KCl, 1 $MgCl_2$, 10 HEPES, 4 MgATP, 0.5 EGTA, pH 7.3. In some cases, guanosine 5′-[β-thio]diphosphate (GDPβS) (Sigma-Aldrich) at the concentration of 0.3 mM was included into the patch pipette to block postsynaptic G-protein-coupled receptors. Recordings were made with a patch-clamp amplifier (Axopatch 200A; Molecular Devices). The access resistance was repetitively monitored every 5 min with a voltage step and was <20 MΩ, and the results were discarded if it changed >15–20%.

Mossy fiber $GABA_A$-mediated postsynaptic currents (GPSCs) were evoked at 0.05 Hz from a holding potential of −70 mV. We used minimal stimulation of the granule cells in the dentate gyrus to activate only one or few presynaptic fibers. According to the technique described by Jonas et al. (1993) and Allen and Stevens (1994), the stimulation intensity was decreased until only a single axon was activated. This was achieved when the mean amplitude of the postsynaptic currents and failure probability remained constant over a range of stimulus intensities near threshold for detecting a response (see Fig. 1*A*,*B*) (Safiulina et al., 2006). An abrupt increase in the mean peak amplitude of synaptic currents was observed when the stimulus intensity was further increased. This all-or-none behavior led us to assume that only a single fiber was stimulated. In addition, the latency and the shape of individual synaptic responses remained constant for repeated stimuli. The monosynaptic nature of synaptic currents was supported by the unimodal and narrow latencies and rise time distributions, which remained constant when the extracellular Ca^{2+}/Mg^{2+} concentration ratio was reduced from 2:1.3 to 1:3 (Safiulina et al., 2006).

When the probability of synaptic failures in response to a first stimulus was near 1 (failures were estimated by visual discrimination), we applied a second pulse at 50 ms interval. If a response to a second stimulus appeared in 15–30 consecutive trials (silent to the first stimulus), we considered this synapse "presynaptically" silent.

MF inputs were identified on the basis of their sensitivity to group III metabotropic glutamate receptor (mGluR) agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4) (10 μM) (Gutiérrez et al., 2003; Kasyanov et al., 2004; Safiulina et al., 2006), their strong paired-pulse facilitation, and short-term frequency-dependent facilitation (Safiulina et al., 2006). GPSCs were blocked by bicuculline or picrotoxin. In contrast to MF inputs, GABAergic inputs from interneurons were insensitive to L-AP4 (Walker et al., 2001; Safiulina et al., 2006). To block G-protein-mediated signals, slices were incubated overnight in a medium containing pertussis toxin (PTx) (5 μg/ml).

In some experiments, antidromic action potentials were recorded from visually identified granule cells in dentate gyrus. In this case, the intracellular solution contained K-gluconate (150 mM) instead of KCl. Extracellular stimuli (at 0.3 Hz; duration, 150 s) were delivered via a stimulation electrode positioned in stratum lucidum ~200 μm away from the granule cell layer.

STDP was induced in current-clamp mode by pairing MF stimulation with postsynaptic spikes. MF-GPSCs were recorded first in voltage-clamp mode for 5–10 min to obtain a stable baseline. Then, STDP was induced by pairing presynaptic stimulation of granule cells in the dentate gyrus with postsynaptic spiking. This sequence (MF stimulation–postsynaptic spike) was repeated 10 times at 0.1 Hz. In the case of positive pairing (pre vs post), a delay of 15 ms was introduced between presynaptic stimulation and postsynaptic spiking, whereas, in the case of negative pairing (post vs pre), the delay between postsynaptic spiking and presynaptic stimulation was 50 ms. Changes in synaptic efficacy were monitored by recording synaptic currents for additional 20–30 min after pairing.

The drugs used were as follows: 6,7-dinitroquinoxaline-2,3-dione (DNQX), L-AP4, picrotoxin (PTX), 4-(8-methyl-9*H*-1,3-dioxolo[4,5-h][2,3]benzodiazepin-5-yl)-benzenamine hydrochloride (GYKI 52466), (*S*)-1-(2-amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione (UBP 302), (*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid (ATPA), D-(−)-2-amino-5-phosphonopentaoic acid (D-AP5), (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), philanthotoxin (PhTx), PTx, 1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) (all purchased from Tocris Bioscience); glutamate scavenger system [glutamic-pyruvic transaminase (GPT) plus pyruvate], 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl] azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS), dihydro-β-erythroidine (DHβE), atropine, GDPβS, baclofen, and 3-[[(3,4-dichlorophenyl) methyl]amino]propyl]diethoxymethyl)phosphinic acid (CGP 52432) were purchased from Sigma-Aldrich.

All drugs were dissolved in water, ethanol, or dimethylsulfoxide (DMSO), as required. UBP 302 was dissolved in 1 eq NaOH. DNQX, GYKI 52466, and LY341495 were dissolved in DMSO. The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of synaptic currents. Drugs were applied in the bath via a three-way tap system, by changing the superfusion solution to one differing only in its content of drug(s). The ratio of flow rate to bath volume ensured complete exchange within 1–2 min.

Data acquisition and analysis. Data were acquired and digitized with an analog-to-digital converter (Digidata 1200; Molecular Devices) and stored on a computer hard disk. Acquisition and analysis of evoked responses were performed with Clampfit 9 (Molecular Devices). Data were sampled at 20 kHz and filtered with a cutoff frequency of 1 kHz. Mean GPSCs amplitude was obtained by averaging successes and failures. The paired-pulse ratio (PPR) was calculated as the mean amplitude of the synaptic response evoked by the second stimulus over that evoked by the first one. The coefficient of variation was calculated as the ratio between the SD of synaptic currents amplitude and the mean. Unless otherwise stated, data are presented as mean ± SEM. Quantitative comparisons were based on Student's paired or unpaired *t* test, as required, and values of $p < 0.05$ were considered to be significant.

Results

Tonic activation of presynaptic GluK1 receptors downregulates GABA release from MF terminals

Whole-cell patch-clamp recordings were performed from CA3 principal cells in hippocampal slices obtained from P2–P5 rats. In agreement with previous studies (Gutiérrez et al., 2003; Kasyanov

et al., 2004; Safiulina et al., 2006; Safiulina and Cherubini, 2009; Sivakumaran et al., 2009), during the first week of postnatal life, minimal stimulation of granule cells in the dentate gyrus elicited low-probability GPSCs, which were completely blocked by bicuculline (10 μM) or picrotoxin (100 μM). Depolarizing the cell to positive potentials (+40 mV) produced bicuculline-sensitive outward currents with latency, onset, and deactivation kinetics similar to those obtained at −70 mV, further indicating that they were mediated by $GABA_A$ receptors (data not shown) (Safiulina et al., 2006). Consistent with previous observations (Safiulina et al., 2006; Safiulina and Cherubini, 2009), bath application of GYKI 52466 (30 μM), which at this concentration selectively blocks AMPA receptors, did not modify the amplitude, the shape, the latency, and rise time of individual responses, indicating that AMPA receptors do not contribute to synaptic currents (Fig. 1*A–D*). On average, on five cells, the amplitude of MF-evoked GPSCs was 40 ± 2 and 39 ± 1 pA ($p = 0.8$), the rise time was 1.8 ± 0.2 and 1.9 ± 0.3 ms ($p = 0.4$), and latency was 2.8 ± 0.2 and 2.8 ± 0.3 ms ($p = 0.8$), before and during GYKI 52466 application, respectively. Interestingly, the addition of DNQX (50 μM) (which blocks both AMPA and kainate receptors) to GYKI 52466 significantly enhanced the amplitude of single fiber-evoked GPSCs, an effect that was associated with an increase in the number of successes (Fig. 2*A*). The time course of DNQX effects on the amplitude of GPSCs (successes plus failures) obtained in eight cells is represented in Figure 2*B*. As summarized in supplemental Figure S1, *A* and *B* (available at www.jneurosci.org as supplemental material), the peak amplitude of GPSCs (successes plus failures) was 30 ± 4 and 53 ± 10 pA before and after DNQX, respectively ($p = 0.01$), whereas the success rate changed from 0.34 ± 0.05 to 0.57 ± 0.08 ($p = 0.006$). These effects were associated with a significant reduction of the PPR (from 1.33 ± 0.23 to 0.67 ± 0.11; $p = 0.001$) (supplemental Fig. S1*C*, available at www.jneurosci.org as supplemental material) and a significant increase in the inverse squared value of the coefficient of variation (CV^{-2}) of responses amplitude (from 0.73 ± 0.15 to 2.28 ± 0.44; $p = 0.03$) (supplemental Fig. S1*D*, available at www.jneurosci.org as supplemental material). Although changes in success rate may be indicative of presynaptic or postsynaptic changes, PPR and CV^{-2} are primarily used to evaluate changes in probability of transmitter release (Zucker and Regehr, 2002). These effects occurred in the absence of any change in input resistance or in the holding current of the recorded neurons. As expected for MF-mediated responses, GPSCs were significantly reduced in amplitude by group III mGluR agonist L-AP4 (10 μM) and completely blocked by picrotoxin (100 μM) (Fig. 2*B*) (Gutiérrez et al., 2003; Kasyanov et al., 2004; Safiulina et al., 2006). Synaptic currents originating from GABAergic interneurons could be readily distinguished from MF-GPSCs since unlike the latter, which exhibited paired-pulse facilitation, they exhibited paired-pulse depression and were insensitive to L-AP4 (the peak amplitude of synaptic currents was 39 ± 8 and 36 ± 9 pA before and during L-AP4, respectively; $p = 0.1$; $n = 7$) (Walker et al., 2001; Safiulina et al., 2006) (data not shown).

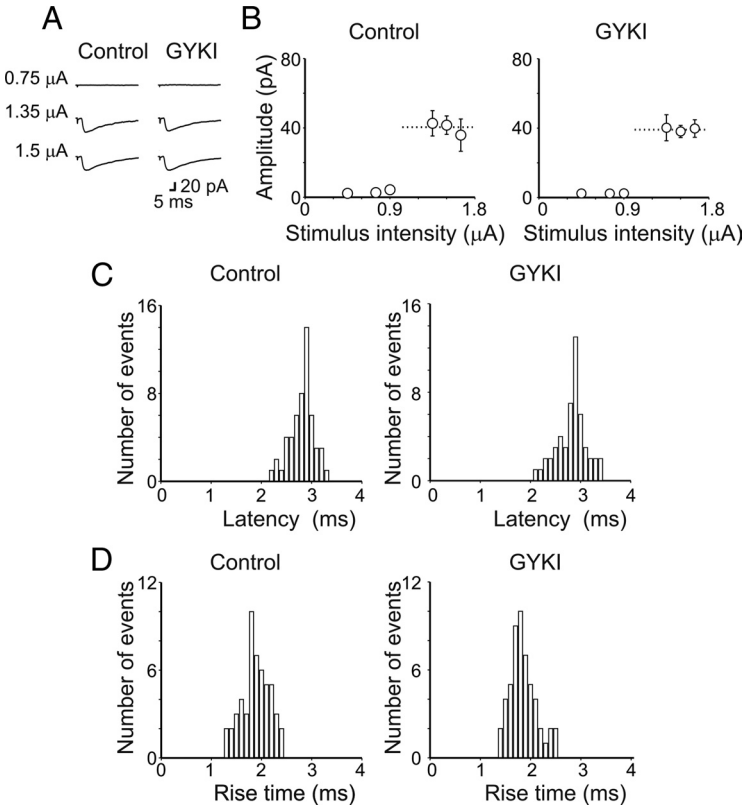

Figure 1. AMPA receptors do not contribute to unitary postsynaptic currents evoked in CA3 principal cells by minimal stimulation of granule cells in the dentate gyrus. ***A***, Unitary synaptic currents evoked in a CA3 pyramidal cell at P4 with different stimulation intensities before (Control) and during application of GYKI 52466 (30 μM). Each trace is the average of 15–20 responses (including failures). ***B***, Peak amplitudes of synaptic currents represented in ***A*** are plotted as a function of stimulus intensities. The bars are SEM, and the dashed lines are the averaged amplitude of GPSCs. Latency (***C***) and rise time (***D***) distributions of individual currents evoked in a CA3 pyramidal cell by minimal stimulation of granule cells in the dentate gyrus in the absence (Control) and in the presence of GYKI 52466. Note that, in the presence of GYKI 52466, the unimodal distribution of latencies and rise times of individual responses did not change.

To exclude the possibility of an indirect effect via kainate-induced modulation of other receptors known to depress transmitter release such as $GABA_B$ receptors (Safiulina and Cherubini, 2009), nicotine and muscarinic acetylcholine receptors (Maggi et al., 2004), purinergic P2Y receptors (Zhang et al., 2003; Safiulina et al., 2005), and mGluRs (Scanziani et al., 1997), in a set of experiments ($n = 18$) DNQX was applied in the presence of CGP 52432 (1 μM), DHβE (50 μM), atropine (1 μM), PPADS (50 μM), and LY341495 (100 μM), selective antagonists for these receptors. Also in this case, DNQX induced a significant increase in GPSCs amplitude similar to that obtained in the absence of the blockers, suggesting a direct effect of DNQX on kainate receptors localized on MF terminals (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

To identify which kainate receptor subtype was involved in the observed effects, we used UBP 302, which is a selective GluK1 kainate receptor antagonist (More et al., 2004; Jane et al., 2009). The rationale behind was that, during postnatal development, GluK1 receptors are highly expressed in the hippocampus (Bettler et al., 1990; Bahn et al., 1994; Ritter et al., 2002) and previous work from immature CA3 pyramidal cells demonstrated the involvement of GluK1 in modulation of spontaneous,

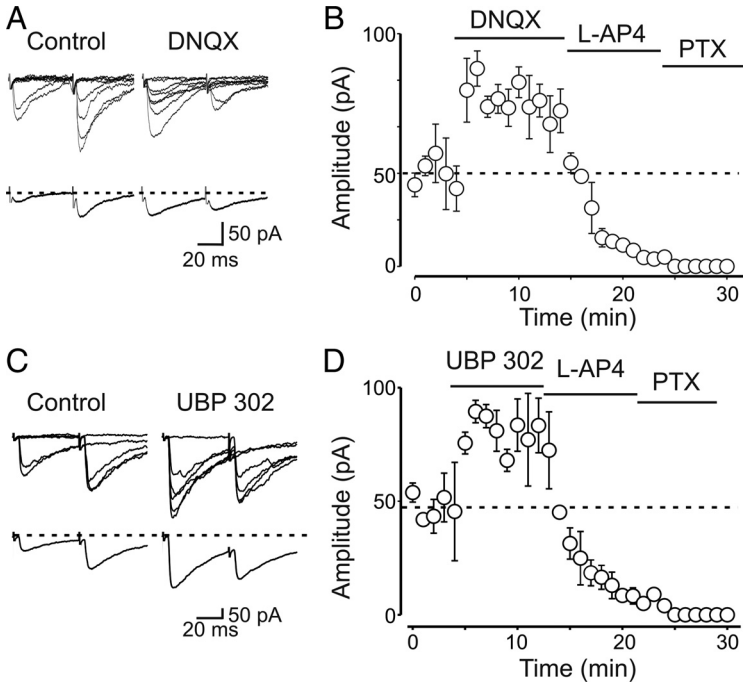

Figure 2. Endogenous activation of presynaptic kainate receptors downregulates MF-GPSCs. ***A***, Superimposed individual traces of MF-GPSCs evoked in the presence of GYKI 52466 (30 μM) and GYKI 52466 plus DNQX (50 μM). Note that, in this and in the following experiments, GYKI 52466 was always present in the bathing solution in control conditions. Below, Averaged traces (successes plus failures). Note that DNQX enhanced the amplitude of the first response and reduced the number of synaptic failures. ***B***, Summary plot showing the mean amplitude of GPSCs obtained in eight cells in the presence of DNQX, L-AP4, and PTX. Vertical bars are SEM. The horizontal dashed line refers to the mean amplitude value measured before DNQX. ***C***, Superimposed individual traces (above) and average traces (below) of MF-GPSCs evoked in the presence of GYKI 52466 (30 μM) and GYKI 52466 plus UBP 302 (10 μM). ***D***, As in ***B***, but in the presence of UBP 302, L-AP4, and PTX ($n = 19$).

network-driven giant depolarizing potentials (GDPs) (Lauri et al., 2005), which are highly expressed in the CA3 area (Ben-Ari et al., 2007). Like DNQX, UBP 302 (10 μM) caused a significant increase in amplitude of GPSCs (Fig. 2*C*,*D*). As summarized in supplemental Figure S3*A* (available at www.jneurosci.org as supplemental material), the peak amplitude of GPSCs was 48 ± 8 and 84 ± 15 pA before and after UBP 302, respectively ($n = 19$; $p = 0.001$). UBP 302 caused also a significant increase in success rate (from 0.4 ± 0.04 to 0.7 ± 0.03; $p = 0.001$) (supplemental Fig. S3*B*, available at www.jneurosci.org as supplemental material), a significant decrease in PPR (from 1.4 ± 0.2 to 0.6 ± 0.1; $n = 16$; $p = 0.001$) (supplemental Fig. S3*C*, available at www.jneurosci.org as supplemental material), and a significant increase in CV^{-2} (from 0.97 ± 0.2 to 3.3 ± 0.9; $p = 0.001$; $n = 19$) (supplemental Fig. S3*D*, available at www.jneurosci.org as supplemental material). In six cases, stimulation of granule cells in the dentate gyrus failed to produce any synaptic response to the first stimulus (over at least 30 consecutive trials). However, occasional responses to the second stimulus suggested that these synapses were "presynaptically" silent (Gasparini et al., 2000; Kasyanov et al., 2004; Safiulina and Cherubini, 2009; Sivakumaran et al., 2009). Application of UBP 302 to "presynaptically" silent cells induced the appearance of synaptic responses to the first stimulus (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). In these cases, the PPR could not be measured because calculated ratios are infinitely large when the mean amplitude of the first response is close to zero. Compatible with a lack of quantal release, in these cases also the CV^{-2} was close to zero.

In contrast to UBP 302, pharmacological activation of GluK1 receptors with ATPA (1 μM), a selective GluK1 agonist (Clarke et al., 1997), reversibly decreased the amplitude of MF-GPSCs (from 54 ± 9 to 22 ± 4 pA; $n = 11$; $p = 0.001$) (Fig. 3*A–C*), an effect that was accompanied with a decrease in the number of successes (0.6 ± 0.1 to 0.35 ± 0.1; $p = 0.001$) (Fig. 3*D*), an increase in PPR (0.8 ± 0.1 to 1.3 ± 0.2; $n = 7$; $p = 0.008$) (Fig. 3*E*), and a decrease in the CV^{-2} (1.5 ± 1.3 to 1.1 ± 0.2; $n = 11$; $p = 0.01$) (Fig. 3*F*). It is worth noting that for ATPA experiments we used cells with high release probability and therefore exhibiting a low PPR (ranging from 0.4 to 1.3). Together, these data strongly indicate that presynaptic GluK1 receptors depress GABA release from immature MF terminals.

Presynaptic GluK1 receptors are endogenously activated by glutamate present in the extracellular space

How could presynaptic GluK1 receptors be activated if, early in postnatal development, the main neurotransmitter released by MF is GABA? One possibility is that these receptors are tonically activated by endogenous glutamate present in the extracellular space. To test this hypothesis, in another set of experiments, we used an enzymatic glutamate scavenger system (GPT plus pyruvate) (Overstreet et al., 1997; Min et al., 1998) on the assumption that enhancing the clearance of glutamate from the extracellular space prevents the activation of presynaptic kainate receptors. GPT catalyzes the conversion of glutamate and pyruvate to α-ketoglutarate and alanine. To exclude any effect via mGluRs (Scanziani et al., 1997), we routinely added in the bathing solution the broad spectrum $mGluR_{1-8}$ antagonist LY341495 (100 μM) (Fitzjohn et al., 1998). The scavenger mimicked in all respects the facilitating effects of UBP 302 on GPSCs (the peak amplitude of GPSCs was 44 ± 8 and 73 ± 10 pA before and after the scavenger, respectively; $n = 7$; $p = 0.003$) (Fig. 4*A*,*B*). The scavenger caused also a significant increase in success rate (from 0.5 ± 0.1 to 0.8 ± 0.1; $p = 0.01$) (Fig. 4*C*), a significant decrease in PPR (from 1.5 ± 0.2 to 0.7 ± 0.1; $n = 7$; $p = 0.008$) (Fig. 4*D*), and a significant increase in CV^{-2} (from 1.02 ± 0.2 to 3.4 ± 0.94; $n = 7$; $p = 0.01$) (Fig. 4*E*), further suggesting a presynaptic site of action. In addition, the facilitating effect of the scavenger on GPSCs was fully occluded by subsequent application of UBP (the peak amplitude of GPSCs was 73 ± 10 and 70 ± 12 pA in the presence of the scavenger and the scavenger plus UBP 302, respectively; $n = 7$) (Fig. 4*B*). Application of the scavenger after UBP 302 was also ineffective ($n = 5$; $p = 0.3$) (data not shown). These results indicate that, early in postnatal life, depression of GABA release from MF terminals occurs via activation of GluK1 receptors by endogenous glutamate present in the extracellular space.

The depressant effect of kainate on MF GPSCs is mediated by G-coupled receptors

At inhibitory synapses, kainate-induced depression of GABA release has been shown to involve the presynaptic activation of a PTx-sensitive $G_{i/o}$-protein-coupled kainate receptor (Lerma,

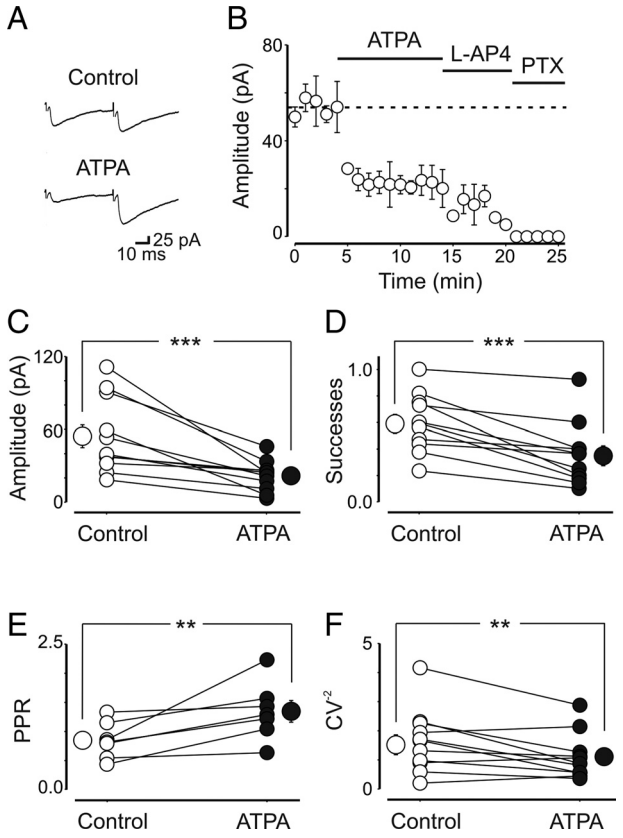

Figure 3. Bath application of the selective GluK1 agonist ATPA mimics the effects of endogenous glutamate on kainate receptors. ***A***, Averaged traces of GPSCs evoked in a CA3 pyramidal cell by stimulation of granule cells in the dentate gyrus, in control condition and after addition of ATPA (1 μM). ***B***, Summary plot for 11 cells. ***C–F***, Amplitude (***C***), successes (***D***), PPR (***E***), and inversed square of CV (***F***) measured in individual cells before and after application of ATPA. $^{**}p < 0.01$; $^{***}p < 0.001$.

2006; Rodríguez-Moreno and Sihra, 2007). G-protein in turn would activate phospholipase C (PLC) leading to the release of calcium from intracellular stores. The concomitant activation of protein kinase C (PKC) would inhibit voltage-dependent N-type calcium channels known to control transmitter release (Castillo et al., 1994; Dunlap et al., 1995; Li et al., 2007). To elucidate whether the effects of kainate on GPSCs involved a G-protein, hippocampal slices were incubated overnight with a solution containing PTx (5 μg/ml). In these conditions, bath application of the $GABA_B$ receptor agonist baclofen (from a holding potential of −60 mV) failed to evoked any outward current (data not shown) (Lauri et al., 2005). UBP 302 applied to nine neurons previously exposed to PTx either did not modify the amplitude of GPSCs (the peak amplitude of GPSCs was 52 ± 11 and 58 ± 13 pA in the absence or in the presence of UBP 302, respectively; $n = 5$; $p = 0.1$) or depressed it (from 68 ± 8 to 42 ± 3 pA; $n = 4$; $p = 0.02$). Pooled data from all cells tested are represented in Figure 5*A*. Similarly, the amplitude of GPSC was unaltered when the scavenger was applied to slices incubated in PTx (on average, in six cells, the peak amplitude of GPSCs was 61 ± 14 and 59 ± 16 pA in the absence and in the presence of the scavenger, respectively; $p = 0.6$) (Fig. 5*B*, closed circles). Incubation with PTx affects G-protein-coupled receptors present on both presynaptic and postsynaptic membranes. To see whether the depression of MF-GPSCs involved G-coupled receptors localized on the presynaptic site, additional experiments ($n = 5$) were performed using GDPβS (0.3 mM) into the patch pipette. This treatment completely blocked postsynaptic $GABA_B$ receptors. Thus, in the presence of GDPβS, the $GABA_B$ receptor agonist baclofen (20 μM) failed to induce outward currents (baclofen evoked-currents were 53 ± 5 and 5 ± 2 pA in the absence or in the presence of GDPβS, respectively; $n = 6$; $p = 0.001$). However, as shown in the example of Figure 5*C*, baclofen, via presynaptic $GABA_B$ receptors, depressed spontaneous ongoing synaptic currents. Bath application of UBP 302 to neurons recorded with GDPβS was still able to enhance GPSCs amplitude (the peak amplitude of GPSCs varied from 29 ± 4 to 43 ± 5 pA; $n = 5$; $p = 0.003$) (Fig. 5*D*,*E*), indicating that the depression of MF-GPSCs is mediated by G-protein-coupled kainate receptors, present on MF terminals.

As already mentioned, the most common transduction signaling pathway stimulated by G-protein involves PLC activation (Rodríguez-Moreno and Lerma, 1998; Rozas et al., 2003). Therefore, in the following experiments, we tried to disrupt the intracellular cascade downstream to G-protein activation with U73122 (10 μM), a selective PLC blocker. Surprisingly, we found that, unlike PTx, application of UBP 302 in the presence of U73122 caused a significant depression of MF-GPSCs, probably unmasking the ionotropic action of GluK1. The amplitude of MF-GPSCs was 50 ± 9 and 29 ± 11 pA in the absence or in the presence of UBP 302, respectively ($n = 6$; $p = 0.01$) (Fig. 6*A*,*B*). In contrast to UBP 302, application of the GluK1 agonist ATPA (1 μM) in the presence of U73122 induced a significant increase in amplitude of GPSCs (from 40 ± 5 to 61 ± 3 pA; $n = 7$; $p = 0.01$) (Fig. 6*C*,*D*). Similarly to UBP 302, the enzymatic glutamate scavenger system applied in the presence of U73122 caused a depressant effect on GPSCs, which was probably dependent on the relief of a tonic action of glutamate on GluK1 receptors (from 70 ± 3 to 30 ± 2 pA; $n = 11$; $p < 0.001$) (Fig. 6*E*,*F*). The lack of UBP 302-induced depression of GPSCs amplitude observed in some neurons exposed to PTx could be explained by the fact that incubating the slices with PTx for several hours may interfere with other processes not directly linked to KAR activation.

Activation of presynaptic kainate receptors enhances MF excitability

In juvenile animals, activation of presynaptic kainate receptors has been found to directly depolarize via cation channels glutamatergic MF (Kamiya and Ozawa, 2000) or GABAergic terminals (Semyanov and Kullmann, 2001), thus lowering the threshold for antidromic action potential generation. To test whether activation of GluK1 receptors localized on immature MF terminals exhibits similar characteristics, we recorded antidromic spikes from single granule cells (held at −70 mV) in response to stimulation of MFs via a stimulation electrode positioned in stratum lucidum. When we used stimuli able to evoke antidromic spikes in >50% of trials, application of UBP 302 (10 μM) reversibly decreased the probability of successes from 0.6 ± 0.05 to 0.15 ± 0.02 ($n = 7$; $p = 0.001$) (Fig. 7*A*,*B*). In contrast, ATPA reversibly increased the probability of successes when antidromic spikes were evoked by weak stimuli (<50% of successes). The success rate was 0.38 ± 0.04 and 0.72 ± 0.04 in the absence or in the presence of ATPA, respectively ($n = 7$; $p = 0.001$) (Fig. 7*C*,*D*). Since GluK1 receptors are known to be highly permeable to calcium (Lauri et al., 2003; Scott et al., 2008), in another set of experiments we applied PhTx (3 μM), which blocks calcium-permeable AMPA/kainate receptor (Fletcher and Lodge, 1996). As UBP 302, PhTx significantly reduced the number of successes from 0.59 ± 0.04 to 0.15 ± 0.05 ($n = 7$; $p = 0.001$) (Fig. 7*E*,*F*). In the presence of PhTx, we increased the stimulation intensity to obtain again >50% of successes, and then we applied UBP 302. In

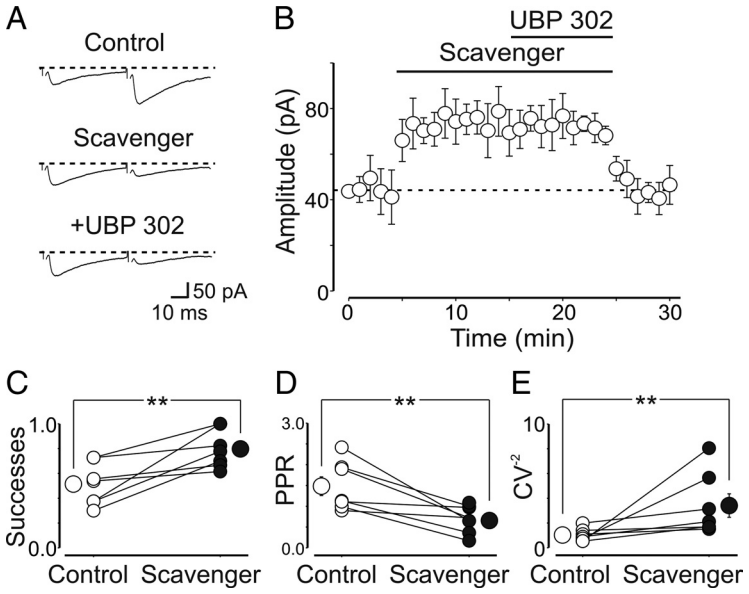

Figure 4. Ambient glutamate activates presynaptic GluK1 kainate receptors on mossy fibers terminals. ***A***, Averaged traces of GPSCs evoked by stimulation of granule cells in the dentate gyrus before and after addition of the glutamate scavenger (GPT plus pyruvate) and the scavenger plus UBP 302. The broad-spectrum $mGluR_{1-8}$ antagonist LY341495 (100 μM) was present throughout the experiments. ***B***, Pooled data from seven cells to show that the glutamate scavenger increased the mean peak amplitude of GPSCs and fully occluded the effects of UBP 302. Error bars indicate SEM. ***C–E***, Successes (***C***), PPR (***D***), and inversed square of CV (***E***) measured in individual cells before and after application of the glutamate scavenger. $^{**}p < 0.01$.

this case, the drug failed to produce any effect. The success rate was 0.58 ± 0.09 and 0.61 ± 0.09 before and during UBP 302, respectively ($n = 7$; $p = 0.08$) (Fig. 7*G*,*H*). In line with an ionotropic type of action, exposing the slices to PTx did not alter UBP 302-induced reduction in the success rate of antidromic spikes (0.62 ± 0.08 and 0.16 ± 0.06; $n = 5$; $p = 0.002$) (data not shown). These data indicate GluK1 increases MF excitability through the activation of calcium-permeable cationic channels and depolarization of MF terminals.

GluK1Rs control the direction of spike time-dependent plasticity

In a previous study, we demonstrated that, depending on the relative timing, pairing afferent stimulation with postsynaptic spiking induced bidirectional changes in synaptic efficacy (Sivakumaran et al., 2009). In particular, a persistent increase in synaptic strength was observed when MF stimulation preceded postsynaptic spiking. To avoid possible contamination with glutamatergic events, experiments were routinely performed in the presence of D-AP5 and DNQX to block NMDA and AMPA/kainate receptors, respectively. Therefore, to see whether GluK1 receptors control spike time-dependent long-term potentiation (LTP), in the present experiments, the pairing procedure was repeated only in the presence of GYKI 52466, to prevent the activation of AMPA receptors. Pairing consisted in correlating (in current-clamp mode) MF stimulation with postsynaptic spikes (10 times at 0.1 Hz) (see Materials and Methods) using, as in previous work, a delay of 15 ms (corresponding to the peak of the synaptic responses) (Fig. 8*A*,*B*). As shown in the representative example of Figure 8*C*, pairing (arrow) induced a reduction in amplitude of GPSCs, which persisted for at least 20 min. On average, the peak amplitude of GPSCs (successes plus failures) was 42 ± 6 and 19 ± 2 pA before and 20 min after pairing, respectively ($p = 0.003$; $n = 11$) (Fig. 8*C*). This effect was associated with a significant decrease in success rate (from 0.46 ± 0.06 to 0.25 ± 0.06; $p = 0.005$), in the inversed square of the coefficient of variation of responses amplitude (from 1.67 ± 0.49 to 0.96 ± 0.29; $p = 0.02$), and a significant increase in PPR (from 0.86 ± 0.27 to 1.97 ± 0.47; $n = 8$; $p = 0.01$). To see whether spike time-dependent depression was mediated via an ionotropic or a metabotropic type of action, similar experiments were repeated in the presence of the selective PLC blocker U73122 (10 μM). In this condition, the pairing procedure failed to produce any effect (the peak amplitude of GPSCs was 60 ± 3 and 58 ± 2 pA before and after pairing, respectively; $n = 7$; $p = 0.5$) (Fig. 8*D*), suggesting the involvement of a G-protein-coupled KAR in STDP. When UBP 302 was added to GYKI 52466, the pairing procedure produced a persistent potentiation of synaptic responses that was in all respects similar to that described previously (Sivakumaran et al., 2009) (Fig. 8*E*). In summary, the amplitude of GPSCs varied from 84 ± 15 to 124 ± 18 pA ($p = 0.001$). This effect was associated with an increase in success rate (from 0.5 ± 0.05 to 0.8 ± 0.06; $p = 0.001$), in CV^{-2} (from 1.19 ± 0.2 to 4.1 ± 1; $p = 0.01$), and a decrease in PPR (from 1.5 ± 0.2 to 0.8 ± 0.2; $n = 9$; $p = 0.004$). In additional experiments, negative pairing (postsynaptic spiking preceding MF stimulation with a delay of 50 ms) induced (in the presence of GYKI 52466) long-term depression similar in all respect to that induced in the presence of DNQX (Sivakumaran et al., 2009), suggesting that KARs are not involved in this form of synaptic plasticity (the peak amplitude of GPSCs was 116 ± 22 and 53 ± 10 pA before and 20 min after pairing, respectively; $n = 7$; $p = 0.004$) (data not shown). These results indicate that presynaptic GluK1 control STD-LTP at immature MF–CA3 synapses.

Discussion

The present experiments from the immature hippocampus have revealed the presence of functional presynaptic kainate receptors on GABAergic MF terminals. Endogenous activation of these receptors by glutamate present in the extracellular medium reduces the probability of GABA release contributing in some cases to synapses silencing. Moreover, GluK1 receptors present on MF terminals dynamically regulate the direction of STDP being able to switch spike time-dependent potentiation into depression.

In previous studies, we provided evidence that, during the first week of postnatal life, the main neurotransmitter released from MF terminals is GABA (Kasyanov et al., 2004; Safiulina et al., 2006; Safiulina and Cherubini, 2009; Sivakumaran et al., 2009). MF-mediated glutamatergic responses start appearing during the second week of postnatal life (Amaral and Dent, 1981). In particular, kainate-mediated EPSCs have been detected only after P6, in coincidence with the appearance of large-amplitude AMPA responses and with the onset of low-frequency facilitation (Marchal and Mulle, 2004). This period tightly correlates with that in which $GABA_A$-mediated synaptic responses shift from the depolarizing to the hyperpolarizing direction (Cherubini et al., 1991; Ben-Ari et al., 2007). In the present experiments, GPSCs originated from MF since they exhibited strong paired-pulse fa-

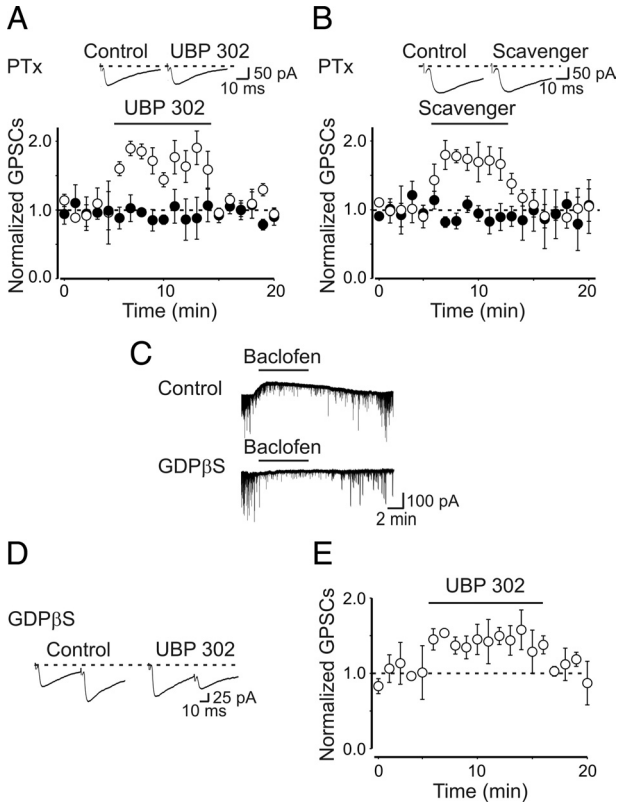

Figure 5. The depressant effect of kainate on GPSCs involves a G-protein-mediated mechanism. ***A***, Summary plot showing the effects of UBP 302 on the peak amplitude of GPSCs in controls (open symbols) and after overnight treatment with PTx (closed symbols). The insets above the graph represent average traces obtained in the presence of GYKI 52466 and GYKI 52466 plus UBP 302. ***B***, As in ***A***, but in the presence of the glutamate scavenger showing that also the effect of the scavenger is dependent on G-protein. ***C***, Application of baclofen (20 μM; bar) induced an outward currents associated with a reduction of spontaneous synaptic events (top trace). Application of baclofen to a cell recorded with a patch pipette containing GDPβS failed to produce an outward current but was still able to depress the ongoing synaptic activity (bottom trace). ***D***, ***E***, GDPβS into the intrapipette solution did not affect UBP 302-induced GPSCs facilitation. ***D***, Representative traces. ***E***, Summary plot from five cells. Error bars indicate SEM.

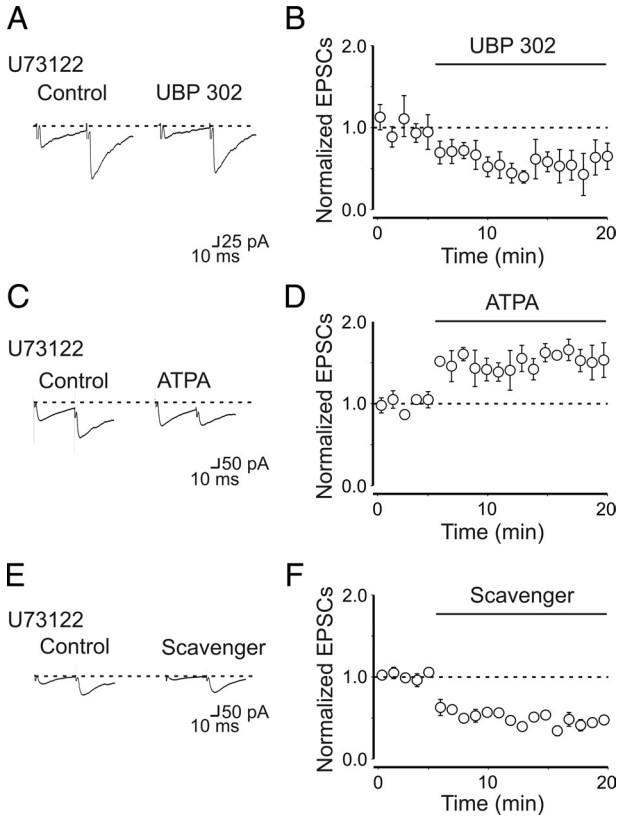

Figure 6. Blocking PLC with U73122 reveals the ionotropic action of GluK1 on MF-GPSCs. ***A***, Averaged traces of MF-GPSCs evoked in a CA3 principal cell in the presence of U73122 (10 μM; Control) and U73122 plus UBP 302 (10 μM). ***B***, Summary plot showing the mean amplitude of GPSCs obtained in six cells in the presence of U73122 before and during application of UBP 302 (bar). The amplitude of synaptic responses is normalized to those obtained before UBP 302 (dashed line). In the presence of UBP 302, the amplitude of the synaptic responses was significantly different from controls ($p = 0.01$). ***C–F***, As in ***A*** and ***B***, but in the presence of ATPA (1 μM) (***C***, ***D***) and the glutamate scavenger (GPT plus pyruvate) (***E***, ***F***). Note that both the ATPA-induced potentiation and the scavenger-induced depression of GPSCs amplitude were significantly different from controls ($p = 0.01$ and $p < 0.001$, respectively). Error bars indicate SEM.

cilitation and were sensitive to group III mGluR agonist L-AP4 (Gutiérrez, 2005; Safiulina et al., 2006). In this respect, they could be easily distinguished from those originating from GABAergic interneurons, which were insensitive to L-AP4 and exhibited paired-pulse depression (Walker et al., 2001; Safiulina et al., 2006). MF-mediated synaptic responses did not carry AMPA-mediated components since GYKI 52466, at the concentration used to block AMPA receptors, failed to modify the amplitude, rise time, or latency of synaptic currents. However, in the presence of GYKI 52466, synaptic currents were reversibly enhanced by DNQX or UBP 302, indicating that presynaptic KARs downregulate GABA release from MF terminals. In this respect, our results are similar to those obtained by Maingret et al. (2005) on $GABA_A$-mediated postsynaptic currents evoked in neonatal CA1 pyramidal neurons by electrical stimulation of GABAergic axons. In the immature hippocampus, presynaptic GluK1 receptors have been well documented (Bahn et al., 1994; Bettler et al., 1990; Ritter et al., 2002), and a recent study has provided evidence that at CA3–CA1 synapses functional GluK1 receptors downregulate glutamate release (Lauri et al., 2006). Moreover, in the CA3 hippocampal region, activation of GluK1 receptors reversibly blocks spontaneous network-driven bursts such as GDPs (Lauri et al., 2005). In our case, the depression of MF-GPSCs by KARs was not mediated indirectly via other signaling molecules known to inhibit GABA release, since DNQX was still able to enhance the amplitude of GPSCs when applied in the presence of various receptor antagonists including those for $GABA_B$, nicotinic, muscarinic, P2Y, and mGlu.

Several lines of evidence suggest that GluK1 receptors localized on MF terminals reduce the probability of GABA release: (1) the increase in amplitude of MF-GPSCs by DNQX or UBP 302 was associated with an enhanced success rate and a reduction in paired-pulse ratio, considered an index of presynaptic release probability (Zucker and Regehr, 2002). (2) The GluK1 agonist ATPA induced a powerful depression of MF-evoked synaptic responses, an effect associated with a significant increase in transmitter failures and in the paired-pulse ratio. (3) Decreasing the concentration of "ambient" glutamate with the scavenger prevented the activation of GluK1 receptors and mimicked the effects of KAR antagonists. Previous studies from different brain structures have demonstrated that physiological modifications in glutamate concentration may cause a switch in KAR function from facilitation to inhibition (Jiang et al., 2001; Delaney and Jahr, 2002; Braga et al., 2003; Youn and Randic, 2004; Lerma, 2006). It is therefore likely that, early in postnatal development, a high level of glutamate in the extracellular space, maintained by a

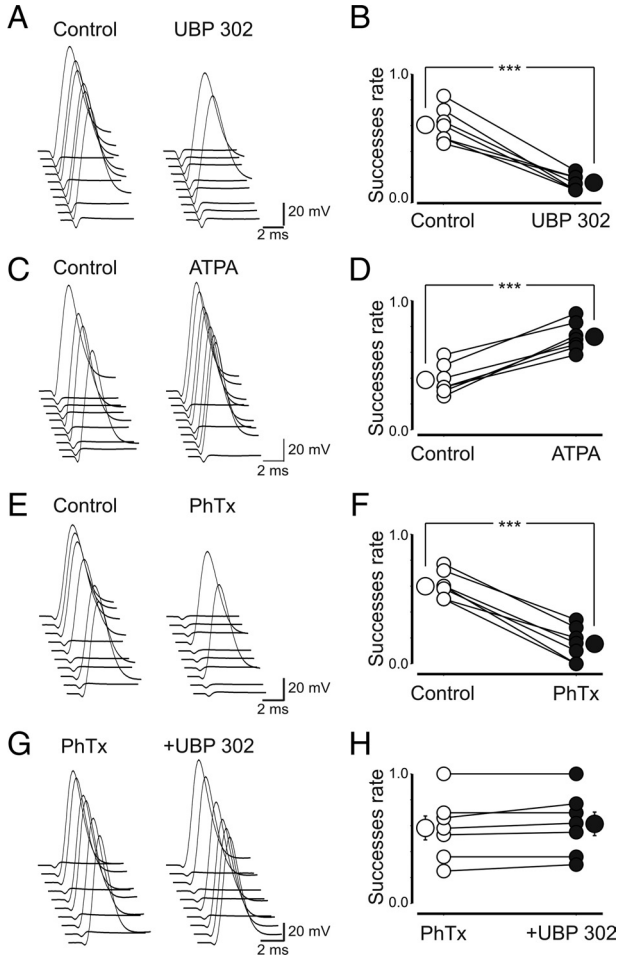

Figure 7. GluK1 receptors sensitive to philantotoxin control MF excitability. ***A***, Consecutive traces showing antidromic spikes recorded in granule cells on stimulation of MF in stratum lucidum before and during application of UBP 302 (note that the stimulus strength was set to obtain >50% of successes). ***B***, Summary plot of UBP 302 effects on success rate ($n = 7$). ***C***, ***D***, In cells with <50% of successes, ATPA enhanced MF excitability and the success rate ($n = 7$). ***E***, ***F***, PhTx (3 μM) mimicked the effects of UBP 302 ($n = 7$). ***G***, ***H***, In the presence of philantotoxin, UBP was not effective ($n = 7$). ***$p < 0.001$. Error bars indicate SEM.

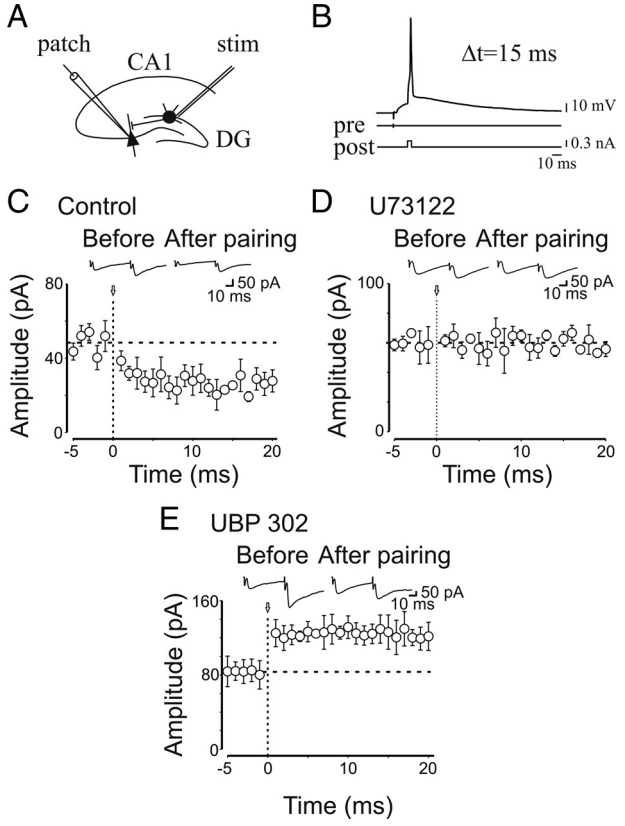

Figure 8. At immature MF–CA3 synapses, presynaptic kainate receptors control the direction of STDP. ***A***, Schematic representation of the experimental design. ***B***, The stimulation of granule cells in the dentate gyrus (pre) preceded the postsynaptic spike (post) by 15 ms (Δt). ***C***, Summary plot of the mean peak amplitude of GPSCs recorded in the presence of GYKI 52466 before and after pairing (arrow at time 0; $n = 11$). The dashed line represents the mean amplitude of GPSCs before pairing. The insets represent averaged GPSCs obtained from a single neuron before and after pairing. Note that pairing induced synaptic depression. ***D***, Summary plot of MF-GPSCs amplitude obtained in the presence U73122 versus time ($n = 7$). Note that blocking PLC with U73122 failed to produce any effect on GPSCs amplitude. ***E***, As in ***C***, but in the presence of UBP 302 ($n = 9$). In this case, pairing induced synaptic potentiation. Error bars indicate SEM.

less efficient glutamate transport mechanism (Danbolt, 2001; Diamond, 2005) and a poorly developed diffusional barrier (Jansson et al., 2000), facilitates the activation of high-affinity KARs with consequent reduction in the probability of transmitter release.

KARs have been shown to exert both an ionotropic and a metabotropic type of action (for review, see Lerma, 2006). In particular, the depression of transmitter release seems to occur via G-protein-coupled KARs (Rodríguez-Moreno and Lerma, 1998; Cunha et al., 2000; Frerking et al., 2001; Lauri et al., 2005, 2006). Also in our case, the depressant effect of kainate on GABA release was likely dependent on a metabotropic type of mechanism since the potentiating effects of both UBP 302 and the glutamate scavenger on GPSCs were prevented by PTx. The signaling pathway likely involved the release of calcium from intracellular stores, the activation of phospholipase C and PKC with consequent inhibition of voltage-dependent calcium channels (Rodríguez-Moreno and Lerma, 1998; Rozas et al., 2003). Interestingly, blocking PLC with U73122, downstream to G-protein activation, unveiled a potentiating effect of GluK1 on MF GPSCs. This was probably dependent on the ionotropic type of action of this receptor and was similar to that observed in some cells exposed to PTx. This type of action was clearly responsible for GluK1-induced increase in MF excitability, in which calcium flux through calcium-permeable KARs depolarized presynaptic terminals and lowered the threshold for antidromic spikes (Kamiya and Ozawa, 2000; Schmitz et al., 2001; Semyanov and Kullmann, 2001; Maingret et al., 2005). How can an increased MF excitability be reconciled with a depression of GABA release? Although, according to Kamiya and Ozawa (2000), a downregulation of transmitter release may occur via inactivation of Na^+/Ca^{2+} channels or electrical shunting, this seems unlikely in view of the recent finding of KA-induced facilitation of action potential evoked calcium entry in MF boutons via a calcium store-dependent mechanism (Scott et al., 2008). In addition, it is worth noting that, unlike adults, immature MFs terminate in very small spherical expansions (Amaral and Dent, 1981) and do not exhibit use-dependent synaptic facilitation yet (Marchal and Mulle, 2004). It is unclear whether the dual signaling pathways (ionotropic and metabotropic), which depend on the common ionotropic GluK1 subunit, are independent or functionally coupled. In a previous study from dorsal root ganglion cells, it has been demonstrated that KA through GluK1 receptors induces a G-protein-dependent rise in $[Ca^{2+}]_i$, favoring its release from the internal stores (Rozas et al., 2003). As a matter of speculation, we favor the

hypothesis that calcium entering through calcium-permeable KARs may directly or indirectly interfere with G-protein-mediated signaling leading to a dominant inhibitory action on MF-GPSCs. The interplay between these two different pathways has been recently shown to account for the PKC-dependent autoregulation of membrane KARs (Rivera et al., 2007). However, much work is needed to elucidate this issue.

Early in postnatal development, G-protein-dependent mechanisms linked to KA activation have been well established particularly at glutamatergic synapses (Lauri et al., 2005, 2006). These receptors have a very high affinity for glutamate, a condition required for being endogenously activated by glutamate present in the extracellular medium. With maturation, presynaptic KARs in an activity-dependent manner would be gradually lost and replaced by low-affinity ones, not longer able to be activated by ambient glutamate (Lauri et al., 2006). In the present experiments, tonic activation of presynaptic KARs by endogenous glutamate accounted for the persistent depression of MF-GPSCs observed after pairing presynaptic MF stimulation with postsynaptic spiking as demonstrated by the possibility to switch spike time-dependent depression into potentiation with UBP 302. Spike time-dependent depression, observed in the absence of AMPA/kainate receptor antagonists, involved the activation of a G-protein-coupled KAR since it was prevented by the selective PLC blocker U73122. In contrast, spike time-dependent potentiation of MF-GPSCs, observed in the presence of UBP 302, was shown to be dependent on intracellular calcium rise and release of BDNF (Sivakumaran et al., 2009). Although the precise mechanisms underlying these phenomena are still unclear, we cannot exclude the possibility that KA-induced synaptic depression may rely on a distinct calcium signal, which in turn may activate a different molecular pathway, as suggested by the calcium hypothesis (Caporale and Dan, 2008).

In conclusion, it is conceivable that, at immature MF–CA3 synapses, KA-induced depression of GABAergic transmission by ambient glutamate limits the excessive activation of the autoassociative CA3 network by the excitatory action of GABA, thus preventing the onset of seizures. These properties are likely to be critical for information processing and for the proper development of the adult hippocampal circuitry.

References

Allen C, Stevens CF (1994) An evaluation of causes for unreliability of synaptic transmission. Proc Natl Acad Sci U S A 91:10380–10383.

Amaral DG, Dent JA (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J Comp Neurol 195:51–86.

Bahn S, Volk B, Wisden W (1994) Kainate receptor gene expression in the developing rat brain. J Neurosci 14:5525–5547.

Ben-Ari Y, Cossart R (2000) Kainate, a double agent that generates seizures: two decades of progress. Trends Neurosci 23:580–587.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215–1284.

Bettler B, Boulter J, Hermans-Borgmeyer I, O'Shea-Greenfield A, Deneris ES, Moll C, Borgmeyer U, Hollmann M, Heinemann S (1990) Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. Neuron 5:583–595.

Braga MF, Aroniadou-Anderjaska V, Xie J, Li H (2003) Bidirectional modulation of GABA release by presynaptic glutamate receptor 5 kainate receptors in the basolateral amygdala. J Neurosci 23:442–452.

Caporale N, Dan Y (2008) Spike timing-dependent plasticity: a hebbian learning rule. Annu Rev Neurosci 31:25–46.

Castillo PE, Weisskopf MG, Nicoll RA (1994) The role of Ca^{2+} channels in hippocampal mossy fiber synaptic transmission and long-term potentiation. Neuron 12:261–269.

Castillo PE, Malenka RC, Nicoll RA (1997) Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. Nature 388:182–186.

Cherubini E, Gaiarsa JL, Ben-Ari Y (1991) GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 14:515–519.

Clarke VR, Ballyk BA, Hoo KH, Mandelzys A, Pellizzari A, Bath CP, Thomas J, Sharpe EF, Davies CH, Ornstein PL, Schoepp DD, Kamboj RK, Collingridge GL, Lodge D, Bleakman D (1997) A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. Nature 389:599–603.

Collingridge GL, Olsen RW, Peters J, Spedding M (2009) A nomenclature for ligand-gated ion channels. Neuropharmacology 56:2–5.

Contractor A, Swanson G, Heinemann SF (2001) Kainate receptors are involved in short- and long-term plasticity at mossy fiber synapses in the hippocampus. Neuron 29:209–216.

Cossart R, Tyzio R, Dinocourt C, Esclapez M, Hirsch JC, Ben-Ari Y, Bernard C (2001) Presynaptic kainate receptors that enhance the release of GABA on CA1 hippocampal interneurons. Neuron 29:497–508.

Cossart R, Epsztein J, Tyzio R, Becq H, Hirsch J, Ben-Ari Y, Crépel V (2002) Quantal release of glutamate generates pure kainate and mixed AMPA/kainate EPSCs in hippocampal neurons. Neuron 35:147–159.

Cunha RA, Malva JO, Ribeiro JA (2000) Pertussis toxin prevents presynaptic inhibition by kainate receptors of rat hippocampal [^{3}H]GABA release. FEBS Lett 469:159–162.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105.

Delaney AJ, Jahr CE (2002) Kainate receptors differentially regulate release at two parallel fiber synapses. Neuron 36:475–482.

Diamond JS (2005) Deriving the glutamate clearance time course from transporter currents in CA1 hippocampal astrocytes: transmitter uptake gets faster during development. J Neurosci 25:2906–2916.

Dunlap K, Luebke JI, Turner TJ (1995) Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neurosci 18:89–98.

Fitzjohn SM, Bortolotto ZA, Palmer MJ, Doherty AJ, Ornstein PL, Schoepp DD, Kingston AE, Lodge D, Collingridge GL (1998) The potent mGlu receptor antagonist LY341495 identifies roles for both cloned and novel mGlu receptors in hippocampal synaptic plasticity. Neuropharmacology 37:1445–1458.

Fletcher EJ, Lodge D (1996) New developments in the molecular pharmacology of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors. Pharmacol Ther 70:65–89.

Frerking M, Schmitz D, Zhou Q, Johansen J, Nicoll RA (2001) Kainate receptors depress excitatory synaptic transmission at CA3→CA1 synapses in the hippocampus via a direct presynaptic action. J Neurosci 21:2958–2966.

Gasparini S, Saviane C, Voronin LL, Cherubini E (2000) Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? Proc Natl Acad Sci U S A 97:9741–9746.

Gutiérrez R (2005) The dual glutamatergic-GABAergic phenotype of hippocampal granule cells. Trends Neurosci 28:297–303.

Gutiérrez R, Romo-Parra H, Maqueda J, Vivar C, Ramìrez M, Morales MA, Lamas M (2003) Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 23:5594–5598.

Jane DE, Lodge D, Collingridge GL (2009) Kainate receptors: pharmacology, function and therapeutic potential. Neuropharmacology 56:90–113.

Jansson A, Lippoldt A, Mazel T, Bartfai T, Ogren SO, Syková E, Agnati LF, Fuxe K (2000) Long distance signalling in volume transmission. Focus on clearance mechanisms. Prog Brain Res 125:399–413.

Jiang L, Xu J, Nedergaard M, Kang J (2001) A kainate receptor increases the efficacy of GABAergic synapses. Neuron 30:503–513.

Jonas P, Major G, Sakmann B (1993) Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. J Physiol 472:615–663.

Kamiya H, Ozawa S (2000) Kainate receptor-mediated presynaptic inhibition at the mouse hippocampal mossy fibre synapse. J Physiol 523:653–665.

Kamiya H, Ozawa S, Manabe T (2002) Kainate receptor-dependent short-term plasticity of presynaptic Ca^{2+} influx at the hippocampal mossy fiber synapses. J Neurosci 22:9237–9243.

Kasyanov AM, Safiulina VF, Voronin LL, Cherubini E (2004) GABA-mediated giant depolarizing potentials as coincidence detectors for enhancing synaptic efficacy in the developing hippocampus. Proc Natl Acad Sci U S A 101:3967–3972.

Kwon HB, Castillo PE (2008) Role of glutamate autoreceptors at hippocampal mossy fiber synapses. Neuron 60:1082–1094.

Lauri SE, Bortolotto ZA, Bleakman D, Ornstein PL, Lodge D, Isaac JT, Collingridge GL (2001a) A critical role of a facilitatory presynaptic kainate receptor in mossy fiber LTP. Neuron 32:697–709.

Lauri SE, Delany C, Clarke VRJ, Bortolotto ZA, Ornstein PL, Isaac JTR, Collingridge GL (2001b) Synaptic activation of a presynaptic kainate receptor facilitates AMPA receptor-mediated synaptic transmission at hippocampal mossy fibre synapses. Neuropharmacology 41:907–915.

Lauri SE, Bortolotto ZA, Nistico R, Bleakman D, Ornstein PL, Lodge D, Isaac JT, Collingridge GL (2003) A role for Ca^{2+} stores in kainate receptor-dependent synaptic facilitation and LTP at mossy fiber synapses in the hippocampus. Neuron 39:327–341.

Lauri SE, Segerstråle M, Vesikansa A, Maingret F, Mulle C, Collingridge GL, Isaac JT, Taira T (2005) Endogenous activation of kainate receptors regulates glutamate release and network activity in the developing hippocampus. J Neurosci 25:4473–4484.

Lauri SE, Vesikansa A, Segerstråle M, Collingridge GL, Isaac JT, Taira T (2006) Functional maturation of CA1 synapses involves activity-dependent loss of tonic kainate receptor-mediated inhibition of glutamate release. Neuron 50:415–429.

Lerma J (2003) Roles and rules of kainate receptors in synaptic transmission. Nat Rev Neurosci 4:481–495.

Lerma J (2006) Kainate receptor physiology. Curr Opin Pharmacol 6:89–97.

Li L, Bischofberger J, Jonas P (2007) Differential gating and recruitment of P/Q-, N-, and R-type Ca^{2+} channels in hippocampal mossy fiber boutons. J Neurosci 7:13420–13429.

Maggi L, Sola E, Minneci F, Le Magueresse C, Changeux JP, Cherubini E (2004) Persistent decrease in synaptic efficacy induced by nicotine at Schaffer collateral-CA1 synapses in the immature rat hippocampus. J Physiol 559:863–874.

Maingret F, Lauri SE, Taira T, Isaac JT (2005) Profound regulation of neonatal CA1 rat hippocampal GABAergic transmission by functionally distinct kainate receptor populations. J Physiol 567:131–142.

Marchal C, Mulle C (2004) Postnatal maturation of mossy fibre excitatory transmission in mouse CA3 pyramidal cells: a potential role for kainate receptors. J Physiol 561:27–37.

Melyan Z, Lancaster B, Wheal HV (2004) Metabotropic regulation of intrinsic excitability by synaptic activation of kainate receptors. J Neurosci 24:4530–4534.

Min MY, Rusakov DA, Kullmann DM (1998) Activation of AMPA, kainate, and metabotropic receptors at hippocampal mossy fiber synapses: role of glutamate diffusion. Neuron 21:561–570.

More JC, Nistico R, Dolman NP, Clarke VR, Alt AJ, Ogden AM, Buelens FP, Troop HM, Kelland EE, Pilato F, Bleakman D, Bortolotto ZA, Collingridge GL, Jane DE (2004) Characterisation of UBP296: a novel, potent and selective kainate receptor antagonist. Neuropharmacology 47:46–64.

Mulle C, Sailer A, Pérez-Otaño I, Dickinson-Anson H, Castillo PE, Bureau I, Maron C, Gage FH, Mann JR, Bettler B, Heinemann SF (1998) Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice. Nature 392:601–615.

Mulle C, Sailer A, Swanson GT, Brana C, O'Gorman S, Bettler B, Heinemann SF (2000) Subunit composition of kainate receptors in hippocampal interneurons. Neuron 28:475–484.

Nicoll RA, Schmitz D (2005) Synaptic plasticity at hippocampal mossy fibre synapses. Nat Rev Neurosci 6:863–876.

Overstreet LS, Pasternak JF, Colley PA, Slater NT, Trommer BL (1997) Metabotropic glutamate receptor mediated long-term depression in developing hippocampus. Neuropharmacology 36:831–844.

Represa A, Tremblay E, Ben-Ari Y (1987) Kainate binding sites in the hippocampal mossy fibers: localization and plasticity. Neuroscience 20:739–748.

Ritter LM, Vazquez DM, Meador-Woodruff JH (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. Brain Res Dev Brain Res 139:227–236.

Rivera R, Rozas JL, Lerma J (2007) PKC-dependent autoregulation of membrane kainate receptors. EMBO J 26:4359–4367.

Rodríguez-Moreno A, Lerma J (1998) Kainate receptor modulation of GABA release involves a metabotropic function. Neuron 20:1211–1218.

Rodríguez-Moreno A, Sihra TS (2007) Kainate receptors with a metabotropic modus operandi. Trends Neurosci 30:630–637.

Rodríguez-Moreno A, Herreras O, Lerma J (1997) Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus. Neuron 19:893–901.

Rozas JL, Paternain AV, Lerma J (2003) Noncanonical signaling by ionotropic kainate receptors. Neuron 39:543–553.

Safiulina VF, Cherubini E (2009) At immature mossy fibers-CA3 connections, activation of presynaptic $GABA_B$ receptors by endogenously released GABA contributes to synapses silencing. Front Cell Neurosci 3:1.

Safiulina VF, Kasyanov AM, Sokolova E, Cherubini E, Giniatullin R (2005) ATP contributes to the generation of network-driven giant depolarizing potentials in the neonatal rat hippocampus. J Physiol 565:981–992.

Safiulina VF, Fattorini G, Conti F, Cherubini E (2006) GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. J Neurosci 26:597–608.

Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385:630–634.

Schmitz D, Mellor J, Frerking M, Nicoll RA (2001) Presynaptic kainate receptors at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 98:11003–11008.

Scott R, Lalic T, Kullmann DM, Capogna M, Rusakov DA (2008) Target-cell specificity of kainate autoreceptor and Ca^{2+}-store-dependent short-term plasticity at hippocampal mossy fiber synapses. J Neurosci 28:13139–13149.

Semyanov A, Kullmann DM (2001) Kainate receptor-dependent axonal depolarization and action potential initiation in interneurons. Nat Neurosci 4:718–723.

Sivakumaran S, Mohajerani MH, Cherubini E (2009) At immature mossy-fiber–CA3 synapses, correlated presynaptic and postsynaptic activity persistently enhances GABA release and network excitability via BDNF and cAMP-dependent PKA. J Neurosci 29:2637–2647.

Vesikansa A, Sallert M, Taira T, Lauri SE (2007) Activation of kainate receptors controls the number of functional glutamatergic synapses in the area CA1 of rat hippocampus. J Physiol 583:145–157.

Vignes M, Collingridge GL (1997) The synaptic activation of kainate receptors. Nature 388:179–182.

Walker MC, Ruiz A, Kullmann DM (2001) Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. Neuron 29:703–715.

Youn DH, Randic M (2004) Modulation of excitatory synaptic transmission in the spinal substantia gelatinosa of mice deficient in the kainate receptor GluR5 and/or GluR6 subunit. J Physiol 555:683–698.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971–982.

Zucker RS, Regehr WG (2002) Short-term synaptic plasticity. Annu Rev Physiol 64:355–405.

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Developmental regulation of CB1-mediated spike-time dependent depression at immature mossy fiber-CA3 synapses

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Early in postnatal life, mossy fibres (MF), the axons of granule cells in the dentate gyrus, release GABA which is depolarizing and excitatory. Synaptic currents undergo spike-time dependent long-term depression (STD-LTD) regardless of the temporal order of stimulation (pre *versus* post and *viceversa*). Here we show that at P3 but not at P21, STD-LTD, induced by negative pairing, is mediated by endocannabinoids mobilized from the postsynaptic cell during spiking-induced membrane depolarization. By diffusing backward, endocannabinoids activate cannabinoid type-1 (CB1) receptors probably expressed on MF. Thus, STD-LTD was prevented by CB1 receptor antagonists and was absent in *CB1*-KO mice. Consistent with these data, in situ hybridization experiments revealed detectable level of CB1 mRNA in the granule cell layer at P3 but not at P21. These results indicate that CB1 receptors are transiently expressed on immature MF terminals where they counteract the enhanced neuronal excitability induced by the excitatory action of GABA.

The axons of granule cells in the dentate gyrus, the mossy fibres (MFs) provide most of the excitatory drive to CA3 principal cells and GABAergic interneurons of the hilus and stratum lucidum[1]. Early in postnatal development MF release GABA which exerts a depolarizing and excitatory action on target neurons[2,3]. Immature MFs are endowed with kainate receptors (KARs) and $GABA_B$ receptors, whose activation reduces GABA release leading in some cases to synapse silencing[4,5].

Immature MF-CA3 synapses undergo activity-dependent modifications of synaptic efficacy which are crucial for learning and memory processes and for the refinement of neuronal circuits. Thus, calcium transients associated with giant depolarizing potentials or GDPs, a hallmark of developmental networks, act as coincident detectors for enhancing synaptic efficacy in an associative manner[6]. In addition, immature MF-CA3 synapses exhibit spike time-dependent long-term depression (STD-LTD)[7], an associative form of learning crucial for information coding. STD-LTD occurs regardless of the temporal order of stimulation (pre *versus* post and *viceversa*). However, STD-LTD induced by positive pairing (pre before post) can be switched to STD-long-term potentiation (LTP) by blocking presynaptic GluK1-containing KARs with selective antagonists, indicating that KARs activated by 'ambient' glutamate control the direction of STD-LTD[5]. However, in the presence of KAR antagonists, negative pairing (postsynaptic spiking before MF stimulation) still induces LTD, suggesting that KARs are not involved in this form of synaptic plasticity. Studies from several brain structures have shown that STD-LTD is controlled by endocannabinoids, which activate presynaptic CB1 receptors *via* retrograde signalling and suppress neurotransmitter release at both excitatory and inhibitory synapses[8,9]. Here we show that, at immature MF-CA3 synapses, the persistent weakening of synaptic strength induced by correlating postsynaptic spiking with presynaptic MF activation involves CB1 signalling. Thus, STD-LTD was prevented by selective CB1 receptor antagonists and was absent in *CB1*-KO mice. In addition, CB1-mediated STD-LTD could not be induced at late developmental stages when MF release glutamate on their targets.

Results

Single MF-evoked $GABA_A$-mediated postsynaptic currents (GPSCs) were recorded at −60 mV from P3–P6 CA3 pyramidal cells in the presence of DNQX (50 μM) and D-AP5 (50 μM) to block AMPA/kainate and NMDA receptors, respectively. Negative pairing (post 50 ms before pre; Fig. 1A and B) induced in 28/44 neurons (64 %) a persistent depression of MF-GPSCs (see also[7]). Thirty minutes after pairing, the mean peak amplitude of GPSCs (successes plus failures) obtained in all neurons tested (exhibiting or not LTD) was 64.4 ± 3.3 % of pre-pairing values (n=44; $p<0.001$; paired *t*-test; Fig. 1C and D). In cells exhibiting LTD (n=28), synaptic depression was associated with a decrease of successes rate (from 0.63 ± 0.05 to 0.39 ± 0.04, before and after pairing, respectively; n=28; $p<0.001$; paired *t*-test), a decrease in the inverse square value of CV (from 2.98 ± 0.68 to 1.12 ± 0.25; n=28; $p<0.001$; paired *t*-test) and an increase in PPR (from 1.09 ± 0.21 to 1.98 ± 0.49; $n=12$; $p<0.001$; paired *t*-test; Fig. 1E), indicating a reduction in the probability of GABA release. To further assess whether STD-LTD results from a reduced GABA release from MF terminals, we analyzed the coefficient of variation before and 30 min after the induction of pairing. Normalized plots of $1/CV^2$ *versus* mean yielded points lying on or below the diagonal, further suggesting that the locus of depression is presynaptic[10] (Fig. 1D). Postsynaptic spiking (10 spikes at 0.1 Hz) in the absence of presynaptic stimulation did not modify MF-GPSCs.

The induction of STD-LTD may require postsynaptic calcium influx through spike-induced membrane depolarization. We tested this possibility by loading the postsynaptic cell with the calcium chelator BAPTA (20 mM). BAPTA prevented the induction of STD-LTD (mean peak amplitude of GPSCs: 95.6 ± 5% of controls, n=13; p=0.7; paired *t*-test; Fig. 2), indicating that this form of synaptic plasticity is due to an increased calcium level in the postsynaptic cell. Calcium influx occurs *via* voltage-dependent calcium channels (VDCC) since STD-LTD was completely blocked by nifedipine (10 μM) a VDCC blocker (after pairing, the mean peak amplitude of GPSCs was 94.9 ± 3.1% of controls; n=8; p=0.1; paired *t*-test; Fig. 2).

Our data demonstrate a postsynaptic induction of STD-LTD, but a presynaptic expression as suggested by the increase in PPR and the decrease in CV^{-2} of MF-GPSCs. The postsynaptic cell must then provide a paracrine retrograde signal to the presynaptic neuron. Possible candidates are endocannabinoids (eCBs), mobilized from principal neurons and known to mediate several forms of retrograde short- and long-term presynaptic depression[9]. Once released, eCBs diffuse to activate CB1 receptors localized on presynaptic neurons and inhibit transmitter release.

To determine whether STD-LTD was CB1-dependent, we applied the selective CB1 antagonist AM251. AM251 (5 μM) *per se* did not modify synaptic activity (see Supplementary Fig. S1 online). However, this compound fully prevented STD-LTD in all cells tested, indicating the involvement of CB1 receptors. In the presence of AM251, the peak amplitude of MF-GPSCs was 97.4 ± 2.7% of controls (n=12; p=0.37; paired *t*-test; Fig. 1C and D). Failure of inducing LTD in a minority of cases (36%) suggests that either endocannabinoids released from principal cells failed to reach MF terminals or that some MF terminals do not express CB1 receptors. In agreement with other studies on different brain structures[11–13], application of AM251 20 min after the induction of STD-LTD did not modify the amplitude of synaptic responses indicating that the activation of CB1 receptors is necessary for the induction, but not for the maintenance of STD-LTD (see Supplementary Fig. S1 online).

To further assess the involvement of CB1 receptors in STD-LTD, the pairing procedure was applied to CA3 principal cells in hippocampal slices obtained from P3–P6 *CB1*-KO mice and WT littermates. As in rats, negative pairing induced a significant and

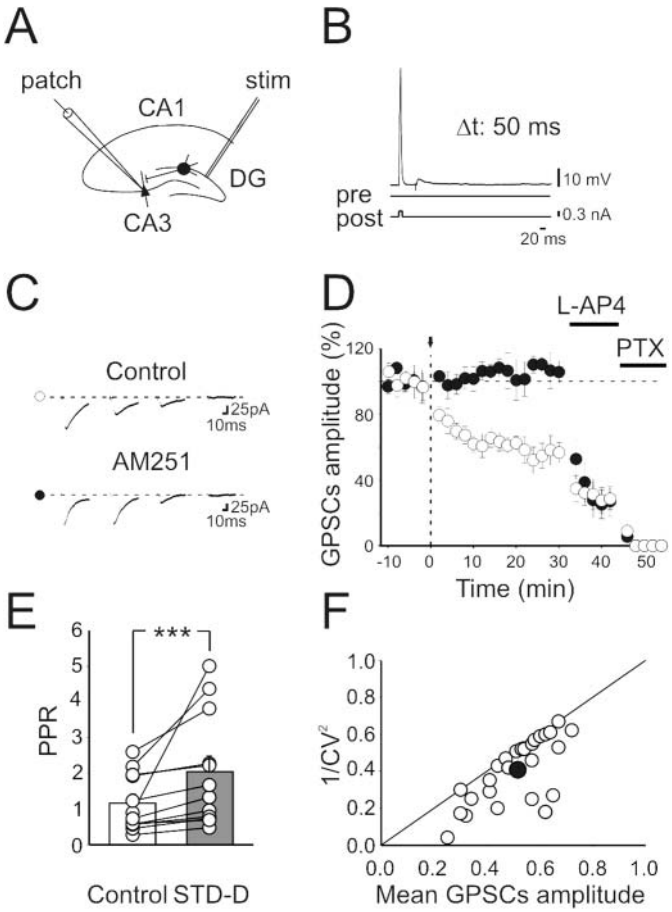

Figure 1 | STD-LTD requires the activation of CB1 receptors. (A) Schematic representation of the experimental design. (B) The stimulation of granule cells in the dentate gyrus followed the postsynaptic spike by 50 ms. (C) MF-GPSCs evoked before, after pairing, after addition of L-AP4 or L-AP4 plus picrotoxin (PTX), in the absence (Control) or in the presence of AM251 (each trace is the average of 30–60 trials including failures). (D) Mean GPSCs amplitude (before and after pairing, arrows at time 0) is plotted against time. Open circles: control (n=44); closed circles: in the presence of AM251(n=12); vertical bars are SEM. (E) Paired-pulse ratio measured before (Control) and after pairing in neurons exhibiting LTD (grey STD-D; n=12). ***$p<0.001$. (F) Plot of $1/CV^2$ *versus* GPSCs amplitude measured after LTD induction and normalized to respective controls. The closed circle indicate the mean (SEM is within the symbols).

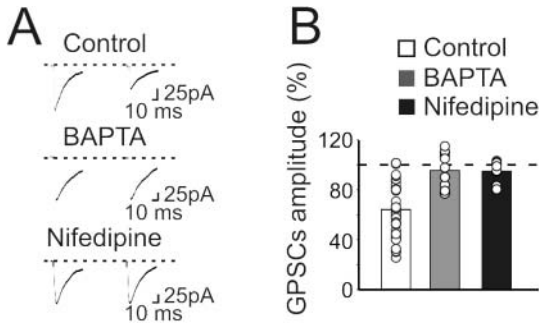

Figure 2 | Pairing-induced LTD requires a calcium influx in the postsynaptic cell *via* voltage-dependent calcium channels. (A) Averaged traces of MF-GPSC (30 to 60 trials including failures) evoked before and 20 min after pairing in control, in neurons loaded with intracellular BAPTA (20 mM) or exposed to nifedipine (10 μM). (B) Pairing-induced changes in the mean amplitude of MF-GPSCs in control (n=44), in cells loaded with BAPTA (n=13) or exposed to nifedipine (black column; n=8).

persistent depression of MF-GPSCs. Pooled data from animals exhibiting (n=9; 69 % of cases) or not (n=4) LTD are reported in Fig. 3. After pairing the mean GPSCc amplitude was 57.8 ± 4.5% of control values (p=0.003; paired t-test; Fig. 3). In cells exhibiting LTD, synaptic depression was associated with a decrease in successes rate (from 0.67 ± 0.07 to 0.33 ± 0.06, before and after pairing, respectively; n=9; p<0.001; paired t-test) and a decrease in the inverse square value of CV (from 3.5 ± 0.58 to 1.6 ± 0.3; n=9; p=0.003; paired t-test). In contrast, in *CB1*-KO mice the pairing protocol did not cause STD-LTD (mean peak amplitude of GPSCs 101.7 ± 4.7% of controls; n=10; p=0.3; paired t-test; Fig. 3), providing further evidence that CB1 receptors are involved in STD-LTD.

If STD-LTD is mediated by CB1 receptors, the possibility to block this form of synaptic plasticity with BAPTA and nifedipine indicates that secretion of eCBs from principal cells is triggered by the elevation of intracellular calcium *via* VDCC. However, signalling *via* group I mGluR might also contribute to intracellular calcium rise *via* PLC, as described for some forms of eCBs-dependent synaptic plasticity[12,14]. Therefore, we tested whether the selective mGluR1 and mGluR5 antagonists LY 367385 and MPEP, respectively were able to prevent STD-LTD. Bath application of LY 367385 (100 μM) and MPEP (5 μM), either alone or in combination, failed to affect STD-LTD. In the presence of LY 367385 plus MPEP, the peak amplitude of MF-GPSCs reached 65 ± 7.3% of control values (n=9; p=0.88; one-way ANOVA; see (see Supplementary Fig. S2 online) indicating that group I mGluR are not involved. Furthermore, STD-LTD did not result from an indirect modulation of eCBs by receptors that depress transmitter release such as $GABA_B$, nicotine and muscarinic acetylcholine receptors, purinergic P2Y and adenosine receptors since the pairing procedure still induced STD-LTD in the presence of the respective antagonists (see Supplementary Fig. S2 online).

Anandamide and 2-arachidonylglycerol (2-AG) are well studied endogenous ligands at cannabinoid receptors[8]. In order to identify which of these two molecules is involved in STD-LTD, the postsynaptic cell was loaded with THL, an inhibitor of diacylglycerol lipase activity[15,16]. THL did not affect STD-LTD: pairing postsynaptic spiking with presynaptic MF stimulation reduced the peak amplitude of MF-GPSCs to 59 ± 8% as in the absence of the drug (n=9; p=0.03; paired t-test; see Supplementary Fig. S3 online). The depressant effect was not significantly different from that obtained in the absence of THL (p=0.76; one-way ANOVA). THL failed to block STD-LTD also when it was applied in the bath at the concentration of 10 μM; 20 min after pairing, the peak amplitude of GPSCs was 51.7 ± 0.12% of controls; n=5; p<0.05; paired t-test; data not shown). These data suggest that 2-AG is not involved in STD-LTD.

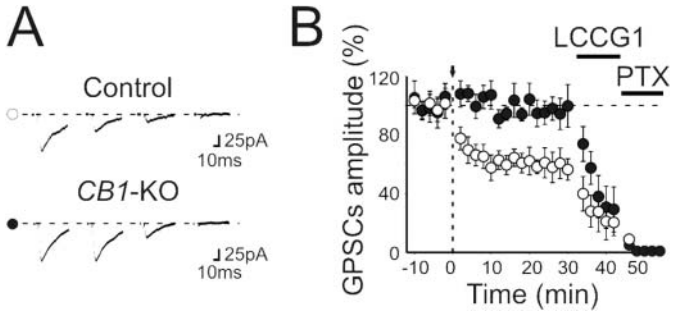

Figure 3 | Lack of STD-LTD in *CB1*-KO mice. (A) MF-GPSCs evoked before, after pairing, after addition of LCCG1 or LCCG1 plus PTX, in WT mice (open circle, Control) or in *CB1*-KO mice (closed circle). (B) Summary plot of mean MF-GPSCs amplitude (before and after pairing, arrows at time 0) *versus* time in WT (open circles; n=9) or in CB1-/- mice (closed circles; n=10). Data from WT animals are pooled between those exhibiting (n=9) or not (n=4) LTD.

To further investigate the endogenous eCB ligand involved in STD-LTD, we next used drugs that alter endocannabinoids degradation. In the brain, anandamide is metabolized by the fatty acid amide hydrolase (FAAH)[17]. Inhibition of FAAH with URB597 (1 μM) did not modify basic synaptic transmission or change the magnitude of STD-LTD. In the presence of URB597, the pairing procedure reduced the peak amplitude of MF-GPSCs to 62 ± 5% of controls (n=7; p<0.05; paired t-test). However, URB597 lengthened up to 100 ms the time window (between postsynaptic spiking and presynaptic stimulation) necessary for the induction of STD-LTD (Fig. 4; see also[12]). Pairing with a delay of 100 ms reduced the peak amplitude of GPSCs to 58 ± 7% of controls (n=9; p=0.03; paired t-test). A further prolongation of the delay between post and presynaptic stimulation to 200 ms failed to induce STD-LTD (99.7 ± 1.2% of control; n=5; p=0.5; paired t-test). Similar data were obtained with AM404[17] (10 μM) which inhibits the carrier responsible for anandamide internalization and degradation (Fig. 4). In contrast, JZL 184 (1 μM), which increases the levels of 2-AG by blocking monoacylglycerol lipase, the enzyme responsible for 2AG degradation, did not alter the time window for LTD induction (Fig. 4). These data show that limiting the uptake or the degradation of anandamide prolongs the time window for the induction of STD-LTD, whereas inhibition of synthesis or degradation of 2-AG does not alter this form of synaptic plasticity. Therefore, these results suggest that anandamide is the main mediator of STD-LTD.

If STD-LTD is mediated by CB1 receptors, CB1 agonists should be able to occlude this form of synaptic plasticity. Overall, in 17 neurons, the CB1 receptor agonist WIN 55,212-2 (2 μM) reduced the peak amplitude of GPSCs to 69 ± 5% of controls (p=0.003; Fig. 5D). These data include also 5 neurons in which WIN 55,212-2 was ineffective. In 12/17 neurons (70.5 %) WIN 55,212-2 reduced the peak amplitude of GPSCs to 57.1 ± 3.4% of controls (p<0.001; paired t-test; Fig. 5A and C). WIN 55,212-2 depressed MF-GPSCs by reducing the probability of GABA release from MF terminals since it decreased the inverse square of CV and increased the PPR, which is inversely correlated with release probability[18] (see Supplementary Fig. S4 online). Importantly, when the pairing protocol was applied after WIN 55,212-2, no further depression of GPSCs was obtained, indicating occlusion (Fig. 5A and C). However, these experiments should be interpreted with caution in view of the reported effects of WIN 55,212-2 on presynaptic calcium channels[19]. Furthermore, STD-LTD induction occluded all effects of WIN 55,212-2 (Fig. 5B and C). Similarly, anandamide (AEA, 30 μM) reduced in 9/13 neurons GPSCs amplitude. The depression of GPSCs amplitude obtained in all cells tested (included those not affected by AEA) was 69.5 ± 5.6 % of controls (n=13; p=0.01; Fig. 5D). In 69.2% of neurons AEA depressed the peak amplitude of GPSCs to 43.3 ± 3% of controls (n=9; p=0.004), an effect that was blocked by AM251 (n=10; p=0.31; paired t-test; Fig. 5D), further indicating that anandamide acts on CB1 receptors. Occlusion was also found between the depressant effects of AEA on GPSCs and STD-LTD (n=5; data not shown). As expected for eCB-induced synaptic depression, a partial recovery of GPSCs amplitude towards pre-drug values (to 76.7 ± 9.4% of control; n=5) was obtained when the CB1 antagonist AM251 was applied 15 minutes after WIN (data not shown).

In the developing hippocampus, CB1-dependent heterosynaptic LTD observed in the CA1 region is associated with a decrease in presynaptic fibres excitability[20]. We asked whether eCBs may affect axonal excitability, by recording antidromic spikes from single granule cells held at −70 mV in the absence or in the presence of the CB1 receptor agonist WIN 55,212-2. MFs were stimulated *via* an electrode positioned in *stratum lucidum* and the stimulus strength was adjusted to evoke antidromic spikes in > 60% of trials. WIN 55,212-2 (2 μM) reduced the probability of evoking antidromic spikes to 54.6 ± 5.7% of controls (n=7; p<0.001; paired t-test; Fig. 6), indicating that

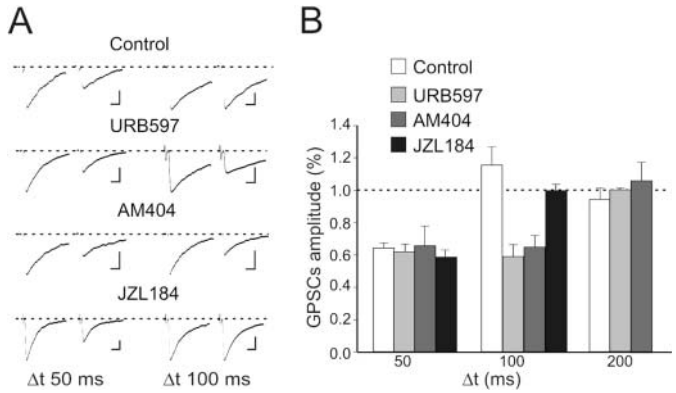

Figure 4 | Blocking anandamide degradation lengthens the time window for STD-LTD induction. (A) MF-GPSCs obtained in control, in the presence of the FAAH inhibitor URB597 (1 μM), of the carrier inhibitor responsible for anandamide internalization and degradation AM404 (10 μM) and of the monoacylglicerol lipase inhibitor JZL 184 (1 μM), the enzyme responsible for 2AG degradation, at Δt of 50 and 100 ms. Calibration: 10 ms and 50 pA. (B) Summary plot of MF-GPSCs obtained in control (n = 44, 5 and 5), in the presence of URB597 (n=7, 9 and 5), AM404 (n=5, 7 and 5) or JZL 184 (n=6, 15) at different time windows (Δt) between postsynaptic spiking and presynaptic stimulation. Note that while URB597 and AM404 prolonged the time windows for inducing STD-LTD, JZL 184 did not.

activation of CB1 receptors probably located on MF terminals reduces MF excitability. This effect was blocked by AM 251 (Fig. 6).

It is well known that CB1 receptors are not expressed on juvenile glutamatergic MF terminals[16,21,22]. Therefore, in the following experiments we tested whether the same pairing protocol used in neonates can induce CB1-dependent STD-LTD at MF-evoked glutamatergic excitatory postsynaptic currents (EPSCs) in CA3 principal cells of P19–P25 old animals. These experiments were routinely performed in the presence of picrotoxin (100 μM) to block $GABA_A$ receptors. Consistent with their MF origin, EPSCs were highly sensitive to group II mGluR agonist DCG-IV[23]. At the concentration of 4 μM this compound induced a 75.8 ± 2.1% reduction in the peak amplitude of EPSCs (Fig. 7). As shown in Fig. 7, negative pairing (post before pre) failed to modify synaptic strength in all neurons tested (n=19; from 6 rats). After pairing the EPSCs amplitude was 104 ± 7% of controls (p=0.84; paired t-test).

Our electrophysiological data support the assumption that CB1 receptors are only transiently expressed on MF terminals in the immediate postnatal period. To validate this hypothesis *in situ* hybridization (ISH) experiments were performed from granule cells at P3 and P21 to assess whether CB1 mRNA expression follows a similar developmental profile. In the hippocampus of adult mice,

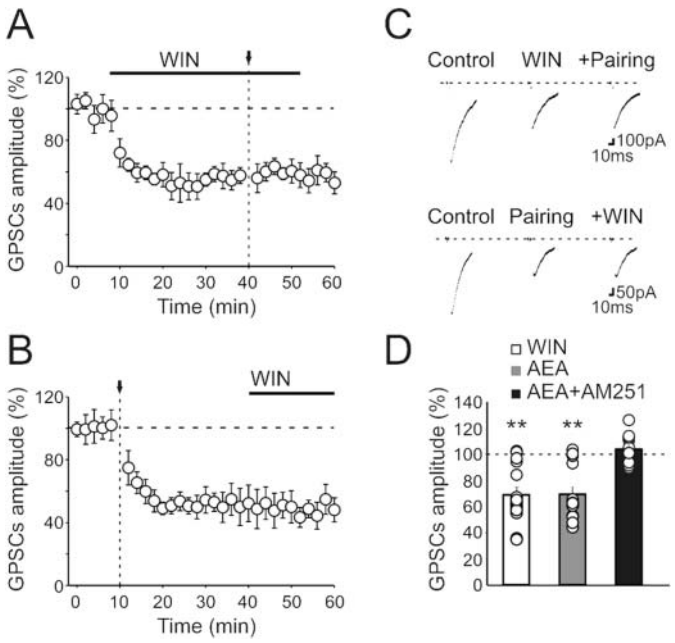

Figure 5 | CB1 receptor agonists reduce the amplitude of MF-GPSCs. (A) The depressant effect of WIN 55,212-2 (2 μM, bar) on MF-GPSCs was occluded by subsequent pairing (arrow, n=12). (B) When WIN 55,212-2 (bar) was applied after pairing (arrow) it did not affect MF-GPSCs (n=8). (C) Individual samples (average of 30 to 60 responses including failures) from A and B respectively. (D) Mean MF-GPSCs amplitude obtained in all cells tested after application of WIN 55,212-2 (n=17), AEA (n=13) or AEA plus AM351 (n=10). Note that AM251 completely antagonized the effects of AEA on MF-GPSCs. **p ≤ 0.01.

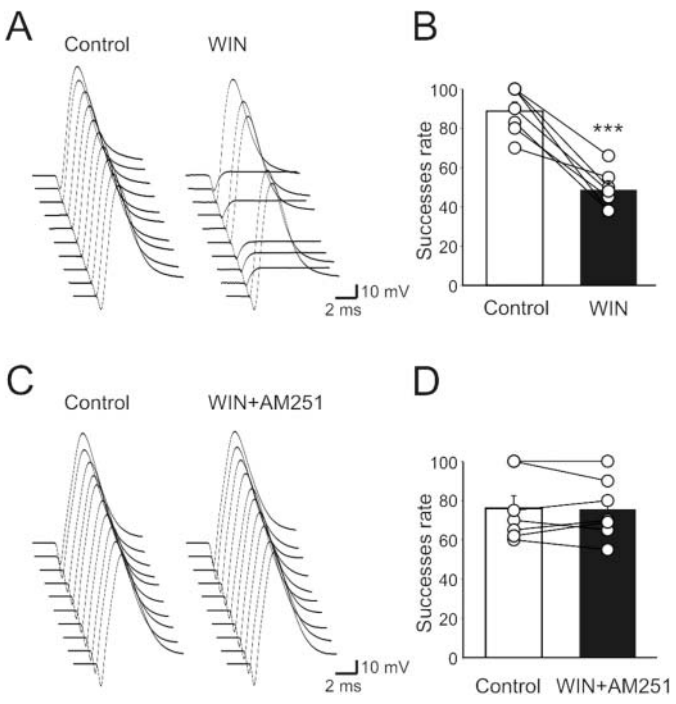

Figure 6 | Activation of CB1 receptors with WIN 55,212-2 reduces MF excitability. (A) Antidromic spikes evoked in granule cells upon stimulation of MF in stratum lucidum before and during bath application of WIN 55,212-2 (note that in this particular case the stimulus strength was set to obtain 100% of successes). (B) Summary plot for the examples shown in A (n=7). C–D As in A–B but in the presence of AM251 (n= 7); ***p<0.001.

CB1 mRNA is detected at very high levels in GABAergic interneurons and at low levels in CA1 and CA3 pyramidal neurons but not in granule cells[21]. Interestingly, in the present experiments at P3 granule cells displayed low, but detectable levels of CB1 mRNA (Fig. 8 A), which were clearly above background levels as determined in sections from *CB1*-KO mice (Fig. 8 A). In line with electrophysiological data however, this expression was lost at P21 mice (Fig. 8 B). Thus, granule cells transiently express CB1 mRNA only at early stages of postnatal development.

Discussion

Both pharmacological (selective CB1 antagonists) and mouse genetic (*CB1*-KO mice) data clearly demonstrate that STD-LTD induced by negative pairing is mediated by activation of $G_{i/o}$-coupled CB1 receptors at immature MF-CA3 synapses. Endocannabinoids are phospholipids that are synthesised and released from brain cells to regulate neuronal activity and synaptic plasticity[8,17]. Our results show the induction of STD-LTD is postsynaptic, triggered by calcium influx *via* voltage-dependent calcium channels activated by spiking-induced membrane depolarization. Thus, the calcium chelator BAPTA may interfere with eCBs production to abolish LTD[24,25]. In agreement with our data, previous studies have demonstrated that, the developmentally regulated expression of eCB-dependent long-term reduction of glutamate release in different brain regions relies on postsynaptic mechanisms including calcium rise *via* VDCC[26,27]. Our results exclude the contribution of many G protein-coupled receptors known to stimulate eCB production in other contexts[9] since STD-LTD was preserved in the presence of specific receptor antagonists.

Anandamide and 2-AG are endocannabinoids known to regulate synaptic transmission mainly through presynaptic CB1 receptors[17]. While 2-AG seems to be rather specific for CB1 receptors, anandamide binds to both CB1 and vanilloid type 1 (TRPV1) receptors[28]. At inhibitory synapses, 2-AG has been proposed to mediate different forms of long- and short-term synaptic plasticity, which are affected by specific inhibitors of synthesis, uptake or degradation of this endocannabinoid[8,9]. However, the other major endocannabinoid has also been proposed to mediate different forms of synaptic plasticity, such as LTD at excitatory synapses in the basal ganglia[27] or LTD at inhibitory synapses in the amygdala[29]. Our data strongly suggest that 2-AG is not involved in STD-LTD, since blockade of 2AG synthesis (by intracellular and bath-applied THL) or degradation (by JZL 184) did not affect pairing-induced synaptic depression. Several lines of evidence support instead the involvement of anandamide in

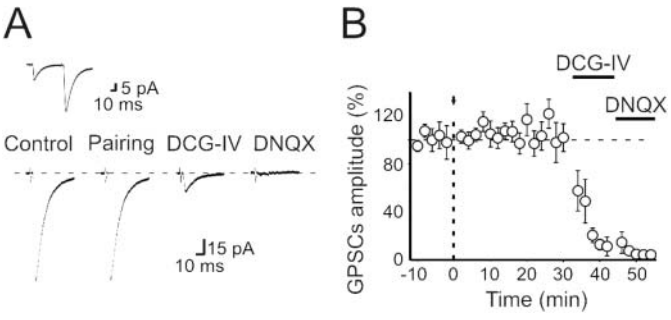

Figure 7 | Lack of CB1-mediated STD-LTD in hippocampal slices obtained from P19–P25 old rats. (A) Glutamatergic MF-EPSCs evoked in the presence of picrotoxin (100 μM) before (Control), after pairing, after addition of DCG-IV or DCG-IV plus DNQX. The inset above the traces shows paired pulse facilitation of MF-EPSCs. (B) Mean amplitude of MF-EPSCs before and after pairing (arrows at time 0) *versus* time (n=19). Note that pairing did not affect synaptic responses. These were strongly reduced by DCG-IV and blocked by DNQX.

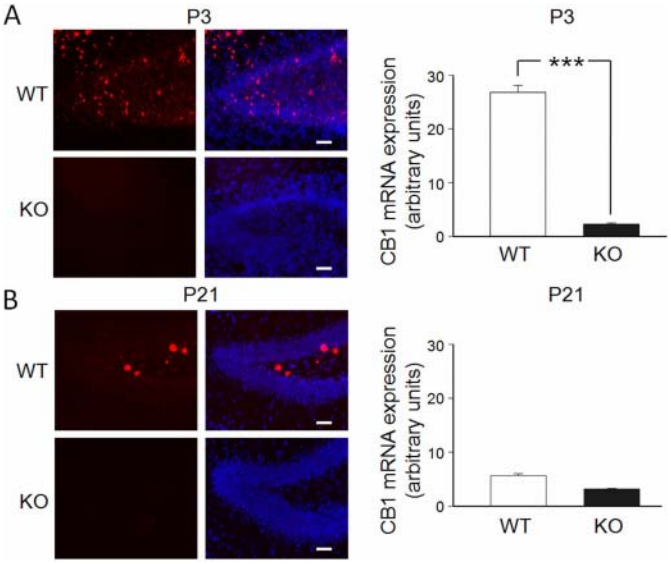

Figure 8 | Developmentally-regulated expression of CB1 mRNA in the granule cell layer of the dentate gyrus. (A) and (B) On the left, photomicrographs representing CB1 mRNA staining (red) alone or together with DAPI nuclear counterstaining (blue) in the dentate gyrus of WT and *CB1*-KO mice at P3 (A) and at P21 (B) animals. Cal. bar: 20 μm. On the right, Each column represents the averaged value of CB1 mRNA expression obtained in 4-8 sections corresponding to dorsal DG of WT or *CB1*-KO mice at P3 and at P21 (2 mice per group; see methods). ***$p<0.001$.

STD-LTD. Thus, inhibiting anandamide transport with AM404[30] or its degradation with URB597[31,32] lengthened the time window for LTD, suggesting that anandamide is the endogenous ligand released by principal cells. Anandamide was acting on CB1 and not TRPV1 receptors since the effects of this molecule on GPSCs were antagonized by the CB1 receptor antagonist AM251 and the pairing protocol failed to induce STD-LTD in animals lacking CB1 receptors. Variation in the rate of eCBs production or inactivation may account for differences in the temporal window for LTD observed at different synapses[12,28]. While STD-LTD is induced postsynaptically, its expression seems to be presynaptic judged by pairing-induced changes in the number of successes, $1/CV^2$ and PPR. In particular, analysis of the CV of synaptic responses indicates a presynaptic expression site presumably associated with a reduced GABA release[10]. The suppression of STD-LTD by CB1 receptor antagonists points to a locus of expression coinciding with CB1 receptors. Possibly, eCBs are produced by postsynaptic neurons and diffuse retrogradely to activate CB1 receptors on MF terminals. Further evidence that CB1 receptors are involved in STD-LTD is given by data showing that the depressant effects of WIN 55,212-2 or AEA on synaptic currents were occluded by STD-LTD, indicating that both STD-LTD and WIN 55,212-2 act on the same targets. As for STD-LTD, the locus of WIN 55,212-2 and AEA action was clearly presynaptic as suggested by the decrease in CV^2 values and the increase in PPR. WIN 55,212-2 may depress GPSCs amplitude by reducing MF excitability, since it decreased the probability of evoking antidromic spikes. Changes in fibre excitability due to potassium channel activation have been implicated in eCB-mediated heterosynaptic depression at Schaffer collateral-CA1 synapses in developing hippocampus[20]. Whatever the mechanisms, our work clearly suggests that, early in postnatal life, CB1 receptors are expressed on the axons of cells projecting from the granule cell layer to stratum lucidum.

Previous reports failed to show the presence of CB1 receptors on adult glutamatergic MF terminals[16,22] or CB1 mRNA in adult granule

cells[21] and in agreement with these findings we failed to induce CB1-mediated STD-LTD at glutamatergic MF-CA3 synapses in the hippocampus of juvenile animals, using the same protocol of neonates. However, it is worth noting that a NMDA-dependent form of STD-LTD can be induced at these synapses using a different stimulating paradigm[23].

Consistent with the involvement of endocannabinoid signalling at immature MF-CA3 synapses, *in situ* hybridization experiments revealed clearly detectable levels of CB1 mRNA in the granule cell layer of the hippocampus of P3 old animals. In older animals instead, the expression of CB1 mRNA was drastically reduced (if not absent; see also Ref 21), suggesting that in this area CB1 expression is developmentally regulated. Although we cannot exclude that, at P3, cells more strongly labelled belongs to GABAergic interneurons, their involvement in STD-LTD seems unlikely since all neurons tested were severely depressed by L-AP4 or LCCG1 while GABAergic interneurons are usually not sensitive to mGluR agonists[2,33]. In addition, the reduced probability of evoking antidromic spikes in granule cells exposed to WIN 55,212-2, a selective CB1 agonist, suggests that CB1 receptors are expressed on cells projecting from the granule cell layer to the stratum lucidum. Whether stimulated axons originate from granule cells or progenitors expressing a GABAergic phenotype is still a matter of debate[2,34]. Alternatively, we cannot exclude the possibility that anandamide binds to CB1 receptors present on different cell types (i.e. astrocytes) whose activation may modulate GABA release from MF terminals. It is know that early in postnatal life GABA can be released in a calcium- and SNARE-independent way by non-conventional release sites such as growth cones and astrocytes and can diffuse away to activate in a paracrine fashion extrasynaptic receptors[35].

What could be the functional role of eCBs-mediated STD-LTD at immature GABAergic MF-CA3 synapses? During postnatal development, activity-dependent changes in synaptic strength are thought to be involved in the refinement of neuronal circuits[6,36]. At early developmental stages eCBs are known to be present, especially in brain areas that control movements, cognition and attention as well as emotion and memory[37–39]. During gestation, CB1 receptors are enriched in axonal growth cones of cortical GABAergic interneurons where they contribute to regulate axonal guidance, selective targeting and synaptogenesis[40]. During postnatal development, changes in CB1 receptor expression may underlie the age-dependent magnitude of eCB-mediated i-LTD an effect that parallels the sensitivity of GABAergic[41] and glutamatergic[42] neurotransmission to eCBs. Also at Schaffer collateral-CA1 synapses, the heterosynaptic eCB-mediated persistent depression of glutamate release observed immediately after birth disappears in the mature hippocampus[20]. Our data suggest that in early postnatal life, eCBs counter the excitatory actions of glutamate and GABA, thus preventing at the network level neuronal hyperexcitability.

Methods

Slice preparation. All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609EEC) and were approved by local veterinary authorities (Dr. Giuseppe Stradaioli). All efforts were made to minimize animal suffering and to reduce the number of animal used. Experiments were performed on hippocampal slices from P3–P6 and P19–P25 old Wistar rats, and from *CB1*-KO mice and WT littermate[43] of the same postnatal age, following a method already described by Gasparini *et al.*[44]. Briefly, animals were decapitated after being anaesthetized with an i.p. injection of urethane (2 g/kg). The brain was quickly removed from the skull and placed in ice-cold ACSF containing (in mM): NaCl 130, KCl 3.5, NaH_2PO_4 1.2, $NaHCO_3$ 27, $MgCl_2$ 1.3, $CaCl_2$ 2, glucose 25, saturated with 95% O_2 and 5% CO_2 (pH 7.3–7.4). Transverse hippocampal slices (400 μm thick) were cut with a vibratome and stored at room temperature (20–24°C) in a holding bath containing the same solution as above. After recovering for at least one hour, single slices were transferred to a recording chamber where they were superfused with oxygenated ACSF at a rate of 2–3 ml/min at 33–35°C.

Electrophysiological recordings. Recordings were made from CA3 pyramidal cells using the whole-cell patch-clamp configuration in current or voltage-clamp mode. Neurons were visualized using an upright microscope (Olympus BX51WI) equipped with differential interference contrast optics and infrared video camera. Patch electrodes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, D). They had a resistance of 4–6 MΩ when filled with an intracellular solution containing (in mM): KCl 140, $MgCl_2$ 1, HEPES 10, MgATP 4, EGTA 0.5 (pH 7.3). In some experiments, recordings were performed with patch pipettes containing the calcium chelator 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA 20 mM, purchased from Sigma, Milan, Italy). In these cases, the KCl pipette concentration was reduced to 120 mM to maintain osmolarity at ~290 mOsm. Recordings were made with a patch clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA). Series resistance was assessed repetitively every five min and in current-clamp recordings compensated at 75% throughout the experiment. Cells exhibiting more than 15–20% changes in series resistance were not analyzed.

$GABA_A$-mediated synaptic potentials or currents (GPSPs or GPSCs) were evoked at 0.05 Hz from a holding potential of −60 mV in the presence of DNQX (50 μM) and D-AP5 (50 μM) to block AMPA- and NMDA-mediated synaptic responses, respectively. We stimulated granule cells minimally in the dentate gyrus[45,46]. Stimulation intensity was decreased until a single axon may have been activated. We judged that a single fibre terminating on the recorded cell was activated when the mean postsynaptic current amplitude and failure probability remained constant over a range of stimulus intensities near threshold for detecting a response[2]. An abrupt increase in the mean peak amplitude of synaptic currents was observed when the stimulus intensity was further increased. This all or none behaviour and the constant latency and shape of individual synaptic responses over time let us assume that only a single fibre was stimulated. The monosynaptic nature of synaptic currents was supported by unimodal and narrow distributions of latency and rise time which remained constant when the extracellular Ca^{2+}/Mg^{2+} concentration ratio was reduced from 2 : 1.3 to 1 : 3[2].

MF inputs were identified on the basis of their sensitivity to the group II and III mGluR agonists 2-amino-4-phosphonobutyric acid (L-AP4), 2S,l′S,2′S)-2-(2′-carboxycyclopropyl)glycine (L-CCG) or dicarboxycyclopro pyl)glycine (DCG-IV), their strong paired pulse facilitation and short-term frequency-dependent facilitation[2]. In neonates, MF-mediated synaptic responses were blocked by bicuculline or picrotoxin, confirming their GABAergic nature. In contrast to MF inputs, GABAergic inputs from interneurons were insensitive to mGluR agonists[2,33].

STD-LTD was induced in current clamp mode by pairing postsynaptic spikes with MF stimulation. MF-GPSCs were recorded first in voltage clamp mode for 5–10 min to obtain a stable baseline. Then a sequence of postsynaptic spike-MF stimulation was repeated ten times at 0.1 Hz with a delay of 50 ms (in some cases also with a delay of 100 and 200 ms) between postsynaptic spiking and presynaptic MF stimulation. Changes in synaptic efficacy were monitored by recording synaptic currents for additional 20–30 min after pairing.

In some experiments, antidromic action potentials were recorded from visually identified granule cells in dentate gyrus. In this case the intracellular solution contained K-gluconate (150 mM) instead of KCl. Extracellular stimuli (at 0.3 Hz, duration 150 μs) were delivered *via* a stimulation electrode positioned in stratum lucidum ~ 200 μm away from the granule cell layer.

Drugs used were: N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), N-(4-Hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (AM404), N-(2-Hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (Anandamide, AEA), atropine, 3-[[(3,4-dichlorophenyl) methyl]amino]propyl]diethoxymethyl)phosphinic acid (CGP 52432), D-(-)-2-amino-5-phosphonopentaoic acid (D-AP5), dihydro-β-erythroidine (DHβE), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), L(+)-2-amino-4-phosphonobutyric acid (L-AP4), dicarboxycyclopro pyl)glycine (DCG-IV), 2S,l′S,2′S)-2-(2′-carboxycyclopropyl)glycine (L-CCGl), (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl)propanoic acid (LY367385), 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), nifedipine, picrotoxin (PTX), tetrahydrolipstatin (THL), R)-(+)-[2,3-Dihydro-5-methyl-3-(4-orpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenyl-methanone mesylate (WIN 55,212-2 mesylate), (all purchased from Tocris Cookson Ltd, Bristol, UK); 4-[[4-formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2pyridinyl] azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS), (from Sigma, Milan, Italy); 4-nitrophenyl-4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL 184), (3′-(aminocarbonyl)[1,1′-biphenyl]-3-yl)-cyclohexylcarbamate (URB597) from Cayman Chemicals. All drugs were dissolved in either distilled water or ethanol, as required, except DNQX, WIN 55,212-2 mesylate, AM251 and URB597 which were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of synaptic currents. Drugs were applied by changing the bath superfusion solution *via* a three-way tap system. Complete exchange was obtained within 1–2 min.

Data acquisition and analysis. Data were acquired and digitized with an A/D converter (Digidata 1200, Molecular Devices) and stored on a computer hard disk. Acquisition and analysis of evoked responses were performed with Clampfit 9 (Molecular Devices). Data were sampled at 20 kHz and filtered with a cut off frequency of 1 kHz. Mean GPSCs amplitude was obtained by averaging successes and failures. The paired pulse ratio (PPR) was calculated as the mean amplitude of the synaptic response evoked by the second stimulus over that evoked by the first one. The coefficient of variation was calculated as the ratio of the standard deviation and the mean synaptic current amplitude. Unless otherwise stated, data are presented as

mean ± SEM. Statistical analysis was done using either Student's paired *t*-test for single comparisons or one-way ANOVA for multiple comparisons. A p value < 0.05 was considered statistically significant.

Fluorescent in situ hybridization (ISH). The brains of P3 and P21 null CB1 mutant mice and wild-type littermates were isolated, quickly frozen on dry ice and stored at −80° C until sectioning in a cryostat (14 µm, Microm HM 500 M, Microm Microtech, France). A DIG-labelled riboprobe against mouse CB1 receptor was used[21] and procedures were as previously described using the TSA amplification System[47]. Slides were analyzed by epifluorescence microscopy at 20X (Leica) and photographed using a Coolsnap HQ2 camera (Roper). In order to detect low but specific levels of labelling, the acquisition parameters were chosen using *CB1*-KO sections in order to exclude any background signal and exactly the same parameters were immediately used to acquire the images from wild-type P3 and P21 sections. Images of CB1 mRNA labelling did not undergo any post-acquisition processing.

Images of CB1 mRNA FISH were acquired in epifluorescence and the levels of expression were evaluated using the program Image J on 4–8 sections corresponding to dorsal DG of wild-type and CB1-KO P3 and P21 mice (2 mice per group). The levels of fluorescent signal were measured by pixel intensity in black and white images after background subtraction for each section (same parameters for all samples). Six regions of interest were randomly chosen for each section corresponding to the granule cell layer of the dentate gyrus, with the only limit to avoid brightly stained cells, presumably corresponding to GABAergic interneurons[21]. The average values of each section represented one data point. Values were also obtained in a similar way from P3 and P21 *CB1*-KO mice.

1. Henze, D. A., Urban, N. N. & Barrionuevo, G. The multifarious hyppocampal mossy fiber pathway: a review. *Neuroscience* **98**, 407–427 (2000).
2. Safiulina, V. F., Fattorini, G., Conti, F. & Cherubini, E. Gabaergic Signaling At Mossy Fiber Synapses In Neonatal Rat Hippocampus. *J. Neurosci.* **26**, 597–608 (2006).
3. Safiulina, V. F. *et al.* Control of GABA release at single mossy fiber-CA3 connections in the developing hippocampus. *Front Syn Neurosci* **2**, 1–11. doi:10.3389/neuro.19.003 (2010).
4. Safiulina, V. F. & Cherubini, E. At immature mossy fibers-CA3 connections, activation of presynaptic $GABA_B$ receptors by endogenously released GABA contributes to synapses silencing. *Front. Neurosci.* **3**, 1–11 (2009).
5. Caiati, M. D., Sivakumaran, S. & Cherubini, E. In the developing rat hippocampus, endogenous activation of presynaptic kainate receptors reduces GABA release from mossy fiber terminals. *J. Neurosci.* **30**, 1750–1759 (2010).
6. Kasyanov, A. M., Safiulina, V. F., Voronin, L. L. & Cherubini, E. GABA-mediated giant depolarizing potentials as coincidence detectors for enhancing synaptic efficacy in the developing hippocampus. *Proc. Natl. Acad. Sci. U S A* **101**, 3967–3972 (2004).
7. Sivakumaran, S., Mohajerani, M. H. & Cherubini, E. At immature mossy fiber-CA3 synapses correlated pre and postsynaptic activity persistently enhances GABA release and network excitability *via* BDNF and cAMP-dependent PKA. *J. Neurosci.* **29**, 2637–2647 (2009).
8. Kano, M., Ohno-Shosaku, T., Hashimotodani, Y., Uchigashima, M. & Watanabe, M. Endocannabinoid-mediated control of synaptic transmission. *Physiol. Rev.* **89**, 309–380 (2009).
9. Heifets, B. D. & Castillo, P. E. Endocannabinoid signaling and long-term synaptic plasticity. *Annu. Rev. Physiol.* **71**, 283–306 (2009).
10. Faber, D. S. & Korn, H. Applicability of the coefficient of variation method for analyzing synaptic plasticity. *Biophys. J.* **60**, 1288–1294 (1991).
11. Chevaleyre, V. & Castillo, P. E. Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. *Neuron* **8**, 461–472 (2003).
12. Sjöström, P. J., Turrigiano, G. G. & Nelson, S. B. Neocortical LTD via coincident activation of presynaptic NMDA and cannabinoid receptors. *Neuron* **39**, 641–654 (2003).
13. Ronesi, J., Gerdeman, G. L. & Lovinger, D. M. Disruption of endocannabinoid release and striatal long-term depression by postsynaptic blockade of endocannabinoid membrane transport. *J. Neurosci.* **24**, 1673–1679 (2004).
14. Lourenço, J. *et al.* Synaptic activation of kainate receptors gates presynaptic CB(1) signaling at GABAergic synapses. *Nat. Neurosci.* **13**, 197–204 (2010).
15. Bisogno, T. *et al.* Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell Biol.* **163**, 463–468 (2003).
16. Katona, I. *et al.* Molecular composition of the endocannabinoid system at glutamatergic synapses. *J. Neurosci.* **26**, 5628–5637 (2006).
17. Piomelli, D. The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* **4**, 873–884 (2003).
18. Zucker, R. S. & Regehr, W. G. Short-term synaptic plasticity. *Annu. Rev. Physiol.* **64**, 355–405 (2002).
19. Németh, B., Ledent, C., Freund, T. F. & Hájos, N. CB1 receptor-dependent and -independent inhibition of excitatory postsynaptic currents in the hippocampus by WIN 55,212-2. *Neuropharmacology* **54**, 51–57 (2008).
20. Yasuda, H., Huang, Y. & Tsumoto, T. Regulation of excitability and plasticity by endocannabinoids and PKA in developing hippocampus. *Proc. Natl. Acad. Sci. U S A* **105**, 3106–3111 (2008).
21. Marsicano, G. & Lutz, B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur. J. Neurosci.* **11**, 4213–4225 (1999).
22. Hofmann, M. E., Nahir, B. & Frazier, C. J. Excitatory afferents to CA3 pyramidal cells display differential sensitivity to CB1 dependent inhibition of synaptic transmission. *Neuropharmacology* **55**, 1140–1146 (2008).
23. Astori, S., Pawlak, V. & Köhr, G. Spike-timing-dependent plasticity in hippocampal CA3 neurons *J. Physiol.* **588**, 4475–4488 (2010).
24. Di Marzo, V. *et al.* Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature* **372**, 686–691 (1994)
25. Kreitzer, A. C. & Regehr, W. G. Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron* **29**, 717–727 (2001).
26. Choi, S. & Lovinger, D. M. Decreased frequency but not amplitude of quantal synaptic responses associated with expression of corticostriatal long-term depression. *J. Neurosci.* **17**, 8613–8620 (1997).
27. Gerdeman, G. L., Ronesi, J. & Lovinger, D. M. Postsynaptic endocannabinoid release is critical to long-term depression in the striatum. *Nat. Neurosci.* **5**, 446–451 (2002).
28. Di Marzo, V. & De Petrocellis, L. Endocannabinoids as regulators of transient receptor potential (TRP) channels: A further opportunity to develop new endocannabinoid-based therapeutic drugs. *Curr. Med. Chem.* **17**, 1430–1449 (2010).
29. Azad, S. C. *et al.* Circuitry for associative plasticity in the amygdala involves endocannabinoid signaling. *J. Neurosci.* **24**, 9953–9961 (2004).
30. Beltramo, M. *et al.* Functional role of high-affinity anandamide transport, as revealed by selective inhibition. *Science* **277**, 1094–1097 (1997).
31. Mor, M. *et al.* Cyclohexylcarbamic acid 3′- or 4′-substituted biphenyl-3-yl esters as fatty acid amide hydrolase inhibitors: Synthesis, quantitative structureactivity relationships, and molecular modeling studies. *J Med Chem* **47**, 4998–5008 (2004).
32. Gobbi, G. *et al.* Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. *Proc. Natl. Acad. Sci. U S A* **102**, 18620–18625 (2005).
33. Walker, M. C., Ruiz, A. & Kullmann, D. M. Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. *Neuron* **29**, 703–715 (2001).
34. Dupuy, S. T. & Houser, C. R. Developmental changes in GABA neurons of the rat dentate gyrus: an in situ hybridization and birth dating study. *J. Comp. Neurol.* **389**, 402–418 (1997).
35. Demarque, M. *et al.* Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation. *Neuron* **36**, 1051–1061 (2002).
36. Mohajerani, M. H., Sivakumaran, S., Zacchi, P., Aguilera, P. & Cherubini, E. Correlated network activity enhances synaptic efficacy *via* BDNF and the ERK pathway at immature CA3-CA1 connections in the hippocampus. *Proc. Natl. Acad. Sci. USA* **104**, 13176–13181 (2007).
37. Fernández-Ruiz, J., Berrendero, F., Hernández, M. L. & Ramos, J. A. The endogenous cannabinoid system and brain development. *Trends Neurosci* **23**, 14–20 (2000).
38. Wang, X., Dow-Edwards, D., Keller, E. & Hurd, Y. L. Preferential limbic expression of the cannabinoid receptor mRNA in the human fetal brain. *Neuroscience* **118**, 681–694 (2003).
39. Zurolo, E. *et al.* CB1 and CB2 cannabinoid receptor expression during development and in epileptogenic developmental pathologies. *Neuroscience* **170**, 28–41 (2010).
40. Berghuis, P. *et al.* Hardwiring the brain: endocannabinoids shape neuronal connectivity. *Science* **316**, 1212–1216 (2007).
41. Kang-Park, M. H., Wilson, W. A., Kuhn, C. M., Moore, S. D. & Swartzwelder, H. S. Differential sensitivity of GABA A receptor-mediated IPSCs to cannabinoids in hippocampal slices from adolescent and adult rats. *J. Neurophysiol.* **98**, 1223–1230 (2007).
42. Al-Hayani, A. & Davies, S. N. Cannabinoid receptor mediated inhibition of excitatory synaptic transmission in the rat hippocampal slice is developmentally regulated. *Br. J. Pharmacol.* **131**, 663–665 (2000).
43. Marsicano, G. *et al.* The endogenous cannabinoid system controls extinction of aversive memories. *Nature* **418**, 530–534 (2002).
44. Gasparini, S., Saviane, C., Voronin, L. L. & Cherubini, E. Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? *Proc. Natl. Acad. Sci. U S A* **97**, 9741–9746 (2000).
45. Jonas, P., Major, G. & Sakmann, B. Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. *J. Physiol.* **472**, 615–663 (1993).2005).
46. Allen, C. & Stevens, C. F. An evaluation of causes for unreliability of synaptic transmission. *Proc. Natl. Acad. Sci. U S A* **91**, 10380–10383 (1994).
47. Bellocchio, L. *et al.* Bimodal control of stimulated food intake by the endocannabinoid system. *Nat Neurosci* **13**, 281–283 (2010).

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Author contributions

MDC, SS, GM, CM, RM, EC designed the study and planned the experiments; MDC, SS, FL, GM, ER and DV performed experiments and analyzed data; EC prepared the manuscript with the help of the other authors.

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PrPC controls via PKA the direction of synaptic plasticity in the immature hippocampus

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PrP^C controls *via* PKA the direction of synaptic plasticity in the immature hippocampus

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Abstract

PrP^{C}, the cellular form of prion protein, is a conserved glycoprotein ubiquitously expressed in almost all tissues, particularly in the brain, where it can be converted into its abnormally folded and aggregated isoform PrP^{Sc} to cause neurodegenerative diseases. The predominant synaptic localization of PrP^{C} suggests a crucial role of this protein in synaptic signaling. Interestingly, PrP^{C} is developmentally regulated and its high expression in the immature brain is thought to be instrumental in regulating neurogenesis and cell proliferation.

Here, *Prnp*$^{0/0}$ mice were used to assess whether, early in postnatal life, PrP^{C} deletion affects activity-dependent synaptic plasticity processes in the hippocampus. To this aim, calcium transients associated with Giant Depolarizing Potentials, a hallmark of developmental networks, were transiently paired with mossy fibers (MF) activation in such a way that the two events were coincident. While this procedure caused long-term potentiation (LTP) of MF-CA3 synapses in wild-type (WT) animals, it caused long-term depression (LTD) in *Prnp*$^{0/0}$ mice. The induction of LTP was postsynaptic and required the activation of cAMP-dependent PKA signaling. The induction of LTD was presynaptic and was reliant on G protein-coupled GluK1 receptor and protein lipase C downstream to G protein activation. In addition, in WT, but not in *Prnp*$^{0/0}$ mice, at emerging CA3-CA1 synapses, pairing Schaffer collateral stimulation with depolarization of CA1 principal cells induced LTP, known to be PKA-dependent.

These data suggest that PrP^{C} plays a crucial role in regulating *via* PKA synaptic plasticity and information processing in the developing hippocampus.

Introduction

The cellular form of the prion protein (PrP^{C}) is a conserved glycoprotein tethered to the cell membrane by a glycosylphosphatidylinositol anchor (Colby and Prusiner, 2011). The ubiquitous expression of PrP^{C} in almost all tissues, most abundantly in the brain, suggests a key role for this protein in several cellular functions. Conversion of PrP^{C} into its abnormally folded isoform PrP^{Sc} causes neurodegenerative disorders in mammals, including Creutzfeldt-Jakob disease in humans (Colby and Prusiner, 2011). Notwithstanding evidence supporting a central role for PrP^{C} in the pathogenesis of transmissible spongiform encephalopathies, its normal physiological function is still unclear. *Prnp*$^{0/0}$ mice lack a consistent overt phenotype (Bueler et al., 1992) though manifesting a variety of rather minor deficits ranging from behavioral to electrophysiological and biochemical alterations (Steele et al., 2007). The predominant synaptic localization of PrP^{C} suggests its function may be related to synaptic transmission and cell excitability. Previous *in vitro* studies on the hippocampus of *Prnp*$^{0/0}$ mice have highlighted reduced $GABA_A$-mediated synaptic inhibition (Collinge et al., 1994), altered glutamatergic excitation (Carleton et al., 2001), impaired long-term potentiation (Collinge et al., 1994; Manson et al., 1995) and a reduced slow afterhyperpolarization (AHP) associated with enhanced cell excitability (Colling et al., 1996; Mallucci et al., 2002; Fuhrmann et al., 2006).

Interestingly, PrP^{C} is developmentally regulated (Manson et al., 1992; Sales et al., 2002; Benvegnu et al., 2010), and its high expression in the immature brain may account for the shorter incubation time of the disease in younger animals (McKinley et al. 1989). Moreove, the high levels of the protein in late pre- and early post-natal life are crucial for

regulating neurogenesis and cell proliferation (Steele et al. 2006). In the hippocampus, developmental changes in PrP^C immunoreactivity parallel those of mossy fiber terminals (MFs), the axons of granule cells in the dentate gyrus (Sales et al., 2002) that reach full maturation around P15-P20 (Amaral and Dent, 1981).

The immature hippocampus is characterized by network-driven Giant Depolarizing Potentials (GDPs) which are generated by the synergistic action of glutamate and GABA, both of which depolarizing and excitatory (Ben-Ari et al., 1989; Ben-Ari et al. 2007). Calcium transients associated with GDPs are thought to be instrumental for enhancing synaptic efficacy at emerging glutamatergic (Mohajerani et al., 2007) and GABAergic (Kasyanov et al., 2004) synapses.

Here, we tested the hypothesis that PrP^C regulates synaptic plasticity processes at immature MF-CA3 synapses, which immediately after birth are mainly GABAergic (Safiulina et al., 2006). The rising phase of GDPs was used to trigger mossy fibers stimulation in such a way that synchronized network activity was coincident with mossy fibers activation. While in WT animals the pairing procedure produced a persistent increase in synaptic strength that depended on postsynaptic PKA signaling, in $Prnp^{0/0}$ mice it caused a persistent weakening of synaptic efficacy that was presynaptic in origin and relied on G protein-coupled GluK1 receptor and protein lipase C downstream to G protein activation.

Materials and Methods

Animals

All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609EEC) and were approved by the local authority veterinary service. All efforts were made to minimize animal suffering and to reduce the number of animals used. Inbred FVB/N wild-type and FVB *Prnp*$^{0/0}$ mice were used in these experiments. The FVB *Prnp*$^{0/0}$ mice were obtained from George A. Carlson, McLaughlin Research Institute, Great Falls, Montana, USA, and were bred by backcrossing with the original *Prnp*$^{0/0}$ mice at least 20 times.

Hippocampal slices

Experiments were performed on hippocampal slices obtained from postnatal (P) 3 to P7 day-old mice as previously described (Le Magueresse et al., 2006). Briefly, animals were decapitated after being anaesthetized with an i.p. injection of urethane (2g/kg). The brain was quickly removed from the skull and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 130, KCl 3.5, NaH_2PO_4 1.2, $NaHCO_3$ 25, $MgCl_2$ 1.3, $CaCl_2$ 2, glucose 25, saturated with 95% O_2 and 5% CO_2 (pH 7.3-7.4). Transverse hippocampal slices (400 µm thick) were cut with a vibratome and stored at room temperature in a holding bath containing the same solution as above. After a recovery period of at least one hour, an individual slice was transferred to the recording chamber where it was continuously superfused with oxygenated ACSF at a rate of 2-3 ml/min at 33-34^{o}C.

Electrophysiological recordings

Electrophysiological experiments were performed mainly from CA3 pyramidal cells using the whole-cell configuration of the patch-clamp technique in current or voltage-clamp mode. Bipolar twisted NiCr-insulated electrodes localized into stratum granulosum of the dentate gyrus were used to evoke synaptic responses in CA3 pyramidal cells. We used minimal stimulation in order to activate only one or few presynaptic fibers. According to the technique described by Jonas et al., (1993) and Allen and Stevens (1994) the stimulation intensity was decreased until only one single axon was activated. This was achieved when the mean amplitude of the postsynaptic currents and failure probability remained constant over a range of stimulus intensities near the threshold for detecting a response. Further enhancing the stimulus intensity led to an abrupt increase in the mean peak amplitude of synaptic currents. This "all-or-none" behavior suggests that only a single granule cell was stimulated. When the stimulation intensity was turned down, the probability of failures in synaptic transmission was near 1. The latency and shape of individual synaptic responses remained constant over repeated stimuli. In most cases paired stimuli were applied at 50 ms interval.

Patch electrodes were pulled from borosilicate glass capillaries (Hingelberg, Malsfeld, D). They had a resistance of 4-6 MΩ when filled with an intracellular solution containing (in mM): K-gluconate 115, KCl 20, disodium phosphocreatine 10, HEPES 10, MgATP 4, GTP 0.3, QX 314 5 (pH 7.3). Recordings were made with a patch clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA). The stability of the patch was checked by repeatedly monitoring (every 5 min) the input and series resistance. Cells exhibiting more than 15% changes in series resistance were excluded from the analysis.

During the first week of postnatal life, MF-CA3 responses are mainly GABAergic since they are insensitive to the AMPA receptor antagonist GYKI 52466 (Caiati et al., 2010) but they are readily and reversibly blocked by bicuculline (20 μM), picrotoxin (100 μM) or gabazine (2μM; see Walker et al., 2001 and Safiulina et al., 2006). MF inputs were identified on the basis of their sensitivity to group III metabotropic glutamate receptor agonist 2-amino-4-phosphonobutyric acid (L-AP4, Gutiererz et al., 2003; Kasyanov et al., 2004), their paired pulse facilitation and their short-term frequency-dependent facilitation (Safiulina et al., 2006). $GABA_A$-mediated synaptic potentials or currents (GPSPs or GPSCs) were evoked at 0.05 Hz from a holding potential of –60 mV. After a control period of 5-10 min, the patch was switched from voltage-clamp to current-clamp mode and MF responses were paired with spontaneously occurring giant depolarizing potentials or GDPs. "Pairing" consisted in triggering MF stimulation with the rising phases of GDPs during a 7-10 min period (Figure 1 A, B). The patch was subsequently switched back to voltage clamp mode and synaptic currents were recorded as in control for additional 20-40 min. In some experiments recordings were performed with patch pipettes containing the calcium chelator 1,2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA, 20 mM; purchased from Sigma, Milan, Italy).

Experiments aimed at assessing synaptic plasticity at glutamatergic CA3-CA1 synapses were performed from CA1 pyramidal cells using patch pipettes filled with a solution containing (in mM): Cs-$MeSO_4$ 120, KCl 20, HEPES 10, EGTA 0.5, Na-GTP 0.3 and Mg-ATP 4 (pH 7.2; osmolality 275-280 mOsm).

Spontaneous miniature GABAergic currents (mGPSCs) were recorded from CA3 principal cells in the presence of DNQX (20 μM) and TTX (1 μM).

Drugs

Drugs used: D-(-)-2-amino-5-phosphonopentaoic acid (D-AP5), 2-amino-4-phosphonobutyric acid (L-AP4), 6,7-dinitroquinoxaline-2,3-dione (DNQX), *S*)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione (UBP 302), 1- [6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), picrotoxin, 6-Imino-3-(4-methoxyphenyl)-1(6*H*)-p yridazinebutanoic acid hydrobromide (gabazine), protein kinase A inhibitor 6-22 amide (PKI 6-22), protein kinase A inhibitor 14-22 amide (PKI 14-22), and forskolin (all purchased from Tocris Cookson Ltd, Bristol, UK); All drugs were dissolved in ACSF, except DNQX, picrotoxin and forskolin which were dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of synaptic currents. Drugs were applied in the bath *via* a three-way tap system, by changing the superfusion solution to one differing only in its content of drug(s). The ratio of flow rate to bath volume ensured complete exchange within 2 min.

Data Analysis

Data were acquired and digitized with an A/D converter (Digidata 1200, Molecular Devices) and stored on a computer hard disk. Acquisition and analysis of evoked responses were performed with Clampfit 9 (Molecular Devices). Data were sampled at 20 kHz and filtered with a cut off frequency of 2 kHz. Mean GPSCs amplitude was obtained by averaging successes and failures. The paired pulse ratio (PPR) was calculated as the mean amplitude of the synaptic response evoked by the second stimulus over that evoked by the first one. The coefficient of variation (CV) of response amplitude was determined

as the ratio between the standard deviation and the mean.

Statistical Analysis

Statistical analysis was carried out using Student's paired *t*-test to compare conditions that allowed recordings from the same neuron before and after drug treatment. The unpaired *t*-test was employed to compare two independent groups and one-way ANOVA was used to compare several independent groups. When the ANOVA resulted in a significant general group effect, Dunnett's *post hoc* test was used to compare different groups versus the control group for single or multiple comparisons. A p value < 0.05 was considered as statistically significant.

Results

Basic synaptic transmission and network activity is unaffected in the hippocampus of *Prnp*$^{0/0}$ mice

Whole cell recordings from CA3 principal cells in P3-P7 hippocampal slices from WT (n=30) and *Prnp*$^{0/0}$ mice (n=27) did not reveal any significant change in resting membrane potential (WT: -55.6 ± 1.55 mV; *Prnp*$^{0/0}$: -52 ± 1.9 mV; $p = 0.1$), membrane capacitance (WT: 64 ± 4.8 pF; *Prnp*$^{0/0}$: 57.4 ± 3.4 pF; $p = 0.5$) and input resistance (WT: 387 ± 27 MΩ; *Prnp*$^{0/0}$: 382 ± 23 MΩ; $p = 0.8$) of the recorded cells. Both WT and *Prnp*$^{0/0}$ mice exhibited similar spike half-width values (WT: 1.6 ± 0.04 ms; *Prnp*$^{0/0}$: 1.6 ± 0.03 ms; $p = 0.6$) and responded to long depolarizing current pulses with repetitive rapidly adapting firing (data not shown). In addition, they exhibited network-driven GDPs at the frequency of 0.056 ± 0.02 Hz and 0.066 ± 0.02 Hz in WT (n=13) and in *Prnp*$^{0/0}$ mice (n=15), respectively ($p>0.05$). In addition, current responses underlying GDPs carried similar charge transfers as indicated by similar values of respective areas (30.7 ± 4.9 pAs^{-1} and 43 ± 7.8 pAs^{-1}, in WT and *Prnp*$^{0/0}$ mice, respectievly; n=12; $p>0.05$; data not shown).

Minimal stimulation of granule cells in the dentate gyrus elicited in CA3 principal cells $GABA_A$-mediated postsynaptic currents (GPSCs) of variable amplitude intermingled with synaptic failures. GPSCs exhibited a similar potency (the mean amplitude of successes without failures was 63.8 ± 8.1 pA in WT animals, n=13 while in *Prnp*$^{0/0}$ mice it was 60.5 ± 9.5 pA, n=23), but slightly different failure rates (0.37 ± 0.06 and 0.57 ± 0.05 in WT and in *Prnp*$^{0/0}$ mice, respectively; $p=0.01$, unpaired *t*-test). In accord with previous data (Safiulina et al., 2006; Sivakumaran et al., 2009; Caiati et al., 2010),

GPSCs were of MF origin as they were sensitive to group III metabotropic glutamate receptor (mGluR) agonist L-AP4. This compound reduced the amplitude of GPSCs to 20.4 ± 3.4% and 18.3 ± 2.8% of controls (pre-drug values) in WT and *Prnp*$^{0/0}$ mice, respectively (p>0.05; paired t-test; data not shown).

Pairing GDPs with MF stimulation induces LTP in WT mice and LTD in *Prnp*$^{0/0}$ mice

After a control period of 10 min, a "pairing" procedure was used to change synaptic efficacy at MF-CA3 synapses (Kasyanov et al., 2004). This method consisted in switching the patch from voltage-clamp to current-clamp mode and in stimulating afferent fibers with the rising phase of GDPs (Fig. 1A and B). The mean number of GDPs recorded during the seven-minute pairing was 18 ± 3 and 20 ± 2 in WT and in *Prnp*$^{0/0}$ mice, respectively (these values were not significantly different, p>0.05; unpaired t-test). After pairing, the patch was switched back to voltage-clamp mode and MF-GPSCs were recorded for additional 20-40 minutes. As illustrated in Fig. 1C, in WT animals the pairing procedure produced a strong and persistent potentiation of MF-GPSCs (255 ± 48.2% of pre-pairing values; p< 0.001; paired t-test; n=13). This effect was associated with a significant increase in the number of successes (from 0.37 ± 0.06 to 0.7 ± 0.06; n=13; p< 0.001; Fig. 2A), in the inverse square value of the coefficient of variation (CV^{-2}) of response amplitude (from 1.09 ± 0.37 to 2.83 ± 0.97; n=13; p< 0.001; Fig. 2A) as well as a significant reduction in paired-pulse ratio (from 2.7 ± 0.6 to 1.5 ± 0.35; n=8; p<0.05; Fig. 2A). This suggests an increased probability of GABA release.

--- Fig. 1near here ---

In *Prnp*$^{0/0}$ mice, on the other hand, the pairing procedure always induced a persistent depression of GPSCs (30 min after pairing the peak amplitude of GPSCs was reduced to 36.3 ± 5.6% of pre-pairing values; n=15; $p<0.001$ paired *t*-test; Fig. 1D). This effect was mainly caused by a decrease in the number of successes (from 0.57 ± 0.06 to 0.21± 0.06, before and after pairing, respectively; n=15; $p<0.001$; Fig. 2B). As expected for a reduction in the probability of GABA release from MF terminals, the increased number of failures was associated with a significant increase in PPR (from 1.05 ± 0.1 to 2.1 ± 0.7; $n=7$; $p= 0.01$; Fig. 2B) and a significant decrease in CV^{-2} (from 3.9 ± 1.3 to 2.2 ± 0.9; $n=15$; $p<0.001$; Fig. 2B).

--- Fig. 2 near here ---

In 6 out of 15 cases, the pairing procedure led to synapse silencing (Fig. 3**;** see Voronin and Cherubini, 2004).

Pairing-induced synaptic depression or silencing was not due to run down of synaptic responses. In fact, GPSCs evoked by a second stimulus (50 ms after the first one) were only slightly affected and in the absence of pairing (just switching from voltage clamp to current clamp mode for 7 min), no changes in GPSCs amplitude with time occurred (after 20 min the amplitude of GPSCs was 108 ± 22% and 102 ± 12% of pre-switching values in WT and *Prnp*$^{0/0}$ mice, respectively; n=5 for both; $p>0.05$; data not shown).

--- Fig. 3 near here ---

In WT mice, LTP induction is postsynaptic whereas in *Prnp*$^{0/0}$ mice LTD is presynaptic

In agreement with earlier data (Kasyanov et al., 2004), loading postsynaptic neurons from WT animals with the calcium chelator BAPTA (20 mM), prevented pairing-induced synaptic potentiation and revealed synaptic depression (the mean amplitude of GPSCs was 47 ± 9% of pre-pairing values; n=7; $p<0.001$, paired *t*-test; Fig. 4A). However, in *Prnp*$^{0/0}$ mice, intracellular BAPTA did not affect pairing-induced synaptic depression (20 min after pairing the peak amplitude of GPSCs was 41 ± 12% of pre-pairing values; n=7; $p<0.05$; Fig. 4B). In BAPTA, MF-GPSCs depression did not significantly differ from that obtained in the absence of BAPTA ($p=0.6$; unpaired *t*-test). This suggests that in *Prnp*$^{0/0}$ mice the induction of LTD is independent of postsynaptic calcium rise and possibly presynaptic in origin. To test this hypothesis in a new set of experiments, the conditioning protocol was applied to cells maintained in voltage clamp mode (at -70 mV). This procedure prevents the depolarization of the postsynaptic cell and calcium influx through high voltage-activated calcium channels and/or NMDA receptors (Malenka and Nicoll, 1999). In line with a presynaptic induction mechanism, this condition did not affect LTD in *Prnp*$^{0/0}$ mice but it prevented synaptic potentiation and caused synaptic depression in WT mice (the mean GPSCs amplitude was 44.6 ± 6.2% of pre-pairing values in WT animals; n=6; $p=0.007$; paired *t*-test and 40.1 ± 6.5% of pre-pairing values in *Prnp*$^{0/0}$ mice; n=9; $p<0.001$; paired *t*-test; Fig. 4C and D). The depressant effect observed in *Prnp*$^{0/0}$ mice was not significantly different from that obtained when pairing was performed in current clamp conditions ($p=0.6$; unpaired t-test).

--- Fig. 4 near here ---

As expected for a presynaptic type of action, LTD was associated with a significant reduction in the number of successes (from 0.74 ± 0.06 to 0.42 ± 0.1; n=7; $p<0.01$ in WT and from 0.67 ± 0.09 to 0.29 ± 0.1; n=8; $p<0.01$ in *Prnp*$^{0/0}$ mice), a significant increase in PPR (from 1.4 ± 0.1 to 2.1 ± 0.3; n=5; $p<0.05$ in WT and from 1.1 ± 0.2 to 1.5 ± 0.3; n=5; $p<0.05$ in *Prnp*$^{0/0}$ mice) and a marked decrease in CV^{-2} (from 3 ± 0.67 to 2.3 ± 0.7; n=7; $p<0.05$ in WT and from 3.3 ± 1.4 to 1.8 ± 1.2; n=6; $p<0.01$ in *Prnp*$^{0/0}$ mice; Fig. 5).

--- Fig. 5 near here ---

In WT animals synaptic depression can thus be unveiled either blocking postsynaptic calcium rise with BAPTA or preventing the opening of voltage-gated calcium channels by clamping the membrane below the resting level.

In WT mice, pairing-induced LTP requires postsynaptic cAMP-dependent PKA activity

Interestingly, at immature glutamatergic Schaffer collateral-CA1 synapses, LTP requires the activation of postsynaptic cyclic AMP-dependent PKA (Yasuda et al., 2003) for its induction . PKA activation is also necessary for spike time-dependent LTP at immature GABAergic MF-CA3 synapses (Sivakumaran et al., 2009), indicating that in the immature hippocampus PKA signaling is critically implicated in activity-dependent synaptic plasticity notwithstanding the nature of the neurotransmitter. To test whether

postsynaptic PKA activity is responsible for pairing-induced LTP at MF-CA3 synapses in WT mice, we loaded the cell with the membrane impermeable PKA inhibitor PKI 6–22, (1 μM). Under these conditions, the pairing procedure failed to induce LTP, instead it revealed a persistent depression of MF-GPSCs (66 ± 9.8 % of pre-pairing values; n=11; p<0.001paired t-test; Fig. 6).

--- Fig. 6 near here ---

These data prove that, in WT animals, PKA activity is crucial for triggering LTP at immature MF-CA3 synapses. To examine whether a postsynaptic deficit of PKA activity accounts for the loss of LTP in *Prnp*$^{0/0}$ mice, we tested forskolin, an activator of PKA, on spontaneous miniature GABAergic currents (mGPSCs), recorded from CA3 pyramidal cells. In WT animals, forskolin (50 μM) only slightly enhanced the frequency of mGPSCs that did not reach a significant level (138.7 ± 23%; n=10; p>0.05 paired t-test; Fig. 7D) but significantly increased their amplitude (128.4 ± 3.3% of control; n=10; p<0.001, paired t-test; Fig. 7A and E; see also Yasuda et al., 2003). The potentiating effects of forskolin on mGPSCs amplitude were prevented by loading the cell with the membrane impermeable PKA inhibitor PKI 6–22, (1 μM; Fig. 7B and E). In *Prnp*$^{0/0}$ mice, forskolin slightly increased the frequency of mGPSCs (124.7 ± 11.8% of control; n=8; p>0.05 paired t-test; Fig. 7D) but failed to enhance their amplitude (92 ± 3.6% of control; n=8; p>0.05 paired t-test; Fig. 7C and E).

--- Fig. 7 near here ---

These experiments provide evidence that PrP^C is associated with the PKA signaling pathway at immature MF-CA3 synapses and this might account for the lack of pairing-induced LTP in PrP^C-deficient mice.

Loss of PKA-dependent LTP at Schaffer collateral-CA1 synapses in the hippocampus of *Prnp*$^{0/0}$ mice

To further assess whether the lack of postsynaptic PKA activity may account for the loss of LTP in *Prnp*$^{0/0}$ mice, a pairing procedure was applied to immature CA1 principal cells known to critically depends on postsynaptic PKA signaling (Yasuda et al., 2003) for LTP. Pairing repetitive stimulation (50 stimuli at 1 Hz) of Schaffer collateral with depolarization of CA1 principal cells to 0 mV (Yasuda et al. 2003) caused in P3-P7 WT animals a persistent increase in synaptic efficacy (30 min after pairing the mean EPSCs amplitude was 143.6 ± 8.9% of controls; n=6; p=0.01, paired t-test; Fig. 8A).

--- Fig. 8 near here ---

This effect was prevented when the postsynaptic cell was loaded with the membrane impermeable PKA inhibitor PKI 6–22 (1 μM; after pairing, the mean EPSCs amplitude was 88 ± 8% of pre-pairing values; n=5; p>0.05; paired t-test; Fig. 8B). In contrast, in *Prnp*$^{0/0}$ mice, the same protocol failed to induce LTP (the mean EPSC amplitude was 85.2 ± 4.6% of control; n=14; p<0.05, paired t-test; Fig. 8). In 5 out of 14 cases, included

in the summary graph of Fig. 8, pairing produced a synaptic depression of 33% of controls; n=5; p=0.003, paired t-test).

In *Prnp*$^{0/0}$ mice, pairing-induced LTD requires the activation of presynaptic GluK1 receptors and PLC

While the present experiments clearly show that in *Prnp*$^{0/0}$ mice PKA-dependent LTP is lost not only at MF-CA3 synapses but also at Schaffer collateral-CA1synapses, they do not clarify the mechanisms responsible for pairing-induced LTD. The PKA signaling pathway was not involved in pairing-induced LTD since application of the membrane permeable PKA inhibitor PKI 14-22 (1 μM) in *Prnp*$^{0/0}$mice failed to prevent LTD (GPSCs amplitude was 42.2 ± 8.2% of controls; n=5; p=0.043, paired t-test; data not shown).

Among presynaptic receptors known to depress GABA release from immature MF terminals, high affinity GluK1 receptors play a crucial role (Caiati et al., 2010). We hypothesized that these receptors could be activated during the pairing procedure by a large amount of glutamate released from network-driven GDPs. In the following experiments from *Prnp*$^{0/0}$ mice pairing was thus performed in the presence of the selective GluK1 antagonist UBP 302 (10 μM). As shown in Fig. 9, this compound fully prevented pairing-induced synaptic depression (on average the peak amplitude of GPSCs was 91.8 ± 4.5% of pre-pairing values; n= 9; p>0.05; paired t-test). This value significantly differed from that obtained in the absence of UBP 302 (p<0.001, one way ANOVA), indicating that GluK1 receptors are indeed responsible for LTD.

--- Fig. 9 near here ---

In the absence of pairing, UBP 302 only slightly enhanced the amplitude of MF-GPSCs (to 121.7 ± 9.8% of controls) without reaching a significant level (p>0.05). This suggests that under resting conditions the amount of glutamate present in the extracellular medium is not sufficient to tonically activate presynaptic GluK1 receptors. However, in agreement with a previous study on rats (Caiati et al., 2010), GluK1 receptors are present on MF terminals of *Prnp*$^{0/0}$ mice since the GluK1 agonist ATPA (1 μM) caused a reduction of MF-GPSC of 41.5 ± 7.2% of controls (n=5; p<0.05; data not shown).

In the developing hippocampus, endogenously released glutamate depresses MF-GPSCs *via* presynaptic $G_{i/o}$ protein-coupled high affinity kainate receptors (Caiati et al., 2010). The most common signaling pathway, downstream to G protein activation, involves phospholipase C (PLC) and intracellular calcium (Lerma, 2006; Rodriguez-Moreno and Sihra, 2007). Therefore in the following experiments from *Prnp*$^{0/0}$ mice we tested whether the PLC inhibitor U73122 is able to block pairing-induced LTD. As shown in Fig. 9B, U73122 (10 μM) prevented pairing-induced synaptic depression (after pairing the mean GPSC amplitude was 89 ± 8.7% of pre-pairing values; n= 8; p>0.05; paired t-test). Data obtained in the presence of U73122 were significantly different from controls (p<0.001; one way ANOVA). Pairing-induced synaptic weakening was not mediated by other receptors known to depress transmitter release, such as $GABA_B$ receptors (Safiulina and Cherubini, 2009), nicotine, muscarinic acetylcholine receptors (Maggi et al., 2004), purinergic P2Y receptors (Zhang et al., 2003; Safiulina et al., 2005), adenosine receptors and mGluRs (Scanziani et al., 1997), since blocking these receptors with a cocktail containing selective antagonists did not modify LTD (Figure S6). In addition, pairing-

induced synaptic depression was still present when NMDA receptors were blocked with D-AP5 (50 μM), indicating that this form of synaptic plasticity is independent of NMDA receptor activation (Fig. 10).

--- Fig. 10 near here ---

Discussion

In this study we describe a previously unknown effect of PrP^{C}, namely its ability to control, at early developmental stages, the direction of synaptic plasticity in the hippocampus. In particular, we show that pairing GDPs-associated calcium transients with stimulation of granule cells in the dentate gyrus triggers synaptic potentiation in WT animals and synaptic depression in *Prnp*$^{0/0}$ mice. As to other forms of synaptic plasticity, calcium entering in the postsynaptic neuron through voltage-dependent calcium channels opening by the depolarizing action of GABA during GDPs Ben_ari et al., 2007) is responsible for LTP. Hence, in agreement with a previous report (Kasyanov et al., 2004), LTP induction was clearly postsynaptic as it could be prevented loading the postsynaptic cell with BAPTA. Interestingly, in the presence of BAPTA, the pairing procedure unveiled LTD, suggesting the two phenomena are tightly correlated (see also Kasyanov et al., 2004; Sivakumaran et al., 2009). While the induction of LTP was clearly postsynaptic, its expression was presynaptic as indicated by pairing-induced decrease in failure rate and paired pulse facilitation, as well as by the increase in CV^{-2}, all traditional indices of presynaptic changes in release probability (Zucker, 1989). In *Prnp*$^{0/0}$ mice, on the other hand, the pairing procedure failed to induce synaptic potentiation yet it caused synaptic depression which in some cases led to synapse silencing. LTD was unaffected

by loading the postsynaptic cell with BAPTA, indicating a possible presynaptic origin. This was further supported by the observation that LTD was not affected when the pairing protocol was delivered to cells kept close to their resting membrane potential to prevent membrane depolarization. However, this procedure unmasked LTD in WT animals, indicating that also in this case LTD was presynaptic. Like LTP, also LTD is likely to involve network-driven GDPs, which, being synchronous over the entire hippocampus, may act as coincident detectors of signals occurring at both post and presynaptic sites.

Similarly to our results, theta burst stimulation of cerebellar mossy fibers was unable to induce LTP in granule cells in slices from juvenile (but not adult) $PrP^{0/0}$ mice (Zurich-I, Bueler et al., 1992), a process probably related to impaired motor control and reinforcement (Prestori et al., 2008). However, while in the cerebellum the lack of LTP probably depended on the reduced cell excitability which prevented granule cells from firing, in our case it was independent of any apparent change in passive membrane properties, neuronal spiking, and synaptic or networking activity.

We also found that in WT animals, LTP required the activation of cAMP-dependent PKA since pairing-induced synaptic potentiation was lost when the postsynaptic cell was loaded with the membrane impermeable PKA inhibitor PKI 6-22. This compound was also able to block the potentiating effect of the adenylate cyclase activator forskolin on the amplitude of miniature GABAergic currents, further indicating that the PKA signaling pathway is crucial for modifying synaptic strength, possibly through phosphorylation of $GABA_A$ receptors. Note that while in neonates forskolin acts mainly at postsynaptic sites (see alsoYasuda et al., 2003), in juvenile animals it affects mainly

presynaptic sites by increasing GABA release (Sciancalepore and Cherubini, 1995). Postsynaptic PKA activity has been involved in LTP induced in the immature hippocampus when MF stimulation is paired with back-propagating action potentials (Sivakumaran et al., 2009). As for spike time-dependent plasticity, also in the present case cAMP-dependent PKA activity is likely to play an essential role in regulating intracellular calcium levels and second messenger cascades (Obrietan and van den Pol, 1997).

Interestingly, PKA together with ERK1/2 signaling pathways are controlled by PrP^{C}, thus contributing to memory consolidation (Coitinho et al., 2006). The loss of pairing-induced potentiation of MF-GPSCs together with the loss of sensitivity of miniature GABAergic currents to forskolin in *Prnp*$^{0/0}$ mice, provide evidence that the prion protein controls synaptic activity by interacting directly or indirectly with postsynaptic PKA activity. The contribution of PrP^{C} to the regulation of hippocampal synaptic plasticity through the PKA signaling pathway was further confirmed by the observation that in *Prnp*$^{0/0}$ mice stimulation of Schaffer collateral failed to induce LTP, known to depend on postsynaptic PKA activation (Yasuda et al., 2003). PrP^{C} is thus involved also in modulating, *via* PKA, synaptic efficacy at glutamatergic connections. It should be stressed that, in the developing brain, PrP^{C} is particularly abundant in plastic regions including the hippocampus where, acting as a growth factor, it contributes to neurogenesis and differentiation *via* different transduction pathways including cAMP-dependent PKA (Kanaani et al., 2005; Steele et al., 2006).

If the loss of postsynaptic PKA activity is responsible for the lack of LTP in *Prnp*$^{0/0}$ mice, what determines LTD at these synapses? Several lines of evidence suggest that this form

of synaptic plasticity is presynaptic in origin. Early in postnatal development, MF are endowed with several neurotransmitter receptors including $GABA_B$ receptors (Safiulina and Cherubini, 2009), GluK1 receptors (Caiati et al., 2010), mGluRs (Scanziani et al., 1997), nAChRs, mAChRs (Maggi et al., 2004), adenosine and purinergic P2Y receptors (Zhang et al., 2003; Safiulina et al., 2005), known to down regulate GABA release. Among these, high affinity GluK1 receptors which are highly expressed in the immature hippocampus mainly at presynaptic sites, play a crucial role (Lauri et al., 2005; Caiati et al., 2010) (see also present data with ATPA). Indeed, blocking these receptors with a selective antagonist, fully prevented LTD induction in *Prnp*$^{0/0}$ mice. This effect was not mediated by other signaling molecules known to inhibit GABA release since the pairing protocol was still able to cause LTD in the presence of various receptor antagonists. LTD was also NMDA-independent as it could be triggered in the presence of D-AP5.

In a previous study on rats we demonstrated that GluK1 receptors localized on MF terminals are activated by a high level of glutamate in the extracellular space, probably maintained by a less efficient glutamate transport system and a poorly developed diffusional barrier (Caiati et al., 2010). Compared to previously reported data (Caiati et al., 2010), the amount of "ambient" glutamate in the extracellular milieu of *Prnp*$^{0/0}$ mice was probably lower because the GluK1 antagonist only slightly enhanced the amplitude of MF-GPSCs. Although we have not ascertained the reason for this apparent discrepancy, it is likely that during network-driven bursts, extracellular glutamate rises to a level sufficient to activate high affinity G-coupled GluK1 receptors present on MF terminals, which are known to exert a ionotropic and metabotropic type of action (Lerma, 2006). In *Prnp*$^{0/0}$ mice, the effect of GluK1 on the persistent depression of GABA release

from MF terminals depended on a metabotropic type of action as LTD was prevented by blocking PLC downstream of G protein activation. The blocking effect of the PLC inhibitor on spike time-dependent depression observed in rats (Caiati et al., 2010) is in line with the present observations. In *Prnp*$^{0/0}$ mice, however, blocking GluK1 did not reveal LTP as in rats (Caiati et al., 2010) due to the loss of postsynaptic PKA signaling.

Recently, the effect of *Prnp* disruption has been studied analyzing alterations of the whole transcriptome during the neurodevelopment of the hippocampus (Benvegnu et al., 2011). Several signal pathways were found to be affected, but with only moderate changes of the gene expression profile. In addition, the genes coding for the proteins involved in the PKA signaling do not localize in the proximity of *Prnp*, thus ruling out that the alteration of this pathway may be due to the genetic manipulation of *Prnp*.

Altogether these observations highlight the "gating" role PrP^{C}-dependent PKA signaling in regulating synaptic plasticity and information processing in the developing hippocampus.

References

Allen C, Stevens CF (1994) An evaluation of causes for unreliability of synaptic transmission. Proc Natl Acad Sci USA 91:10380-10383.

Amaral DG, Dent JA (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J Comp Neurol 195:51-86.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303-325.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87: 1215-1284.

Benvegnu S, Poggiolini I, Legname G (2010) Neurodevelopmental Expression and Localization of the Cellular Prion Protein in the Central Nervous System of the Mouse. J Comp Neurol 518:1879-1891.

Benvegnu S, Roncaglia P, Agostini F, Casalone C, Corona C, Gustincich S, Legname G (2011) Developmental influence of the cellular prion protein on the gene expression profile in mouse hippocampus. Physiol Genomics 43:711-725.

Bueler H, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C (1992) Normal development and behavior of mice lacking the neuronal cell-surface Prp protein. Nature 356:577-582.

Caiati MD, Sivakumaran S, Cherubini E (2010) In the developing rat hippocampus, endogenous activation of presynaptic kainate receptors reduces GABA release from mossy fiber terminals. J Neurosci 30:1750-1759.

Carleton A, Tremblay P, Vincent JD, Lledo PM (2001) Dose-dependent, prion protein (PrP)-mediated facilitation of excitatory synaptic transmission in the mouse hippocampus. Pflugers Archiv 442:223-229.

Coitinho AS, Freitas ARO, Lopes MH, Hajj GNM, Roesler R, Walz R, Rossato JI, Cammarota M, Izquierdo I, Martins VR, Brentani RR (2006) The interaction between prion protein and laminin modulates memory consolidation. Eur J Neurosci 24:3255-3264.

Colby DW, Prusiner SB (2011) Prions. Cold Spring Harb PerspectBiol 3.

Colling SB, Collinge J, Jefferys JG (1996) Hippocampal slices from prion protein null mice: disrupted Ca(2+)-activated K+ currents. Neurosci Lett 209:49-52.

Collinge J, Whittington MA, Sidle KCL, Smith CJ, Palmer MS, Clarke AR, Jefferys JG (1994) Prion protein is necessary for normal synaptic function. Nature 370:295-297.

Fuhrmann M, Bittner T, Mitteregger G, Haider N, Moosmang S, Kretzschmar H, Herms J (2006) Loss of the cellular prion protein affects the Ca2+ homeostasis in hippocampal CA1 neurons. J Neurochem 98:1876-1885.

Gutierrez R, Romo-Parra H, Maqueda J, Vivar C, Ramirez M, Morales MA, Lamas M (2003) Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 23:5594-5598.

Jonas P, Major G, Sakmann B (1993) Quantal components of unitary EPSCs at the mossy fiber synapse on CA3 pyramidal cells of rat hippocampus. J Physiol 472:615-663.

Kanaani J, Prusiner SB, Diacovo J, Baekkeskov S, Legname G (2005) Recombinant prion protein induces rapid polarization and development of synapses in embryonic rat hippocampal neurons in vitro. J Neurochem 95:1373-1386.

Kasyanov AM, Safiulina VF, Voronin LL, Cherubini E (2004) GABA-mediated giant depolarizing potentials as coincidence detectors for enhancing synaptic efficacy in the developing hippocampus. Proc Natl Acad Sci USA 101:3967-3972.

Lauri SE, Segerstrale M, Vesikansa A, Maingret F, Mulle C, Collingridge GL, Isaac JT, Taira T (2005) Endogenous activation of kainate receptors regulates glutamate release and network activity in the developing hippocampus. J Neurosci 25:4473-4484.

Le Magueresse C, Safiulina V, Changeux JP, Cherubini E (2006) Nicotinic modulation of network and synaptic transmission in the immature hippocampus investigated with genetically modified mice. J Physiol 576:533-546.

Lerma J (2006) Kainate receptor physiology. Curr Opin Pharmacol 6:89-97.

Maggi L, Sola E, Minneci F, Le Magueresse C, Changeux JP, Cherubini E (2004) Persistent decrease in synaptic efficacy induced by nicotine at Schaffer collateral-CA1 synapses in the immature rat hippocampus. J Physiol 559:863-874.

Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? Science 285:1870-1874.

Mallucci GR, Ratte S, Asante EA, Linehan J, Gowland I, Jefferys JG, Collinge J (2002) Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. EMBO J 21:1240-1240.

Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J (1992) The prion protein gene: a role in mouse embryogenesis? Development 115:117-122.

Manson JC, Hope J, Clarke AR, Johnston A, Black C, MacLeod N (1995) PrP gene dosage and long term potentiation. Neurodegeneration 4:113-114.

McKinley MP, DeArmond SJ, Torchia M, Mobley WC, Prusiner SB (1989) Acceleration of scrapie in neonatal Syrian hamsters. Neurology 39:1319-1324.

Mohajerani MH, Sivakumaran S, Zacchi P, Aguilera P, Cherubini E (2007) Correlated network activity enhances synaptic efficacy via BDNF and the ERK pathway at immature CA3-CA1 connections in the hippocampus. Proc Natl Acad Sci USA 104:13176-13181.

Obrietan K, van den Pol AN (1997) GABA activity mediating cytosolic Ca2+ rises in developing neurons is modulated by cAMP-dependent signal transduction. J Neurosci 17:4785-4799.

Prestori F, Rossi P, Bearzatto B, Laine J, Necchi D, Diwakar S, Schiffmann SN, Axelrad H, D'Angelo E (2008) Altered neuron excitability and synaptic plasticity in the cerebellar granular layer of juvenile prion protein knock-out mice with impaired motor control. J Neurosci 28:7091-7103.

Rodriguez-Moreno A, Sihra TS (2007) Metabotropic actions of kainate receptors in the CNS. J Neurochem 103:2121-2135.

Safiulina VF, Kasyanov AM, Sokolova E, Cherubini E, Giniatullin R (2005) ATP contributes to the generation of network-driven giant depolarizing potentials in the neonatal rat hippocampus. J Physiol 565:981-992.

Safiulina VF, Fattorini G, Conti F, Cherubini E (2006) GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. J Neurosci 26:597-608.

Safiulina VF, Cherubini E (2009) At immature mossy fibers-CA3 connections, activation of presynaptic GABA(B) receptors by endogenously released GABA contributes to synapses silencing. Front Cell Neurosci 3 Epub 2009 Feb 26.

Sales N, Hassig R, Rodolfo K, Di Giamberardino L, Traiffort E, Ruat M, Frétier P, Moya KL (2002) Developmental expression of the cellular prion protein in elongating axons. Eur J Neurosci 15:1163-1177.

Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385:630-634.

Sciancalepore M, Cherubini E (1995) PKA-dependent increase in frequency of miniature GABAergic currents in rat CA3 hippocampal neurons. Neurosci Lett 187:91-94.

Sivakumaran S, Mohajerani MH, Cherubini E (2009) At immature mossy-Fiber-CA3 synapses, correlated presynaptic and postsynaptic activity persistently enhances GABA release and network excitability via BDNF and cAMP-dependent PKA. J Neurosci 29:2637-2647.

Steele AD, Emsley JG, Ozdinler PH, Lindquist S, Macklis JD (2006) Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. Proc Natl Acad Sci USA 103:3416-3421.

Steele AD, Lindquist S, Aguzzi A (2007) The prion protein knockout mouse: a phenotype under challenge. Prion 1:83-93.

Voronin LL, Cherubini E (2004) 'Deaf, mute and whispering' silent synapses: their role in synaptic plasticity. J Physiol 557:3-12.

Walker MC, Ruiz A, Kullmann DM (2001) Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. Neuron 29:703-715.

Yasuda H, Barth AL, Stellwagen D, Malenka RC (2003) A developmental switch in the signaling cascades for LTP induction. Nat Neurosci 6:15-16.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40:971-982.

Zucker RS (1989) Short-term synaptic plasticity. Annu Rev Neurosci 12:13-31.

Figure Legends

Figure 1. Pairing GDPs with MF stimulation induces LTP in WT mice and LTD in *Prnp*$^{0/0}$ mice. (A) Diagram of the hippocampus showing a CA3 pyramidal neuron receiving synaptic input from the MF. The stimulating electrode (stim) was positioned in stratum granulosum of the dentate gyrus. (B) GDPs recorded from a CA3 principal cell in current clamp mode; the inset below represents the GDP (marked with an asterisk) on an expanded time scale. Note the absence of spikes riding on top of GDPs due to the block of the sodium channel with intracellular QX 314. The rising phase of GDPs (between the dashed lines) was used to trigger synaptic stimulation (stim). (C) The mean amplitude of MF-GPSCs recorded in WT animals (n=13) before and after pairing (arrow at time 0) is plotted versus time, in the absence or in the presence (bars) of L-AP4 and PTX. Insets above the graphs represent individual traces of GPSCs evoked before and after pairing, after addition of L-AP4 and PTX. (D) As in **c** but from *Prnp*$^{0/0}$ mice (n=15). In this and in the following Figures vertical bars refer to SEM.

Figure 2. Presynaptic expression of LTP and LTD in WT and *Prnp*$^{0/0}$ mice. (A, B) Summary plot (columns) of successes, inverse square of CV, PPR of MF-GPSCs measured in individual cells before (Control, white) and after pairing (Pairing, black) in WT (A) and in *Prnp*$^{0/0}$ animals (B). ***p<0.001.

Figure 3. Pairing-induced synapse silencing in CA3 principal cells from *Prnp*$^{0/0}$ mice. A. Amplitudes of synaptic responses (insets above the graphs) evoked in a P4 CA3 principal cell by two stimuli (50 ms apart; GPSCs evoked by the first stimulus: open symbols; by the second stimulus: closed symbols) obtained before and after pairing

(arrows at time 0) are plotted against time. B. Summary plots for six cells silenced after pairing. Amplitudes and successes measured in individual cells before (open symbols) and after pairing (closed symbols).

Figure 4. By loading the cells with BAPTA or by maintaining them close to their resting values during pairing blocks LTP and unveils LTD in WT animals. (A, C) Plot of mean MF-GPSCs amplitude from CA3 pyramidal cells loaded with BAPTA (n=7; A) or voltage clamped at -70 mV (n=6; C) before and after pairing (arrows at time 0) versus time. Insets above the graphs represent individual traces of GPSCs evoked before and after pairing. Note pairing-induced LTD instead of LTP. (B, D) As in A, C but from *Prnp*$^{0/0}$ mice (n=7 in B and n=9 in D).

Figure 5. Presynaptic expression of pairing-induced LTD in CA3 pyramidal cells loaded with BAPTA. A and B. Summary plots of successes, inverse square of CV, PPR of MF-GPSCs measured in individual cells before (Control, white) and after pairing (Pairing, black) in WT (A, n=7) and in *Prnp*$^{0/0}$ animals (B, n=7). *p<0.05; **p<0.01.

As expected for a presynaptic type of action, pairing-induced LTD was associated with a significant reduction in the number of successes (from 0.74 ± 0.06 to 0.42 ± 0.1; n=7; p<0.01 in WT and from 0.67 ± 0.09 to 0.29 ± 0.1; n=8; p<0.01 in *Prnp*$^{0/0}$ mice), a significant increase in PPR (from 1.4 ± 0.1 to 2.1 ± 0.3; n=5; p<0.05 in WT and from 1.1 ± 0.2 to 1.5 ± 0.3; n=5; p<0.05 in *Prnp*$^{0/0}$ mice) and a marked decrease in CV^{-2} (from 3 ±

0.67 to 2.3 ± 0.7; n=7; $p<0.05$ in WT and from 3.3 ± 1.4 to 1.8 ± 1.2; n=6; $p<0.01$ in *Prnp*$^{0/0}$ mice).

Figure 6. Inhibiting PKA in the postsynaptic neuron prevents pairing-induced LTP in WT mice. (A) Sample traces of a P5 CA3 pyramidal cell (loaded with the membrane impermeable PKA inhibitor PKI 6–22) obtained before (Control) and 30 min after pairing. (B) Summary plot of MF-GPSCs amplitudes measured in individual cells loaded with PKI 6–22 before (Control, white) and after pairing (Pairing, black). ***$p<0.001$.

Figure 7. In WT but not in *Prnp*$^{0/0}$ mice, forskolin enhances the amplitude of miniature GPSCs. (A) Amplitude distribution of miniature GPSCs recorded in CA3 principal cells from WT animals under control conditions and during application of forskolin (n=10). (B) As in (A) but with the membrane impermeable PKA inhibitor PKI 6–22 into the patch pipette (n=8). (C) As in (A) but from *Prnp*$^{0/0}$ mice (n=8). Note reduction of larger amplitude events with forskolin in B and C. Bin width: 5 pA. (D) and (E) Summary plots of forskolin-induced increase in frequency (D) or in amplitude (E) of miniature GABAergic events in cells from WT animals (white columns); in cells from WT animals but loaded with PKI 6–22 (gray columns) or in cells from *Prnp*$^{0/0}$ mice (black columns). ***$p<0.001$ (respect to pre-pairing values; paired *t*-test); values for WT+PKI and *Prnp*$^{0/0}$ versus WT were significantly different ($p<0.001$, one way ANOVA).

Figure 8. Lack of PKA-dependent LTP at Schaffer collateral-CA1 synapses in the hippocampus of *Prnp*$^{0/0}$ mice. A. The mean EPSC amplitude recorded before and after pairing (arrow at time 0) is plotted against time in WT (open symbols; n=6) and in *Prnp*$^{0/0}$ mice (closed symbols; n=14). Insets above the graph represent sample traces of Schaffer collateral-CA1 EPSCs obtained in WT and *Prnp*$^{0/0}$ mice before (left) and after pairing (right). B. Blocking postsynaptic PKA with the membrane impermeable PKA inhibitor PKI 6–22 prevents LTP induction. Each column represents pairing-induced changes of EPSCs amplitude (normalized to pre-pairing values) in CA1 pyramidal cells from WT animals (WT, white, n=6), from WT animals in the presence of PKI 6–22 (+PKI, grey, n=5), and from *Prnp*$^{0/0}$ mice (*Prnp*$^{0/0}$, black, n=14). Data obtained from WT animals in the presence of PKI 6–22 and from *Prnp*$^{0/0}$ versus WT were significantly different (p<0.001; one way ANOVA).

Figure 9. In *Prnp*$^{0/0}$ mice, at MF-CA3 synapses, pairing induced LTD is prevented by blocking G-protein coupled GluK1 receptors or phospholipase C downstream to G-protein activation. (A) The mean amplitude of MF-GPSCs recorded in *Prnp*$^{0/0}$ mice (n=9) in the presence of the GluK1 antagonist UBP 302 before and after pairing (arrow at time 0) is plotted versus time. Insets above the graph represent individual traces of MF-GPSCs evoked before (Control) and after pairing. (B) Each column represents the mean MF-GPSC amplitude obtained 30 min after pairing and normalized to pre-pairing values in control (white, n=15), in the presence of UBP 302 (black, n=9) and PLC inhibitor U73122 (grey, n=8). Data obtained in the presence of UBP 302 and U73122 versus controls were significantly different (p<0.001; one way ANOVA).

Figure 10. At MF-CA3 synapses in slices from *Prnp*$^{0/0}$ mice LTD results from the direct effect of pairing on GluK1 receptors. Each column represents the mean MF-GPSC amplitude obtained 30 min after pairing and normalized to pre-pairing values in control (white, n=15), in the presence of different receptor antagonists (Antag, grey, n=6) and in the presence of D-AP5 (black, n= 7). Receptor antagonists comprise: CGP 52432, 3 µM; atropine 1 µM, DHβE 50 µM; DPCPX, 10 µM; PPADS, 50 µM; LY341495, 100 µM to block $GABA_B$, muscarinic, nicotinic adenosine, purinergic P2Y and mGluR receptors, respectively. Data obtained in the presence of different receptor antagonists and D-AP5 were not significantly different from controls: p=0.73 and 0.44, respectively (one way ANOVA).

Figure 1

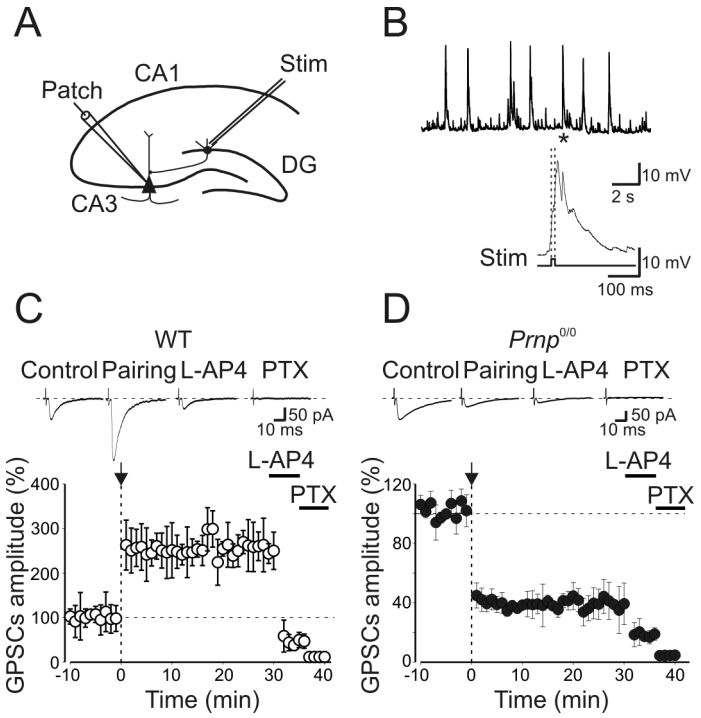

A
Patch
CA1
Stim
DG
CA3
B
10 mV
2 s
Stim
10 mV
100 ms
C
WT
Control Pairing L-AP4 PTX
50 pA
10 ms
L-AP4
PTX
GPSCs amplitude (%)
400
300
200
100
0
-10
0
10
20
30
40
Time (min)
D
Prnp0/0
Control Pairing L-AP4 PTX
50 pA
10 ms
L-AP4
PTX
GPSCs amplitude (%)
120
80
40
0
-10
0
10
20
30
40
Time (min)

Figure 2

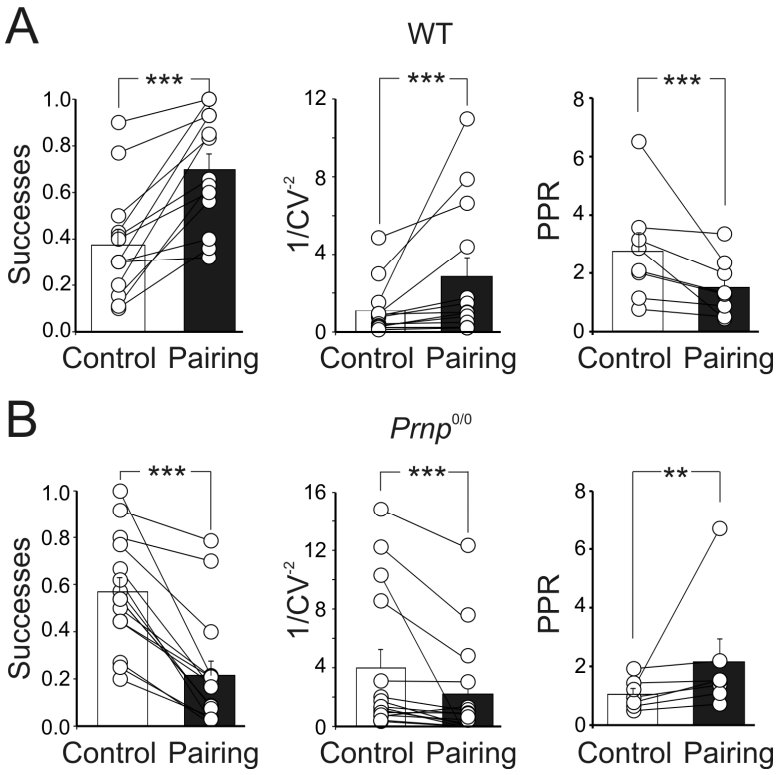

A
WT
***
***
***
Successes
1/CV-2
PPR
Control Pairing
Control Pairing
Control Pairing
B
Prnp0/0
***
***
**
Successes
1/CV-2
PPR
Control Pairing
Control Pairing
Control Pairing

Figure 3

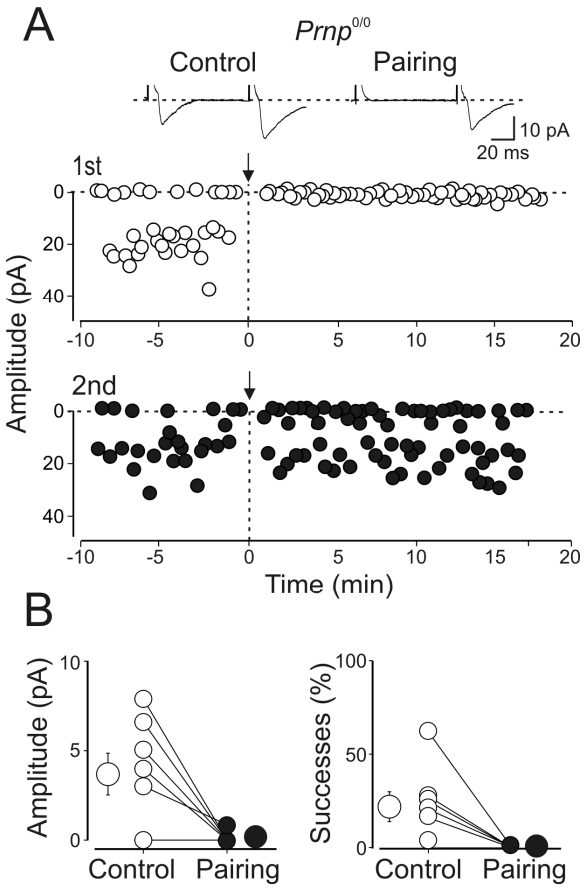

A
Prnp0/0
Control
Pairing
10 pA
20 ms
1st
2nd
Amplitude (pA)
Time (min)
B
Amplitude (pA)
Successes (%)
Control
Pairing

Figure 4

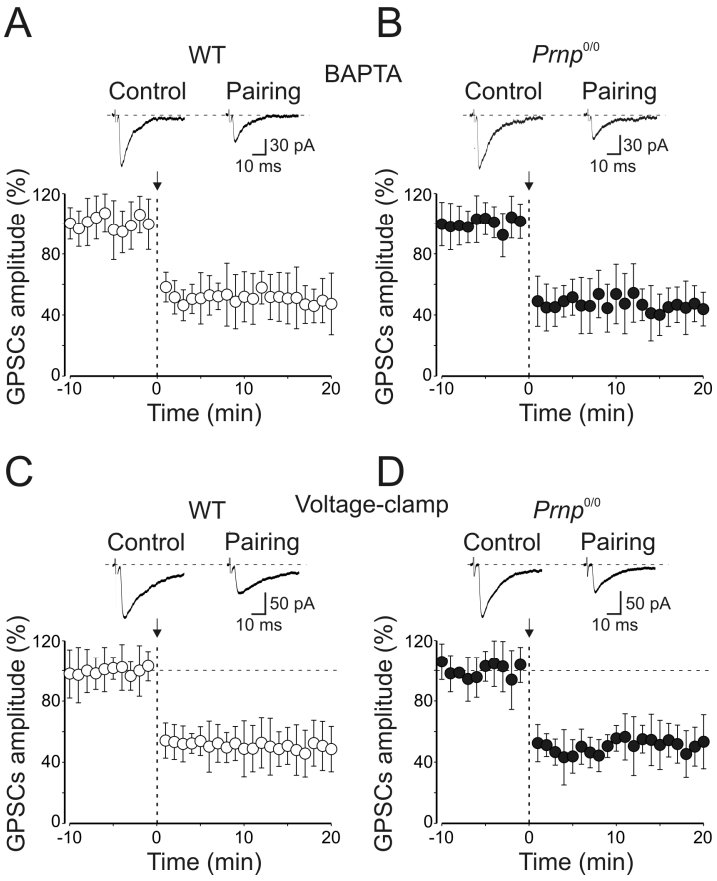

A
WT
BAPTA
Control
Pairing
30 pA
10 ms
GPSCs amplitude (%)
Time (min)
B
Prnp0/0
C
Voltage-clamp
50 pA
D

Figure 5

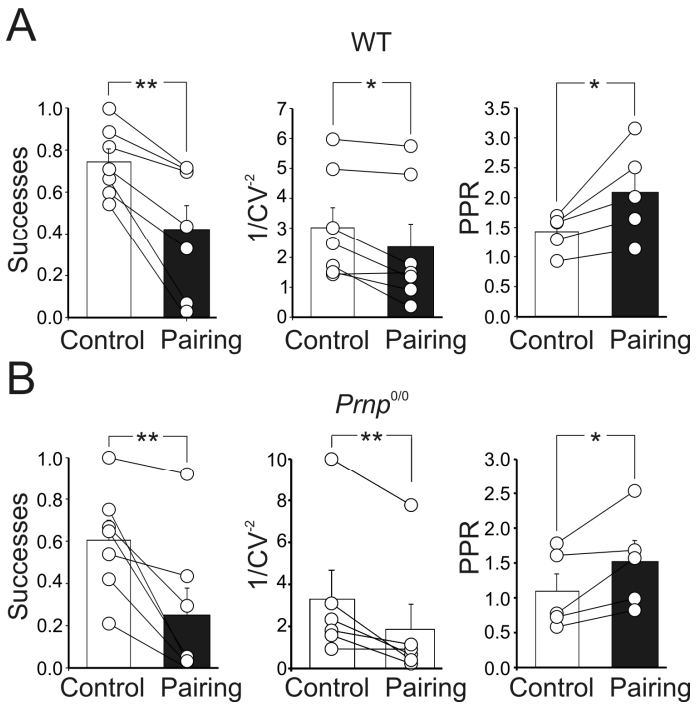

A
WT
**
*
*
Successes
1/CV-2
PPR
Control
Pairing
B
Prnp0/0
**
**
*
Successes
1/CV-2
PPR
Control
Pairing

Figure 6

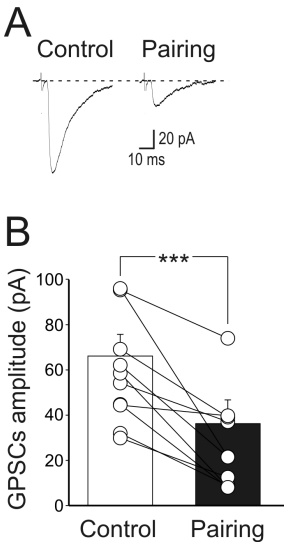

A
Control
Pairing
20 pA
10 ms
B
***
GPSCs amplitude (pA)
100
80
60
40
20
0
Control
Pairing

Figure 7

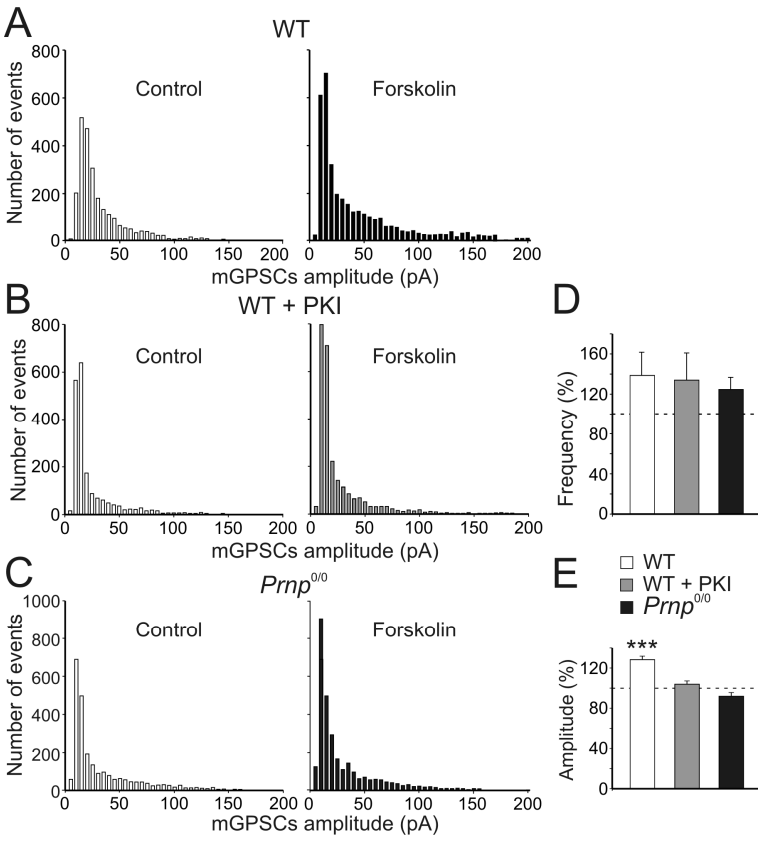

A
WT
Control
Forskolin
Number of events
mGPSCs amplitude (pA)
B
WT + PKI
Control
Forskolin
Number of events
mGPSCs amplitude (pA)
C
Prnp0/0
Control
Forskolin
Number of events
mGPSCs amplitude (pA)
D
Frequency (%)
E
WT
WT + PKI
Prnp0/0
***
Amplitude (%)

Figure 8

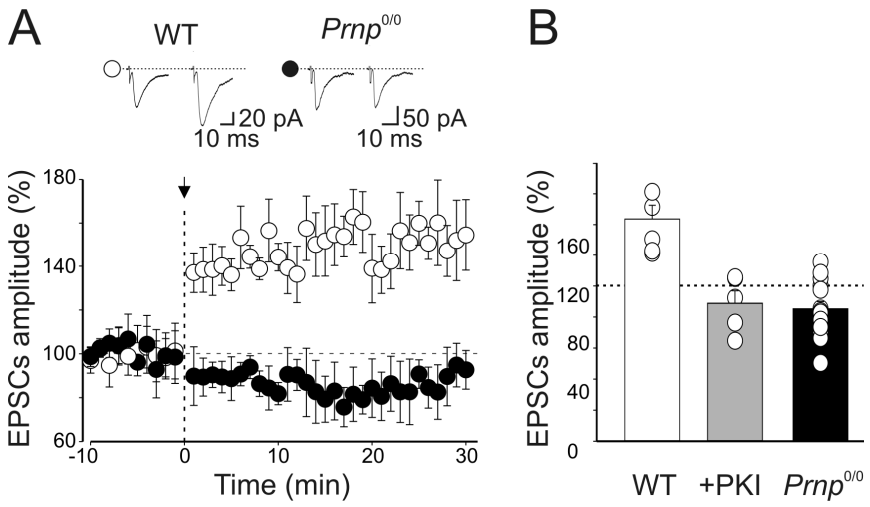

A
WT
Prnp0/0
20 pA
10 ms
50 pA
10 ms
EPSCs amplitude (%)
180
140
100
60
-10
0
10
20
30
Time (min)
B
EPSCs amplitude (%)
160
120
80
40
0
WT
+PKI
Prnp0/0

Figure 9

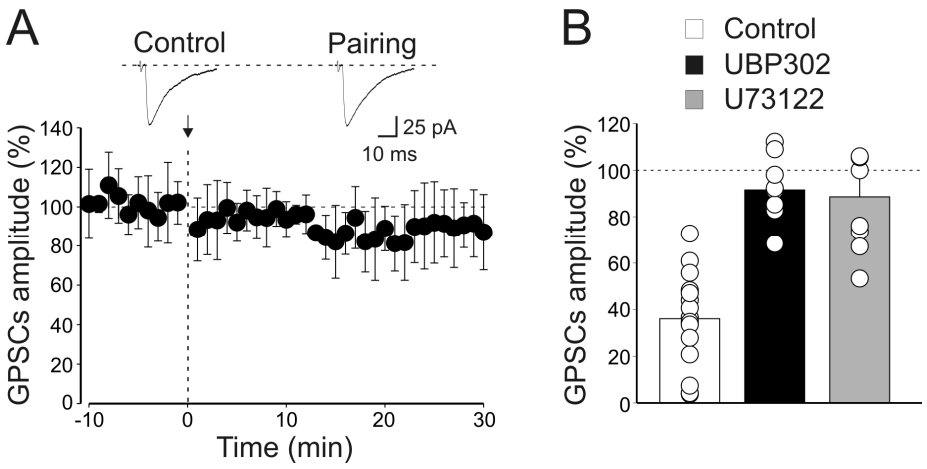
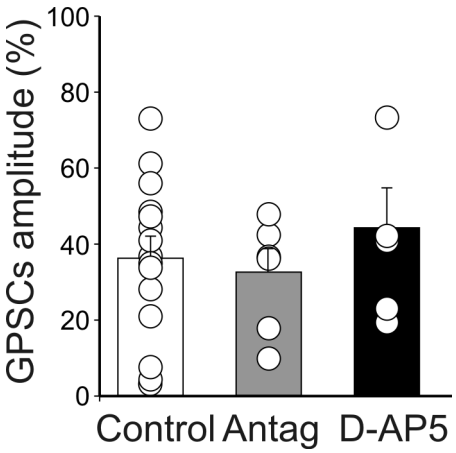

A
Control
Pairing
25 pA
10 ms
GPSCs amplitude (%)
140
120
100
80
60
40
20
0
-10
0
10
20
30
Time (min)
B
Control
UBP302
U73122
GPSCs amplitude (%)
120
100
80
60
40
20
0

Figure 10



GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The present results have revealed novel mechanisms of activity-dependent modulation of neurotransmitter release at immature MF-CA3 synapses. In particular, I reported that in young animals pre-synaptic G-coupled-GluK1 and CB1 receptors, whose expression and function appear to be developmentally regulated, reduce the release of GABA from MF terminals.

In the first study, I demonstrated that presynaptic GluK1 KARs down regulate GABA release from MF terminals *via* a metabotropic type of action. The fundamental question to be addressed was how presynaptic GluK1 receptors could be activated if the main neurotransmitter released from MFs is GABA. One possibility is that these receptors are constitutively activated by glutamate present in the extracellular medium which in neonates is maintained at high levels by a less efficient glutamate transport mechanism and a poorly developed diffusional barrier. Indeed, by enhancing the clearance of glutamate from the extracellular space, using an enzymatic glutamate scavenger system prevented the activation of presynaptic KARs by glutamate and similarly to UBP 302 induced an increase in amplitude of MF-GPSCs.

I also showed that the depressant effect of GluK1 on GABA release was dependent on PTx-sensitive G protein-coupled KARs since it was prevented when hippocampal slices were incubated in the presence of a solution containing PTx which blocks $G_{i/o}$ protein-coupled receptors. This effect was presynaptic since application of UBP 302 to cells patched with a solution containing GDPβS still potentiated synaptic responses. In addition, the depressant effect of GluK1 on GABA release was prevented by U73122, which selectively inhibits phospholipase C, downstream to G protein activation. Interestingly, U73122, enhanced the probability of GABA release, thus unveiling the ionotropic type of action of kainate receptors.

Furthermore, I found that GluK1 KARs control the direction of STDP at immature MF-CA3 synapses. When positive pairing (15 ms delay) was delivered in the presence of the selective GluK1 antagonist UBP 302, a shift from STD-LTD to STD-LTP was detected. This activity-dependent form of synaptic plasticity involves the activation of a G-protein coupled receptors and PLC since it was prevented by the selective PLC blocker U73122. However, in the presence of GluK1 KAR antagonists, negative pairing still induced LTD, suggesting that KARs are not involved in this form of synaptic plasticity.

In the second study, I demonstrated that STD-LTD induced by negative pairing is mediated by CB1 receptors, "switched on" by endocannabinoids released from CA3 principal cells upon spiking-induced membrane depolarization. The endogenous ligand responsible for STD-LTD appears to be anandamide since inhibiting its transport or its degradation, lengthened the time window for LTD.
An intriguing question is where CB1 receptors are localized, since previous reports failed to show the presence of CB1 receptors on adult glutamatergic MF terminals. *In situ* hybridization experiments performed from Dr. Marsicano's group in Bordeaux, have clearly revealed detectable levels of CB1 mRNA in the granule cell layer of the hippocampus of P3 old but not juvenile animals, suggesting that in this area the expression of CB1 receptors is developmentally regulated.

Finally, in the third study I showed that the PrP^C protein controls the direction of synaptic plasticity at immature GABAergic MF-CA3 synapses. I found that at MF-CA3 synapses, the deletion of PrP^C in $Prnp^{0/0}$ mice prevents the induction of LTP but causes LTD. LTP was postsynaptic in origin and required the activation of cAMP-dependent PKA signaling while LTD was presynaptic and was reliant on G protein-coupled GluK1 receptor and protein lipase C downstream to G protein activation.

In addition, at emerging CA3-CA1 synapses of PrPC-deficient mice, stimulation of Schaffer collateral failed to induce LTP, known to be PKA-dependent. Since LTD has been associated to synaptic pruning, these findings raise the intriguing possibility that similar structural changes may occur in the hippocampus of *Prnp* $^{0/0}$ mice. In future studies it would be of interest to investigate whether *Prnp* $^{0/0}$ mice exhibit structural changes in spine density and morphology which may explain deficits in spatial memory (Steele et al., 2006).

As already mentioned, immediately after birth, GABA released from MF terminals exerts a depolarizing and sometimes excitatory type of action on targeted cells. Hence, GluK1 and CB1 receptors may play an important role in shaping MF-CA3 connections during a critical period of MF maturation. However, several crucial issues still remain to be solved in order to fully understand and estimate the functional impact that GABA release and its regulation by several neurotransmitter may have on the excitability of the entire hippocampal circuit.

Although several lines of evidence suggest that early in postnatal development MFs release GABA onto their targets (Walker *et* al. 2003; Safiulina *et* al. 2006; Gutierrez, 2005; Beltan and Gutiererz, 2012), a direct evidence is still missing. Although monosynaptic GABAergic currents, elicited by minimal stimulation of granule cells in the dentate gyrus were identified as belonging to MFs according to classical criteria, they could have been originated from the activation of axon collaterals of local GABAergic interneurons, as sugegsted by Uchigashima *et* al. (2007).
Recently, using organotypic hippocampal cultures prepared from P10-P20 GAD67-GFP transgenic mice, Cabezas *et* al. (2012) have demonstrated that granule cells expressing GAD67 in a subpopulation of immature granule cells, are purely glutamatergic and make glutamatergic monosynaptic contacts with CA3 pyramidal cells. While this approach is interesting, a direct comparison with data gained from acute hippocampal slices is impossible due to the difficulty of estimating the exact developmental stage of MF in organotypic slices prepared from juvenile animals.

The most straightforward way to address this issue would be to perform pair recordings from granule cells and CA3 pyramidal neurons in pups at very early stages of postnatal development. However, since the probability of finding connected granule-CA3 pairs is extremely low, other strategies should be experimented. Though technically challenging, the optogenetic approach of injecting *in utero* a retroviral vector carrying Channelrhodopsin-2 (ChR-2) would represent, indeed, a feasible stratagem to selectively introduce ChR-2 into dividing granule cells progenitors. Immediately after birth, activation of ChR-2 with light, would allow identifying without doubts the nature of the neurotransmitter released by immature granule cells into patched CA3 principal cells. The MF nature of ChR-2 driven synaptic responses would be further ascertained by the sensitivity of the latter to group II and III mGluR agonists.

It should be stressed however, that one of the most serious limitation of this approach is represented by the relative long time (two-three weeks) required by Channelrhodopsin-2 to be successfully expressed in injected animals since granule cells have been shown to release GABA or GABA plus glutamate during a relatively short time window. The simplest way to circumvent this problem could be the development of a mouse line constitutively expressing ChR-2 in hippocampal progenitors.

As alternative to the optogenetic technique, two-photon uncaging of glutamate into granule cells in hippocampal slices obtained from GFP+ mice could be used. This method, recently developed in Yuste's lab (Nikolenko *et* al., 2007), would offer the advantage of activating relatively quickly (<5 min) and with single-cells resolution a large number of granule cells (up to 1.000) and test from connected CA3 principal cells whether or not they are GABAergic. Both the optogenetic and two-photon photo-stimulation approaches will allow to selectively stimulate granule cells as well as to overcome the difficulty in finding connected pairs of neurons.

Another important point to be addressed is whether GABA released from MF terminals can modulate its own release not only through $GABA_B$ but also through $GABA_A$ autoreceptors. In organotypic cell cultures, Cabezas *et* al. (2012) have shown that GABA released from a subset of immature granule cells can modulate its own release and reduce MF excitability *via* presynaptic $GABA_B$ receptors and a previous study from newborn hippocampal slices has demonstrated that the constitutive activation of $GABA_B$ receptors by spillover of GABA from MF terminals and/or neighboring GABAergic interneurons contributes to synaptic depression or synaptic silencing (Safiulina and Cherubini, 2009).

The optogenetic approach could be used also to study whether GABA released from MF terminals is able to change *via* $GABA_A$ receptors MF excitability. To this aim, we shall antidromically activate MF terminals in stratum lucidum while recording antidromic spikes in dentate gyrus granule cells before and after focal light-driven activation of ChR-2-positive positive axon terminals or synaptic boutons. The possibility to prevent changes in probability of eliciting antidromic spikes with selective $GABA_A$ receptor antagonists would allow characterizing the functional role exerted by $GABA_A$ autoreceptors on GABA release and on the excitability of the local network. The pharmacological blockade of GABA uptake will permit to enhance GABA action at presynaptic level. This method would allow also establishing whether GABA released from MF terminals enhances or decreases the probability of evoking antidromic spikes since ChR-2 does not modify the intracellular chloride concentration in axon terminals.

A final fundamental question is why should immature MF use GABA instead of glutamate as a neurotransmitter?

In a recent study (Safiulina *et* al., 2010), the relative strength of CA3 pyramidal cells output in relation to their MF GABAergic or glutamatergic inputs using a realistic model which takes into account different parameters, including unitary conductance of MF-GPSCs or MF-EPSCs, quantal content, and kinetics of GABA- or AMPA-mediated responses has been compared. The model has been simplified, implementing the macroscopic overall conductance changes observed during the experiments.

It did not include the expression of postsynaptic NMDA receptors or more detailed mechanisms such as postsynaptic receptor density, neurotransmitter diffusion, reuptake, etc. Some of adult MF-EPSCs parameters have been taken from Jonas et al. (1993), while the firing frequency of immature granule cells was estimated from the firing rate of spikes occurring at the top of spontaneous GDPs. In addition, we estimated the number of MF inputs impinging into CA3 principal cells equal to 30 on the assumption that during the first week of postnatal life more than one third of MF reach their targets (Gaarskjaer, 1985). In adulthood, each principal cell receives ~50 MF inputs from granule cells. Thirty synapses were randomly distributed on the proximal apical dendrites (within 100 μm from the soma) of a realistic CA3 model neuron (Hemond et al., 2008). The average number of spikes generated by a train of 10 random (poissonian) activations of GABAergic or glutamatergic inputs, at an average frequency of 20 or 40 Hz, was then calculated from 10 simulations.
The model predicts that more positive reversal potential of glutamatergic synapses can depolarize more easily CA3 pyramidal cells and transiently hinder the membrane repolarization process, hence inactivating Na channels and saturating the cell. In addition to elicit less spikes, with obvious consequences for frequency-dependent synaptic plasticity mechanisms, this effect could generate a number of other problems, ranging from excitotoxicity caused by a much higher elevation in intracellular calcium concentration to receptor desensitization. Therefore, it appears that, despite its shunting action, GABA released from MF terminals is still able to elicit action potentials in targeted neurons and to convey information into the auto-associative CA3 network. GABA-induced excitation would provide the basis for enhancing synaptic efficacy at emerging synapses in a Hebbian type of way (spike time-dependent plasticity), hence contributing to synaptogenesis and to the establishment of the adult neuronal circuit.

In conclusion, I would like to highlight, as a key aspect in the study of the modulation of the activity-dependent processes at immature MF-CA3 synapse, that major milestones of neuronal development are highly conserved during adult neurogenesis.
In view of the ever increasing body of evidence in favor of the participation of adult neurogenesis in several neuropsychiatric disorders, it would be important to explore whether mechanisms similar to those observed at immature MF also take place also in adult born granule cells.

BIBLIOGRAPHY

Acsady L, Kamondi A, Sik A, Freund T, Buzsaki G (1998) GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. J Neurosci 18:3386-403.

Acsády L, Katona I, Martínez-Guijarro FJ, Buzsáki G, Freund TF (2000) Unusual target selectivity of perisomatic inhibitory cells in the hilar region of the rat hippocampus. J Neurosci 20(18):6907-19.

Aguzzi A, Polymenidou M (2004) Mammalian prion biology: one century of evolving concepts. Cell 116(2):313-27.

Alle H, Geiger JR (2007) GABAergic spill-over transmission onto hippocampal mossy fiber boutons. J Neurosci 27(4):942-50.

Allen C, Stevens CF (1994) An evaluation of causes for unreliability of synaptic transmission. Proc Natl Acad Sci U S A 91(22):10380-3.

Altman J, Das GD (1965) Post-natal origin of microneurones in the rat brain. Nature 207(5000):953-6.

Amaral DG (1978) A Golgi study of cell types in the hilar region of the hippocampus in the rat. J Comp Neurol 182:851-914.

Amaral DG, Dent JA (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J Comp Neurol 195(1):51-86.

Amico C, Marchetti C, Nobile M, Usai C (1995) Pharmacological types of calcium channels and their modulation by baclofen in cerebellar granules. J Neurosci 15:2839-48.

Armstrong JN, Saganich MJ, Xu NJ, Henkemeyer M, Heinemann SF, Contractor A (2006) B-ephrin reverse signaling is required for NMDA-independent long-term potentiation of mossy fibers in the hippocampus. J Neurosci 26(13):3474-81.

Austin JE, Buckmaster PS (2004) Recurrent excitation of granule cells with basal dendrites and low interneuron density and inhibitory postsynaptic current frequency in the dentate gyrus of macaque monkeys. J Comp Neurol 476(3):205-18.

Bahn S, Volk B, Wisden W (1994) Kainate receptor gene expression in the developing rat brain. J Neurosci 14(9):5525-47.

Bakker A, Kirwan CB, Miller M, Stark CE (2008) Pattern separation in the human hippocampal CA3 and dentate gyrus. Science 319(5870):1640-2.

Barnes CA, McNaughton BL, Mizumori SJ, Leonard BW, Lin LH (1990) Comparison of spatial and temporal characteristics of neuronal activity in sequential stages of hippocampal processing. Prog Brain Res 83:287-300.

Bayer SA (1980a) Development of the hippocampal region in the rat. I. Neurogenesis examined with 3H-thymidine autoradiography. J Comp Neurol 190:87-114.

Bayer SA (1980b) Development of the hippocampal region in the rat. II. Morphogenesis during embryonic and early postnatal life. J Comp Neurol 190:115-34.

Behr J, Lyson KJ, Mody I (1998) Enhanced propagation of epileptiform activity through the kindled dentate gyrus. J Neurophysiol 79(4):1726-32.

Bellocchio L, Lafenêtre P, Cannich A, Cota D, Puente N, Grandes P, Chaouloff F, Piazza PV, Marsicano G (2010) Bimodal control of stimulated food intake by the endocannabinoid system. Nat Neurosci 13(3):281-3.

Beltrán JQ, Gutiérrez R (2012) Co-release of glutamate and GABA from single, identified mossy fibre giant boutons. J Physiol590 (Pt 19):4789-800. doi: 10.1113/jphysiol.2012.236372. Epub 2012 Jun 18.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303-25.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215-84.

Benvegnù S, Poggiolini I, Legname G (2010) Neurodevelopmental expression and localization of the cellular prion protein in the central nervous system of the mouse. J Comp Neurol 518(11):1879-91.

Bergersen L, Ruiz A, Bjaalie JG, Kullmann DM, Gundersen V (2003) GABA and $GABA_A$ receptors at hippocampal mossy fibre synapses. Eur J Neurosci 18:931-41.

Berghuis P, Rajnicek AM, Morozov YM, Ross RA, Mulder J, Urbán GM, Monory K, Marsicano G, Matteoli M, Canty A, Irving AJ, Katona I, Yanagawa Y, Rakic P, Lutz B, Mackie K, Harkany T (2007) Hardwiring the brain: endocannabinoids shape neuronal connectivity. Science 316(5828):1212-6.

Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004) Molecular structure and physiological functions of GABA(B) receptors. Physiol Rev 84:835-67.

Bhattacharyya BJ, Banisadr G, Jung H, Ren D, Cronshaw DG, Zou Y, Miller RJ (2008) The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. J Neurosci 28(26):6720-30.

Blaesse P, Airaksinen MS, Rivera C, Kaila K (2009) Cation-chloride cotransporters and neuronal function. Neuron 61:820-38.

Breustedt J, Schmitz D (2004) Assessing the role of GLUK5 and GLUK6 at hippocampal mossy fiber synapses. J Neurosci 24(45):10093-8.

Brown TH, Johnston D (1983) Voltage-clamp analysis of mossy fiber synaptic input to hippocampal neurons. J Neurophysiol 50(2):487-507.

Buckmaster PS, Dudek FE (1999) In vivo intracellular analysis of granule cell axon reorganization in epileptic rats. J Neurophysiol 81(2):712-21.

Buzsaki G, Draguhn A (2004) Neuronal oscillations in cortical networks. Science 304:1926-9.

Cabezas C, Irinopoulou T, Gauvain G, Poncer JC (2012) Presynaptic But Not Postsynaptic GABA Signaling at Unitary Mossy Fiber Synapses. J Neurosci 32(34):11835-40.

Caiati MD, Sivakumaran S, Cherubini E (2010) In the developing rat hippocampus, endogenous activation of presynaptic kainate receptors reduces GABA release from mossy fiber terminals. J Neurosci 30(5):1750-9.

Cancedda L, Fiumelli H, Chen K, Poo MM (2007) Excitatory GABA action is essential for morphological maturation of cortical neurons in vivo. J Neurosci 27:5224-35.

Castillo PE, Malenka RC, Nicoll RA (1997) Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. Nature 388(6638):182-6.

Chen G, Trombley PQ, van den Pol AN (1996) Excitatory actions of GABA in developing rat hypothalamic neurones. J Physiol 494:451‑464.

Cherubini E, Conti F (2001) Generating diversity at GABAergic synapses. Trends Neurosci 24:155-62.

Chicurel ME, Harris KM (1992) Three-dimensional analysis of the structure and composition of CA3 branched dendritic spines and their synaptic relationships with mossy fiber boutons in the rat hippocampus. J Comp Neurol 325(2):169-82.

Choi SH, Veeraraghavalu K, Lazarov O, Marler S, Ransohoff RM, Ramirez JM, Sisodia SS (2008) Non-cell-autonomous effects of presenilin 1 variants on enrichment-mediated hippocampal progenitor cell proliferation and differentiation. Neuron 59(4):568-80.

Christian K, Song H, Ming GL (2010) Adult neurogenesis as a cellular model to study schizophrenia. Cell Cycle 9(4):636-7.

Claiborne BJ, Amaral DG, Cowan WM (1986) A light and electron microscopic analysis of the mossy fibers of the rat dentate gyrus. J Comp Neurol 246(4):435-58.

Collinge J, Whittington MA, Sidle KC, Smith CJ, Palmer MS, Clarke AR, Jefferys JG (1994) Prion protein is necessary for normal synaptic function. Nature 370(6487):295-7.

Contractor A, Rogers C, Maron C, Henkemeyer M, Swanson GT, Heinemann SF (2002) Trans-synaptic Eph receptor-ephrin signaling in hippocampal mossy fiber LTP. Science 296(5574):1864-9.

Contractor A, Swanson G, Heinemann SF (2001) Kainate receptors are involved in short- and long-term plasticity at mossy fiber synapses in the hippocampus. Neuron 29(1):209-16.

Contractor A, Swanson GT, Sailer A, O'Gorman S, Heinemann SF (2000) Identification of the kainate receptor subunits underlying modulation of excitatory synaptic transmission in the CA3 region of the hippocampus. J Neurosci 20(22):8269-78.

Couve A, Moss SJ, Pangalos MN (2000) GABAB receptors: a new paradigm in G protein signaling. Mol Cell Neurosci 16:296-312.

Dammerman RS, Flint AC, Noctor S, Kriegstein AR (2000) An excitatory GABAergic plexus in developing neocortical layer 1. J Neurophysiol 84:428-34.

Dan Y, Poo MM (2006) Spike timing-dependent plasticity: from synapse to perception. Physiol Rev 86(3):1033-48.

Danzer SC (2012), Depression, stress, epilepsy and adult neurogenesis. Exp Neurol 233(1):22-32. Epub 2011 Jun 12.

Demarque M, Represa A, Becq H, Khalilov I, Ben-Ari Y, Aniksztejn L (2002) Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation. Neuron 36:1051-61.

Dobrunz LE, Stevens CF (1999) Response of hippocampal synapses to natural stimulation patterns. Neuron 22(1):157-66.

Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H (2007)

Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. Cell 130(6):1146-58.

Dudek FE, Sutula TP (2007) Epileptogenesis in the dentate gyrus: a critical perspective. Prog Brain Res 163:755-73.

Durand GM, Kovalchuk Y, Konnerth A (1996) Long-term potentiation and functional synapse induction in developing hippocampus. Nature 381:71-5.

Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ (2005) NKCC1 transporter facilitates seizures in the developing brain. Nat Med 11:1205-13.

Eilers J, Plant TD, Marandi N, Konnerth A (2001) GABA-mediated Ca2+ signalling in developing rat cerebellar Purkinje neurones. J Physiol 536:429-37.

Ellgren M, Artmann A, Tkalych O, Gupta A, Hansen HS, Hansen SH, Devi LA, Hurd YL (2008) Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. Eur Neuropsychopharmacol 18(11):826-34.

Espósito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, Pitossi FJ, Schinder AF (2005) Neuronal differentiation in the adult hippocampus recapitulates embryonic development. J Neurosci 25(44):10074-86.

Faulkner RL, Jang MH, Liu XB, Duan X, Sailor KA, Kim JY, Ge S, Jones EG, Ming GL, Song H, Cheng HJ (2008) Development of hippocampal mossy fiber synaptic outputs by new neurons in the adult brain. Proc Natl Acad Sci U S A 105(37):14157-62.

Feller MB, Butts DA, Aaron HL, Rokhsar DS, Shatz CJ (1997) Dynamic processes shape spatiotemporal properties of retinal waves. Neuron 19:293-306.

Feng R, Rampon C, Tang YP, Shrom D, Jin J, Kyin M, Sopher B, Miller MW, Ware CB, Martin GM, Kim SH, Langdon RB, Sisodia SS, Tsien JZ (2001) Deficient neurogenesis in forebrain-specific presenilin-1 knockout mice is associated with reduced clearance of hippocampal memory traces. Neuron 32(5):911-26.

Fournier JG, Escaig-Haye F, Billette de Villemeur T, Robain O (1995) Ultrastructural localization of cellular prion protein (PrPc) in synaptic boutons of normal hamster hippocampus. C R Acad Sci III 318(3):339-44.

Frerking M, Ohliger-Frerking P (2002) AMPA receptors and kainate receptors encode different features of afferent activity. J Neurosci 22(17):7434-43.

Freund TF, Katona I, Piomelli D (2003) Role of endogenous cannabinoids in synaptic signaling. Physiol Rev. 2003 Jul;83(3):1017-66.

Gaarskjaer FB (1985) The development of the dentate area and the hippocampal mossy fiber projection of the rat. J Comp Neurol 241(2):154-70.

Gage FH (2000) Mammalian neural stem cells. Science 287(5457):1433-8.

Gaiarsa JL, Tseeb V, Ben-Ari Y (1995) Postnatal development of pre- and postsynaptic GABAB-mediated inhibitions in the CA3 hippocampal region of the rat. J Neurophysiol. 1995 Jan;73(1):246-55.

Gall C (1984) The distribution of cholecystokinin-like immunoreactivity in the hippocampal formation of the guinea pig: localization in the mossy fibers. Brain Res 306(1-2):73-83.

Galve-Roperh I, Harkany T (2008) Endocannabinoid signaling controls pyramidal cell specification and long-range axon patterning. Proc Natl Acad Sci U S A 105(25):8760-5.

Garaschuk O, Hanse E, Konnerth A (1998) Developmental profile and synaptic origin of early network oscillations in the CA1 region of rat neonatal hippocampus. J Physiol 507:219-36.

Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589-93.

Ge S, Sailor KA, Ming GL, Song H (2008) Synaptic integration and plasticity of new neurons in the adult hippocampus. J Physiol 586(16):3759-65.

Ge S, Yang CH, Hsu KS, Ming GL, Song H (2006) A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron 54(4):559-66.

Geiger JR, Jonas P (2000) Dynamic control of presynaptic Ca(2+) inflow by fast-inactivating K(+) channels in hippocampal mossy fiber boutons. Neuron 28(3):927-39.

Geiger JR, Lübke J, Roth A, Frotscher M, Jonas P (1997) Submillisecond AMPA receptor-mediated signaling at a principal neuron-interneuron synapse. Neuron 18(6):1009-23.

Gilbert PE, Kesner RP, Lee I (2001) Dissociating hippocampal subregions: double dissociation between dentate gyrus and CA1. Hippocampus 11(6):626-36.

Gillespie DC, Kim G, Kandler K (2005) Inhibitory synapses in the developing auditory system are glutamatergic. Nat Neurosci. 2005 Mar;8(3):332-8.

Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH (2010) Mechanisms underlying inflammation in neurodegeneration. Cell 140(6):918-34.

Gloveli T, Schmitz D, Heinemann U (1998) Interaction between superficial layers of the entorhinal cortex and the hippocampus in normal and epileptic temporal lobe. Epilepsy Res 32(1-2):183-93.

Golgi C (1886) Sulla fina anatomia degli organi centrali del sistema nervoso. U. Hoepli, Milano.

Gubellini P, Ben-Ari Y, Gaiarsa JL (2005) Endogenous neurotrophins are required for the induction of GABAergic long-term potentiation in the neonatal rat hippocampus. J Neurosci 25:5796-802.

Gutiérrez R (2002) Activity-dependent expression of simultaneous glutamatergic and GABAergic neurotransmission from the mossy fibers in vitro. J Neurophysiol 87(5):2562-70.
Gutierrez R (2005) The dual glutamatergic-GABAergic phenotype of hippocampal granule cells. Trends Neurosci 28:297-303.

Gutiérrez R, Heinemann U (2001) Kindling induces transient fast inhibition in the dentate gyrus-CA3 projection. Eur J Neurosci 13(7):1371-9.

Gutierrez R, Romo-Parra H, Maqueda J, Vivar C, Ramirez M, Morales MA, Lamas M (2003) Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 23:5594-8.

Gutierrez R, Romo-Parra H, Maqueda J, Vivar C, Ramirez M, Morales MA, Lamas M (2003) Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 23:5594-8.

Hafting T, Fyhn M, Molden S, Moser MB, Moser EI (2005) Microstructure of a spatial map in the entorhinal cortex. Nature 436(7052):801-6.

Hahn TT, Sakmann B, Mehta MR (2007) Differential responses of hippocampal subfields to cortical up-down states. Proc Natl Acad Sci U S A 104(12):5169-74.

Harkany T, Guzmán M, Galve-Roperh I, Berghuis P, Devi LA, Mackie K (2007) The emerging functions of endocannabinoid signaling during CNS development. Trends Pharmacol Sci 28(2):83-92.

Harkany T, Mackie K, Doherty P (2008) Wiring and firing neuronal networks: endocannabinoids take center stage. Curr Opin Neurobiol 18(3):338-45.

Hasselmo ME, Schnell E, Barkai E (1995) Dynamics of learning and recall at excitatory recurrent synapses and cholinergic modulation in rat hippocampal region CA3. J Neurosci 15(7 Pt 2):5249-62.

Hattiangady B, Rao MS, Shetty AK (2004) Chronic temporal lobe epilepsy is associated with severely declined dentate neurogenesis in the adult hippocampus. Neurobiol Dis 17(3):473-90.

Hattiangady B, Shetty AK (2010) Decreased neuronal differentiation of newly generated cells underlies reduced hippocampal neurogenesis in chronic temporal lobe epilepsy. Hippocampus 20(1):97-112.

Heinemann U, Beck H, Dreier JP, Ficker E, Stabel J, Zhang CL (1992) The dentate gyrus as a regulated gate for the propagation of epileptiform activity. Epilepsy Res Suppl 7:273-80.

Hemond P, Epstein D, Boley A, Migliore M, Ascoli GA, Jaffe DB (2008) Distinct classes of pyramidal cells exhibit mutually exclusive firing patterns in hippocampal area CA3b. Hippocampus 18(4):411-24.

Heng L, Beverley JA, Steiner H, Tseng KY (2011) Differential developmental trajectories for CB1 cannabinoid receptor expression in limbic/associative and sensorimotor cortical areas. Synapse 65(4):278-86. doi: 10.1002/syn.20844.

Heng L, Beverley JA, Steiner H, Tseng KY (2011) Differential developmental trajectories for CB1 cannabinoid receptor expression in limbic/associative and sensorimotor cortical areas. Synapse 65(4):278-86. doi: 10.1002/syn.20844.

Hennou S, Khalilov I, Diabira D, Ben-Ari Y, Gozlan H (2002) Early sequential formation of functional GABA(A) and glutamatergic synapses on CA1 interneurons of the rat foetal hippocampus. Eur J Neurosci 16:197-208.

Henze DA, Urban NN, Barrionuevo G (2000) The multifarious hippocampal mossy fiber pathway: a review. Neuroscience 98:407-27.

Henze DA, Wittner L, Buzsáki G (2002) Single granule cells reliably discharge targets in the hippocampal CA3 network in vivo. Nat Neurosci 5(8):790-5.

Hofmann ME, Nahir B, Frazier CJ (2008) Excitatory afferents to CA3 pyramidal cells display differential sensitivity to CB1 dependent inhibition of synaptic transmission. Neuropharmacology 55(7):1140-6.

Hosokawa Y, Sciancalepore M, Stratta F, Martina M, Cherubini E (1994) Developmental changes in spontaneous GABAA-mediated synaptic events in rat hippocampal CA3 neurons. Eur J Neurosci 6:805-13.

Jaffe DB, Gutiérrez R (2007) Mossy fiber synaptic transmission: communication from the dentate gyrus to area CA3. Prog Brain Res 163:109-32.

Jakubs K, Nanobashvili A, Bonde S, Ekdahl CT, Kokaia Z, Kokaia M, Lindvall O (2006) Environment matters: synaptic properties of neurons born in the epileptic adult brain develop to reduce excitability. Neuron 52(6):1047-59.

Jang IS, Nakamura M, Ito Y, Akaike N (1996) Presynaptic GABAA receptors facilitate spontaneous glutamate release from presynaptic terminals on mechanically dissociated rat CA3 pyramidal neurons. Neuroscience 138(1):25-35.

Jonas P, Bischofberger J, Sandkühler J (1998) Corelease of two fast neurotransmitters at a central synapse. Science 281(5375):419-24.

Jonas P, Major G, Sakmann B (1993) Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. J Physiol 472:615-63.

Jonas P, Major G, Sakmann B (1993) Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. J Physiol 472:615-63.

Jung MW, McNaughton BL (1993) Spatial selectivity of unit activity in the hippocampal granular layer. Hippocampus 3(2):165-82.

Kamiya H, Ozawa S (1999) Dual mechanism for presynaptic modulation by axonal metabotropic glutamate receptor at the mouse mossy fibre-CA3 synapse. J Physiol 518 (Pt 2):497-506.

Kamiya H, Ozawa S, Manabe T (2002) Kainate receptor-dependent short-term plasticity of presynaptic Ca2+ influx at the hippocampal mossy fiber synapses. J Neurosci 22:9237-9243.

Kamiya H, Shinozaki H, Yamamoto C (1996) Activation of metabotropic glutamate receptor type 2/3 suppresses transmission at rat hippocampal mossy fibre synapses. J Physiol 493 (Pt 2):447-55.

Kano M, Ohno-Shosaku T, Hashimotodani Y, Uchigashima M, Watanabe M (2009) Endocannabinoid-mediated control of synaptic transmission. Physiol Rev 89(1):309-80.

Kasyanov AM, Safiulina VF, Voronin LL, Cherubini E (2004) GABA-mediated giant depolarizing potentials as coincidence detectors for enhancing synaptic efficacy in the developing hippocampus. Proc Natl Acad Sci U S A. 101:3967-72.

Katona I, Urbán GM, Wallace M, Ledent C, Jung KM, Piomelli D, Mackie K, Freund TF (2006) Molecular composition of the endocannabinoid system at glutamatergic synapses. J Neurosci 26(21):5628-37.

Khazipov R, Esclapez M, Caillard O, Bernard C, Khalilov I, Tyzio R, Hirsch J, Dzhala V, Berger B, Ben-Ari Y (2001) Early development of neuronal activity in the primate hippocampus in utero. J Neurosci 21:9770-81.

Khirug S, Huttu K, Ludwig A, Smirnov S, Voipio J, Rivera C, Kaila K, Khiroug L (2005) Distinct properties of functional KCC2 expression in immature mouse hippocampal neurons in culture and in acute slices. Eur J Neurosci 21:899-904.

Kobayashi K (2009), Targeting the hippocampal mossy fiber synapse for the treatment of psychiatric disorders. Mol Neurobiol 39(1):24-36.

Kobayashi K, Manabe T, Takahashi T (1996) Presynaptic long-term depression at the hippocampal mossy fiber-CA3 synapse. Science 273(5275):648-50.

Komuro H, Rakic P (1998) Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca2+ fluctuations. J Neurobiol 37:110-30.

Kreitzer AC, Regehr WG (2002) Retrograde signaling by endocannabinoids. Curr Opin Neurobiol 12(3):324-30.

Kron MM, Zhang H, Parent JM (2010) The developmental stage of dentate granule cells dictates their contribution to seizure-induced plasticity. J Neurosci 30(6):2051-9.

Kuczewski N, Langlois A, Fiorentino H, Bonnet S, Marissal T, Diabira D, Ferrand N, Porcher C, Gaiarsa JL (2008) Spontaneous glutamatergic activity induces a BDNF-dependent potentiation of GABAergic synapses in the newborn rat hippocampus. J Physiol 586:5119-28.

Lanthorn TH, Ganong AH, Cotman CW (1984) 2-Amino-4-phosphonobutyrate selectively blocks mossy fiber-CA3 responses in guinea pig but not rat hippocampus. Brain Res 290(1):174-8.

Lassalle JM, Bataille T, Halley H (2000) Reversible inactivation of the hippocampal mossy fiber synapses in mice impairs spatial learning, but neither consolidation nor memory retrieval, in the Morris navigation task. Neurobiol Learn Mem 73(3):243-57.

Lauri SE, Bortolotto ZA, Bleakman D, Ornstein PL, Lodge D, Isaac JT, Collingridge GL (2001) A critical role of a facilitatory presynaptic kainate receptor in mossy fiber LTP. Neuron 32(4):697-709.

Lauri SE, Bortolotto ZA, Nistico R, Bleakman D, Ornstein PL, Lodge D, Isaac JT, Collingridge GL (2003a) A role for Ca2+ stores in kainate receptor-dependent synaptic facilitation and LTP at mossy fiber synapses in the hippocampus. Neuron 39(2):327-41.

Lauri SE, Lamsa K, Pavlov I, Riekki R, Johnson BE, Molnar E, Rauvala H, Taira T (2003b) Activity blockade increases the number of functional synapses in the hippocampus of newborn rats. Mol Cell Neurosci 22(1):107-17.

Lauri SE, Vesikansa A, Segerstråle M, Collingridge GL, Isaac JT, Taira T (2006) Functional maturation of CA1 synapses involves activity-dependent loss of tonic kainate receptor-mediated inhibition of glutamate release. Neuron 50(3):415-29.

Lawrence JJ, Grinspan ZM, McBain CJ (2004) Quantal transmission at mossy fibre targets in the CA3 region of the rat hippocampus. J Physiol 554(Pt 1):175-93.

Lee I, Kesner RP (2004) Encoding versus retrieval of spatial memory: double dissociation between the dentate gyrus and the perforant path inputs into CA3 in the dorsal hippocampus. Hippocampus 14(1):66-76.

Lei S, McBain CJ (2003) GABA B receptor modulation of excitatory and inhibitory synaptic transmission onto rat CA3 hippocampal interneurons. J Physiol 546(Pt 2):439-53.

Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R (1997) Ca2+ oscillations mediated by the synergistic excitatory actions of GABA(A) and NMDA receptors in the neonatal hippocampus. Neuron 18:243-55.

Lerma J (2003) Roles and rules of kainate receptors in synaptic transmission. Nat Rev Neurosci 4(6):481-95.

Lerma J (2006) Kainate receptor physiology. Curr Opin Pharmacol 6(1):89-97.

Leutgeb JK, Leutgeb S, Moser MB, Moser EI (2007) Pattern separation in the dentate gyrus and CA3 of the hippocampus. Science 315(5814):961-6.

Lorente de Nó R (1934) Studies of the structure of the cerebral cortex. II. Continuation of the study of the ammonic system. J. Psychol Neurol 46: 113-177.

LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR (1995) GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 15:1287-98.

Lovinger DM (2010) Neurotransmitter roles in synaptic modulation, plasticity and learning in the dorsal striatum. Neuropharmacology 58(7):951-61.

Luhmann HJ, Prince DA (1991) Postnatal maturation of the GABAergic system in rat neocortex. J Neurophysiol 65:247-63.

Luscher C, Jan LY, Stoffel M, Malenka RC, Nicoll RA (1997) G protein-coupled inwardly rectifying K+ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. Neuron 19:687-95.

Maggi L, Sola E, Minneci F, Le Magueresse C, Changeux JP, Cherubini E (2004) Persistent decrease in synaptic efficacy induced by nicotine at Schaffer collateral-CA1 synapses in the immature rat hippocampus. J Physiol 559(Pt 3):863-74. Epub 2004 Jul 22.

Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J (1992) The prion protein gene: a role in mouse embryogenesis? Development 115(1):117-22.

Marchal C, Mulle C (2004) Postnatal maturation of mossy fibre excitatory transmission in mouse CA3 pyramidal cells: a potential role for kainate receptors. J Physiol 561:27-37.

Marchionni I, Omrani A, Cherubini E (2007) In the developing rat hippocampus a tonic GABAA-mediated conductance selectively enhances the glutamatergic drive of principal cells. J Physiol 581:515-28.

Maric D, Liu QY, Maric I, Chaudry S, Chang YH, Smith SV, Sieghart W, Fritschy JM, Barker JL (2001) GABA expression dominates neuronal lineage progression in the embryonic rat neocortex and facilitates neurite outgrowth via GABA(A) autoreceptor/Cl- channels. J Neurosci 21:2343-60.

Marín-Burgin A, Mongiat LA, Pardi MB, Schinder AF (2012) Unique processing during a period of high excitation/inhibition balance in adult-born neurons. Science 335(6073):1238-42. Epub 2012 Jan 26.

Marr D (1971) Simple memory: a theory for archicortex. Philos Trans R Soc Lond B Biol Sci 262(841):23-81.

Marsicano G, Lutz B (1999) Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. Eur J Neurosci 11(12):4213-25.

McGinty JF, Henriksen SJ, Goldstein A, Terenius L, Bloom FE (1983) Dynorphin is contained within hippocampal mossy fibers: immunochemical alterations after kainic acid administration and colchicine-induced neurotoxicity. Proc Natl Acad Sci U S A 80(2):589-93.

McNaughton BL, Barnes CA, O'Keefe J (1983) The contributions of position, direction, and velocity to single unit activity in the hippocampus of freely-moving rats. Exp Brain Res 52(1):41-9.

McNaughton, BL, and Morris RGM (1987) Hippocampal synaptic enhancement and information storage within a distributed memory system. Trends Neurosci 10, 408-415.

Mellor J, Nicoll RA (2001) Hippocampal mossy fiber LTP is independent of postsynaptic calcium. Nat Neurosci 4(2):125-6.

Menendez de la Prida L, Sanchez-Andres JV (2000) Heterogeneous populations of cells mediate spontaneous synchronous bursting in the developing hippocampus through a frequency-dependent mechanism. Neuroscience 97:227-41.

Mercer A, Trigg HL, Thomson AM (2007) Characterization of neurons in the CA2 subfield of the adult rat hippocampus. J Neurosci 27(27):7329-38.

Miles R, Tóth K, Gulyás AI, Hájos N, Freund TF (1996) Differences between somatic and dendritic inhibition in the hippocampus. Neuron 16(4):815-23.

Miles R, Wong RK (1983) Single neurones can initiate synchronized population discharge in the hippocampus. Nature 306(5941):371-3.

Min MY, Rusakov DA, Kullmann DM (1998) Activation of AMPA, kainate, and metabotropic receptors at hippocampal mossy fiber synapses: role of glutamate diffusion. Neuron 21(3):561-70.

Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-50.

Mintz IM, Bean BP (1993) GABAB receptor inhibition of P-type Ca2+ channels in central neurons. Neuron 10:889-98.

Mironov A Jr, Latawiec D, Wille H, Bouzamondo-Bernstein E, Legname G, Williamson RA, Burton D, DeArmond SJ, Prusiner SB, Peters PJ (2003) Cytosolic prion protein in neurons. J Neurosci 23(18):7183-93.

Mohajerani MH, Cherubini E (2005) Spontaneous recurrent network activity in organotypic rat hippocampal slices. Eur J Neurosci 22:107-18.

Mohajerani MH, Sivakumaran S, Zacchi P, Aguilera P, Cherubini E (2007) Correlated network activity enhances synaptic efficacy via BDNF and the ERK pathway at immature CA3 CA1 connections in the hippocampus. Proc Natl Acad Sci U S A 104(32):13176-81.

Mongiat LA, Schinder AF (2011) Adult neurogenesis and the plasticity of the dentate gyrus network. Eur J Neurosci 33(6):1055-61.

Moore KA, Nicoll RA, Schmitz D (2003) Adenosine gates synaptic plasticity at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 100(24):14397-402.

Mori M, Abegg MH, Gähwiler BH, Gerber U A frequency-dependent switch from inhibition to excitation in a hippocampal unitary circuit. Nature 431(7007):453-6.

Mulder J, Aguado T, Keimpema E, Barabás K, Ballester Rosado CJ, Nguyen L, Monory K, Marsicano G, Di Marzo V, Hurd YL, Guillemot F, Mackie K, Lutz B, Guzmán M, Lu HC, Nadler JV (2003) The recurrent mossy fiber pathway of the epileptic brain. Neurochem Res 28(11):1649-58.

Nakamura M, Sekino Y, Manabe T (2007) GABAergic interneurons facilitate mossy fiber excitability in the developing hippocampus. J Neurosci 27(6):1365-73.

Nicoll RA, Schmitz D (2005) Synaptic plasticity at hippocampal mossy fibre synapses. Nat Rev Neurosci 6(11):863-76.

Nikolenko V, Poskanzer KE, Yuste R (2007) Two-photon photostimulation and imaging of neural circuits. Nat Methods 4(11):943-50.

Nissant A, Bardy C, Katagiri H, Murray K, Lledo PM (2009) Adult neurogenesis promotes synaptic plasticity in the olfactory bulb. Nat Neurosci 12(6):728-30.

O'Donovan MJ (1999) The origin of spontaneous activity in developing networks of the vertebrate nervous system. Curr Opin Neurobiol 9:94-104.

O'Keefe J, Dostrovsky J (1971) The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Res 34(1):171-5.

O'Malley DM, Masland RH (1989) Co-release of acetylcholine and gamma-aminobutyric acid by a retinal neuron. Proc Natl Acad Sci U S A 86(9):3414-8.

O'Reilly RC, McClelland JL (1994) Hippocampal conjunctive encoding, storage, and recall: avoiding a trade-off. Hippocampus 4(6):661-82.

Overstreet-Wadiche LS, Bensen AL, Westbrook GL (2006a) Delayed development of adult-generated granule cells in dentate gyrus. J Neurosci 26(8):2326-34.

Overstreet-Wadiche LS, Westbrook GL (2006b) Functional maturation of adult-generated granule cells. Hippocampus 16(3):208-15.

Owens DF, Boyce LH, Davis MB, Kriegstein AR (1996) Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci 16:6414-23.

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-27.

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-27.

Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH (2006) Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. Ann Neurol 59(1):81-91.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17(10):3727-38.

Pathak HR, Weissinger F, Terunuma M, Carlson GC, Hsu FC, Moss SJ, Coulter DA (2007) Disrupted dentate granule cell chloride regulation enhances synaptic excitability during development of temporal lobe epilepsy. J Neurosci 27(51):14012-22.

Pierce JP, Melton J, Punsoni M, McCloskey DP, Scharfman HE (2005) Mossy fibers are the primary source of afferent input to ectopic granule cells that are born after pilocarpine-induced seizures. Exp Neurol 196(2):316-31.

Pinheiro PS, Lanore F, Veran J, Artinian J, Blanchet C, Crépel V, Perrais D, Mulle C (2012) Selective Block of Postsynaptic Kainate Receptors Reveals Their Function at Hippocampal Mossy Fiber Synapses. Cereb Cortex [Epub ahead of print].

Pinheiro PS, Perrais D, Coussen F, Barhanin J, Bettler B, Mann JR, Malva JO, Heinemann SF, Mulle C (2007) GluR7 is an essential subunit of presynaptic kainate autoreceptors at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 104(29):12181-6.

Poncer JC, McKinney RA, Gahwiler BH, Thompson SM (1997) Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. Neuron 18:463- 72.

Poncer JC, McKinney RA, Gähwiler BH, Thompson SM (1997) Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. Neuron 18(3):463-72.

Prusiner SB (1998) Prions. Proc Natl Acad Sci U S A 95(23):13363-83.

Quirk GJ, Muller RU, Kubie JL, Ranck JB Jr (1990) The positional firing properties of medial entorhinal neurons: description and comparison with hippocampal place cells. J Neurosci 12(5):1945-63.

Ramírez M, Gutiérrez R (2001) Activity-dependent expression of GAD67 in the granule cells of the rat hippocampus. Brain Res 917(2):139-46.

Ramón y Cajal S (1911) Histologie du Systeme Nerveux de l'Homme et des Vertebretes. Paris: A. Maloine.

Rao A, Cha EM, Craig AM (2000) Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. J Neurosci 20(22):8344-53.

Rebola N, Carta M, Lanore F, Blanchet C, Mulle C (2011) NMDA receptor-dependent metaplasticity at hippocampal mossy fiber synapses. Nat Neurosci 14(6):691-3.

Ribak CE, Tran PH, Spigelman I, Okazaki MM, Nadler JV (2000) Status epilepticus-induced hilar basal dendrites on rodent granule cells contribute to recurrent excitatory circuitry. J Comp Neurol 428(2):240-53.

Ritter LM, Vazquez DM, Meador-Woodruff JH (2002) Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. Brain Res Dev Brain Res 139(2):227-36.

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K (1999) The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-5.

Rodríguez-Moreno A, Sihra TS (2004) Presynaptic kainate receptor facilitation of glutamate release involves protein kinase A in the rat hippocampus. J Physiol 557(Pt 3):733-45.

Rodríguez-Moreno A, Sihra TS (2011) Kainate receptors. Novel signaling insights. Adv Exp Med Biol 717:vii-xi, xiii.

Ruiz A, Fabian-Fine R, Scott R, Walker MC, Rusakov DA, Kullmann DM (2003) GABAA receptors at hippocampal mossy fibers. Neuron 39(6):961-73.

Ruiz A, Sachidhanandam S, Utvik JK, Coussen F, Mulle C (2005) Distinct subunits in heteromeric kainate receptors mediate ionotropic and metabotropic function at hippocampal mossy fiber synapses. J Neurosci 25(50):11710-8.

Sachidhanandam S, Blanchet C, Jeantet Y, Cho YH, Mulle C (2009) Kainate receptors act as conditional amplifiers of spike transmission at hippocampal mossy fiber synapses. J Neurosci 29(15):5000-8.

Safiulina VF, Caiati MD, Sivakumaran S, Bisson G, Migliore M, Cherubini E (2010) Control of GABA Release at Mossy Fiber-CA3 Connections in the Developing Hippocampus. Front Synaptic Neurosci 2:1

Safiulina VF, Cherubini E (2009) At immature mossy fibers-CA3 connections, activation of presynaptic GABA(B) receptors by endogenously released GABA contributes to synapses silencing. Front Cell Neurosci 3:1.

Safiulina VF, Fattorini G, Conti F, Cherubini E (2006) GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. J Neurosci 26:597-608.

Safiulina VF, Kasyanov AM, Giniatullin R, Cherubini E (2005) Adenosine down-regulates giant depolarizing potentials in the developing rat hippocampus by exerting a negative control on glutamatergic inputs. J Neurophysiol 94(4):2797-804.

Safiulina VF, Zacchi P, Taglialatela M, Yaari Y, Cherubini E (2008) Low expression of v7/M channels facilitates intrinsic and network bursting in the developing rat hippocampus. J Physiol 586(Pt 22):5437-53.

Sala L (1891) Zur feineren Anatomie des grossen Seepferdefusses. Z. Swiss. Zool. 52, 18-45.
Salès N, Hässig R, Rodolfo K, Di Giamberardino L, Traiffort E, Ruat M, Frétier P, Moya KL (2002) Developmental expression of the cellular prion protein in elongating axons. Eur J Neurosci 15(7):1163-77.

Salin PA, Scanziani M, Malenka RC, Nicoll RA (1996) Distinct short-term plasticity at two excitatory synapses in the hippocampus. Proc Natl Acad Sci U S A 93:13304-9.

Sanabria ER, Su H, Yaari Y (2001) Initiation of network bursts by Ca2+-dependent intrinsic bursting in the rat pilocarpine model of temporal lobe epilepsy. J Physiol 532:205-16.

Sandler R, Smith AD (1991) Coexistence of GABA and glutamate in mossy fiber terminals of the primate hippocampus: an ultrastructural study. J Comp Neurol 303(2):177-92.

Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385(6617):630-4.

Scharfman HE, Goodman JH, Sollas AL (2000) Granule-like neurons at the hilar/CA3 border after status epilepticus and their synchrony with area CA3 pyramidal cells: functional implications of seizure-induced neurogenesis. J Neurosci 20(16):6144-58.

Scharfman HE, Sollas AE, Berger RE, Goodman JH, Pierce JP (2003) Perforant path activation of ectopic granule cells that are born after pilocarpine-induced seizures. Neuroscience 121(4):1017-29.

Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429(6988):184-7.

Schmitz D, Mellor J, Frerking M, Nicoll RA (2001) Presynaptic kainate receptors at hippocampal mossy fiber synapses. Proc Natl Acad Sci U S A 98(20):11003-8.

Schwarzer C, Sperk G (1995) Hippocampal granule cells express glutamic acid decarboxylase-67 after limbic seizures in the rat. Neuroscience 69(3):705-9.

Scott R, Lalic T, Kullmann DM, Capogna M, Rusakov DA (2008) Target-cell specificity of kainate autoreceptor and Ca2+-store-dependent short-term plasticity at hippocampal mossy fiber synapses. J Neurosci 28(49):13139-49.

Scott R, Rusakov DA (2006) Main determinants of presynaptic Ca2+ dynamics at individual mossy fiber-CA3 pyramidal cell synapses. J Neurosci 26(26):7071-81.

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. J Neurosci 30(19):6507-14.

Shao LR, Dudek FE (2011) Repetitive perforant-path stimulation induces epileptiform bursts in minislices of dentate gyrus from rats with kainate-induced epilepsy. J Neurophysiol 105(2):522-7.

Shapiro LA, Ribak CE (2006) Newly born dentate granule neurons after pilocarpine-induced epilepsy have hilar basal dendrites with immature synapses. Epilepsy Res 69(1):53-66.

Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17(19):7503-22.

Shimizu H, Fukaya M, Yamasaki M, Watanabe M, Manabe T, Kamiya H (2008) Use-dependent amplification of presynaptic Ca2+ signaling by axonal ryanodine receptors at the hippocampal mossy fiber synapse. Proc Natl Acad Sci U S A 105(33):11998-2003.

Simuni T, Sethi K (2008) Nonmotor manifestations of Parkinson's disease. Ann Neurol 64 Suppl 2:S65-80.

Sipila ST, Huttu K, Soltesz I, Voipio J, Kaila K (2005) Depolarizing GABA acts on intrinsically bursting pyramidal neurons to drive giant depolarizing potentials in the immature hippocampus. J Neurosci 25:5280-9.

Sipila ST, Schuchmann S, Voipio J, Yamada J, Kaila K (2006) The cation-chloride cotransporter NKCC1 promotes sharp waves in the neonatal rat hippocampus. J Physiol 573:765-73.

Sivakumaran S, Mohajerani MH, Cherubini E (2009) At immature mossy-fiber-CA3 synapses, correlated presynaptic and postsynaptic activity persistently enhances GABA release and network excitability via BDNF and cAMP-dependent PKA. J Neurosci 29:2637-47.

Sperk G, Marksteiner J, Gruber B, Bellmann R, Mahata M, Ortler M Functional changes in neuropeptide Y- and somatostatin-containing neurons induced by limbic seizures in the rat. Neuroscience 50(4):831-46.

Spitzer NC (2002) Activity-dependent neuronal differentiation prior to synapse formation: the functions of calcium transients. J Physiol Paris 96:73-80.

Squire LR (1992) Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychol Rev 99(2):195-231.

Steele AD, Emsley JG, Ozdinler PH, Lindquist S, Macklis JD (2006) Prion protein (PrPc) positively regulates neural precursor proliferation during developmental and adult mammalian neurogenesis. Proc Natl Acad Sci U S A 103(9):3416-21.

Szabo B, Schlicker E (2005) Effects of cannabinoids on neurotransmission. Handb Exp Pharmacol (168):327-65.

Tauck DL, Nadler JV (1985) Evidence of functional mossy fiber sprouting in hippocampal formation of kainic acid-treated rats. J Neurosci 5(4):1016-22.

Thompson SM, Haas HL, Gähwiler BH (1992) Comparison of the actions of adenosine at pre- and postsynaptic receptors in the rat hippocampus in vitro. J Physiol 451:347-63.

Toni N, Laplagne DA, Zhao C, Lombardi G, Ribak CE, Gage FH, Schinder AF (2008) Neurons born in the adult dentate gyrus form functional synapses with target cells. Nat Neurosci 11(8):901-7.

Toth K, Suares G, Lawrence JJ, Philips-Tansey E, McBain CJ (2000) Differential mechanisms of transmission at three types of mossy fiber synapse. J Neurosci 20(22):8279-89.

Treves A, Rolls ET (1992) Computational constraints suggest the need for two distinct input systems to the hippocampal CA3 network. Hippocampus 2(2):189-99.

Treves A, Rolls ET (1994) Computational analysis of the role of the hippocampus in memory. Hippocampus 4(3):374-91.

Tyzio R, Represa A, Jorquera I, Ben-Ari Y, Gozlan H, Aniksztejn L (1999) The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. J Neurosci 19:10372-82.

Tzounopoulos T, Janz R, Südhof TC, Nicoll RA, Malenka RC (1998) A role for cAMP in long-term depression at hippocampal mossy fiber synapses. Neuron 21(4):837-45.

Uchigashima M, Fukaya M, Watanabe M, Kamiya H (2007) Evidence against GABA release from glutamatergic mossy fiber terminals in the developing hippocampus. J Neurosci 27:8088-100.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-4.

Veeraraghavalu K, Choi SH, Zhang X, Sisodia SS (2010) Presenilin 1 mutants impair the self-renewal and differentiation of adult murine subventricular zone-neuronal progenitors via cell-autonomous mechanisms involving notch signaling. J Neurosci 30(20):6903-15.

Vignes M, Collingridge GL (1997) The synaptic activation of kainate receptors. Nature 388(6638):179-82.

Vogt KE, Nicoll RA (1999) Glutamate and gamma-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. Proc Natl Acad Sci U S A 96(3):1118-22.

Voronin LL, Cherubini E (2004) 'Deaf, mute and whispering' silent synapses: their role in synaptic plasticity. J Physiol 557:3-12.

Walker MC, Ruiz A, Kullmann DM (2001) Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. Neuron 29:703-15.

Walter C, Murphy BL, Pun RY, Spieles-Engemann AL, Danzer SC (2007) Pilocarpine-induced seizures cause selective time-dependent changes to adult-generated hippocampal dentate granule cells. J Neurosci 27(28):7541-52.

Wang DD, Kriegstein AR (2008) GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. J Neurosci 28:5547-58.

Wang J, Reichling DB, Kyrozis A, MacDermott AB (1994) Developmental loss of GABA- and glycine-induced depolarization and Ca2+ transients in embryonic rat dorsal horn neurons in culture. Eur J Neurosci 6:1275-80.

Weisskopf MG, Castillo PE, Zalutsky RA, Nicoll RA (1994) Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. Science 265(5180):1878-82.

Weisskopf MG, Zalutsky RA, Nicoll RA (1993) The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. Nature 362(6419):423-7.

Winner B, Kohl Z, Gage FH (2011) Neurodegenerative disease and adult neurogenesis. Eur J Neurosci 33(6):1139-51. doi: 10.1111/j.1460-9568.2011.07613.x.

Wisden W, Seeburg PH (1993) A complex mosaic of high-affinity kainate receptors in rat brain. J Neurosci 13(8):3582-98.

Wittner L, Miles R (2007) Factors defining a pacemaker region for synchrony in the hippocampus. J Physiol 584(Pt 3):867-83.

Yeckel MF, Kapur A, Johnston D (1999) Multiple forms of LTP in hippocampal CA3 neurons use a common postsynaptic mechanism. Nat Neurosci 2(7):625-33.

Yokoi M, Kobayashi K, Manabe T, Takahashi T, Sakaguchi I, Katsuura G, Shigemoto R, Ohishi H, Nomura S, Nakamura K, Nakao K, Katsuki M, Nakanishi S (1996) Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. Science 273(5275):645-7.

Yuste R, Katz LC (1991) Control of postsynaptic Ca2+ influx in developing neocortex by excitatory and inhibitory neurotransmitters. Neuron 6:333-44.

Zander JF, Münster-Wandowski A, Brunk I, Pahner I, Gómez-Lira G, Heinemann U, Gutiérrez R, Laube G, Ahnert-Hilger G (2010) Synaptic and vesicular coexistence of VGLUT and VGAT in selected excitatory and inhibitory synapses. J Neurosci 30(22):7634-45.

Zhan RZ, Timofeeva O, Nadler JV (2010) High ratio of synaptic excitation to synaptic inhibition in hilar ectopic granule cells of pilocarpine-treated rats. J Neurophysiol 104(6):3293-304.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. Neuron 40(5):971-82.

Zhao C, Teng EM, Summers RG Jr, Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci. 26(1):3-11.

ANNEX

frontiers in
SYNAPTIC NEUROSCIENCE

REVIEW ARTICLE
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Control of GABA release at mossy fiber-CA3 connections in the developing hippocampus

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In this review some of the recent work carried out in our laboratory concerning the functional role of GABAergic signalling at immature mossy fibres (MF)-CA3 principal cell synapses has been highlighted. While in adulthood MF, the axons of *dentate gyrus* granule cells release onto CA3 principal cells and interneurons glutamate, early in postnatal life they release GABA, which exerts into targeted cells a depolarizing and excitatory action. We found that $GABA_A$-mediated postsynaptic currents (MF-GPSCs) exhibited a very low probability of release, were sensitive to L-AP4, a group III metabotropic glutamate receptor agonist, and revealed short-term frequency-dependent facilitation. Moreover, MF-GPSCs were down regulated by presynaptic $GABA_B$ and kainate receptors, activated by spillover of GABA from MF terminals and by glutamate present in the extracellular medium, respectively. Activation of these receptors contributed to the low release probability and in some cases to synapses silencing. By pairing calcium transients, associated with network-driven giant depolarizing potentials or GDPs (a hallmark of developmental networks thought to represent a primordial form of synchrony between neurons), generated by the synergistic action of glutamate and GABA with MF activation increased the probability of GABA release and caused the conversion of silent synapses into conductive ones suggesting that GDPs act as coincident detector signals for enhancing synaptic efficacy. Finally, to compare the relative strength of CA3 pyramidal cell output in relation to their MF glutamatergic or GABAergic inputs in adulthood or in postnatal development, respectively, a realistic model was constructed taking into account different biophysical properties of these synapses.

Keywords: developing hippocampus, mossy fibres, $GABA_A$-mediated post synaptic currents, presynaptic $GABA_B$ receptors, presynaptic GluK1 receptors, activity-dependent plasticity, realistic modelling, quantal analysis

GABAergic PHENOTYPE OF HIPPOCAMPAL GRANULE CELLS

The axons of granule cells in the dentate gyrus, the mossy fibres (MFs) are part of the classical glutamatergic trisynaptic circuit in the hippocampus. They provide most of excitatory inputs to CA3 principal cells and GABAergic interneurons present in the *hilus* and in *stratum lucidum* (Frotscher et al., 2006). Other inputs to CA3 principal cells include fibres from the *enthorinal cortex* and from other CA3 pyramidal cells *via* recurrent collaterals. MFs were called "mossy" by Ramon y Cajal (Ramon y Cajal, 1911) because of their particular shape which is reminiscent of that of the moss on a tree. Thus, in adulthood they form large (3–5 μm diameter) *en passant* swellings and terminal expansions on CA3 principal neurons or hilar mossy cells, seen as giant boutons at the electron microscopic level. The presynaptic swellings adapt very well to specialized postsynaptic elements present on proximal dendrites of CA3 principal cells, called *thorny excrescences*. The MF synaptic complex contains multiple active zones (up to 50) associated with postsynaptic densities. Unlikely principal cells, GABAergic interneurons in *stratum lucidum*, which constitute the large majority of postsynaptic MF targets, are innervated by small *en passant* boutons or filopodial extensions comprising only one or few release sites (Acsády et al., 1998). The small number of release sites and the relatively high release probability endows these synapses with distinct functional properties as compared to MF-CA3 synapses (Nicoll and Schmitz, 2005; Bischofberger et al., 2006; McBain, 2008).

Interestingly, at principal cell synapses, giant boutons develop gradually during the first weeks of postnatal life. Light and electron microscopic studies have shown that immediately after birth immature axons terminate in very small spherical expansions which establish both symmetric and asymmetric contacts with proximal dendrites of pyramidal cells (Stirling and Bliss, 1978; Amaral and Dent, 1981). During this period, pyramidal cell dendrites show a marked lateral growth and fingers which began indenting into MF expansions. Moreover, at this developmental stage, the main neurotransmitter released from MF terminals is GABA which exerts into targeted cells a depolarizing and excitatory action (Safiulina et al., 2006; Sivakumaran et al., 2009).

In this paper we will review recent findings obtained in our laboratory on immature MF-CA3 connections highlighting also new data on GABAergic signalling at these synapses. In particular, we will discuss these results in view of their possible implications in network excitability and information processing.

EARLY IN POSTNATAL LIFE THE MAIN NEUROTRANSMITTER RELEASED FROM MOSSY FIBRE (MF) TERMINALS IS GABA

The presence of GABA in *stratum lucidum* was initially described by Ottersen and Storm-Mathisen (1984) who concluded that this neurotransmitter is stored in MF terminals together with glutamate. In addition, evidence has been provided that MFs express GAD65 and GAD67 (Schwarzer and Sperk, 1995; Sloviter et al., 1996) as well as the mRNA for the vesicular GABA transporter, VGAT (Lamas et al., 2001; Gómez-Lira et al., 2005). Moreover, hippocampal pyramidal neurons are able to express, in addition to glutamate, "mistargeted" $GABA_A$ receptors which, in particular conditions may become functional (Rao et al., 2000). This suggests that MFs can use GABA as a neurotransmitter since they posses all the machinery for synthesising, storing, releasing and sensing it. Indeed, in juvenile animals, it has been shown that stimulation of MFs elicits in CA3 pyramidal neurons AMPA and $GABA_A$-mediated synaptic currents (Walker et al., 2001; Gutierrez et al., 2003). In addition, immunogold experiments have demonstrated that AMPA and $GABA_A$ receptors are co-localized on the same synapse in close apposition to MF terminals (Bergersen et al., 2003). In adulthood, markers of the GABAergic phenotype disappear but they can be transiently expressed during seizures (Gutierrez and Heinemann, 2001; Romo-Parra et al., 2003). Here the release of GABA would counteract the enhanced excitability induced by epileptic activity (Jaffe and Gutiérrez, 2007).

In the immediate postnatal period, GABA appears to be the only neurotransmitter released from MF terminals. We used minimal stimulation of granule cells in the dentate gyrus to activate only one or a few presynaptic fibres (Jonas et al., 1993; Allen and Stevens, 1994). With this technique, a small stimulating electrode is placed on the granule cell layer and the stimulation intensity is decreased until only a single axon is activated. This is achieved when the mean amplitude of the postsynaptic currents and failure probability remain constant over a range of stimulus intensities near threshold for detecting a response. Small movements of the stimulating electrode 20–30 μm away from the initial location, lead to the loss of the evoked response. The example of **Figure 1A** (left) illustrates averaged traces (successes plus failures) of synaptic currents evoked in a CA3 principal cell in response to activation of granule cells in the *dentate gyrus* with different stimulation intensities. An abrupt increase in the mean peak amplitude of synaptic currents could be detected by increasing stimulation strength (**Figures 1A,B**; left).

This all or none behaviour suggests that only a single fibre was stimulated. In addition, the latency and the shape of individual synaptic responses remained constant for repeated stimuli and when the extracellular Ca^{2+}/Mg^{2+} concentration ratio was reduced from 2:1.3 to 1:3 further supporting the monosynaptic nature of synaptic currents (Safiulina et al., 2006). Unitary responses elicited

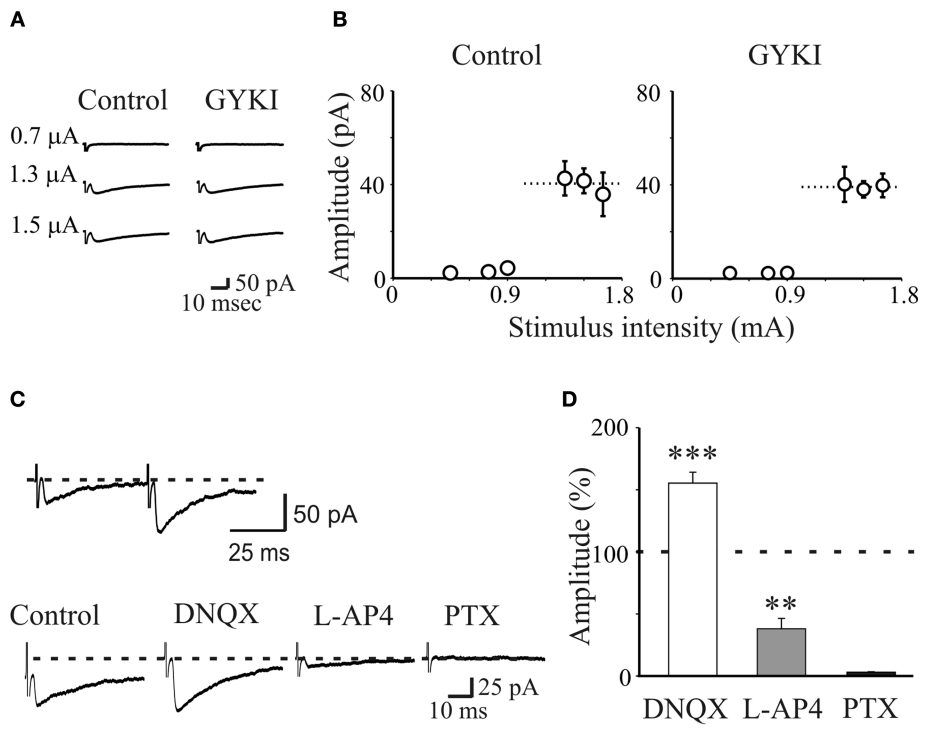

FIGURE 1 | AMPA receptors do not contribute to unitary postsynaptic currents evoked in CA3 principal cells by minimal stimulation of granule cells in the dentate gyrus. (A) Unitary synaptic currents evoked in a CA3 pyramidal cell at P4 with different stimulation intensities before (Control) and during application of GYKI 52466 (30 μM). Each trace is the average of 15–20 responses (including failures). **(B)** Peak amplitudes of synaptic currents represented in **(A)** are plotted as a function of stimulus intensities. Bars are SEM, dashed lines represent the averaged amplitude of GPSCs. **(C)** upper trace: MF-GPSCs evoked in a P3 principal cell by minimal stimulation of stratum granulosum in the dentate gyrus exhibit paired-pulse facilitation. Lower traces: MF-GPSCs recorded in the presence of GYKI were enhanced in amplitude by DNQX (50 μM), reduced by L-AP4 (10 μM) and abolished by PTX (100 μM). Each trace is the average of 30 traces. **(D)** Summary data obtained from 15 neurons. $^{**}p < 0.01$; $^{***}p < 0.001$. Modified from Caiati et al., 2010.

by stimulation of *stratum granulosum* in the dentate gyrus could be attributed to MF inputs since they exhibited strong paired-pulse facilitation in response to two stimuli applied 50 ms apart and short-term frequency-dependent facilitation (Salin et al., 1996; **Figures 1C and 2A,C**). However, while paired-pulse facilitation was a constant finding (many cells did not respond to the first stimulus but exhibited only few responses to the second one implying that they were "presynaptically" silent), the magnitude of frequency-dependent facilitation was dependent on the age of the animals. While at P2–P3 this was rather mild reaching at maximum 200% of controls, at P5–P6 it reached 400% (**Figure 2**).

MF-evoked synaptic currents were also identified on the basis of their sensitivity to group III mGluR agonist 2-amino-4-phosphonobutyric acid (L-AP4; **Figures 1C,D**). In this regard, neonatal rats behave differently from adult animals which are sensitive to (2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl) glycine (DCG-IV; Kamiya et al., 1996) but insensitive to L-AP4 (Lanthorn et al., 1984). It should be stressed however, that both group II and III mGluRs have been found on rat MF terminals: while group III mGluRs are located predominantly near presynaptic active zones, group II in preterminal rather than terminal portions of axons (Shigemoto et al., 1997). The inability of DCG-IV in modulating MF responses in immature neurons could be attributed to the different expression and/or location of group II/III mGluRs early in postnatal development.

MF-evoked unitary synaptic currents were insensitive to GYKI (30 μM) which at this concentration completely blocks AMPA receptors, indicating that activation of these receptors does not contribute to synaptic currents (**Figures 1A,B**). However, $GABA_A$-mediated postsynaptic currents (GPSCs) were reversibly enhanced by DNQX (50 μM; **Figures 1C,D**) which blocks both AMPA and kainate receptors. GPSCs were completely blocked by $GABA_A$ receptor antagonists picrotoxin (100 μM), bicuculline (10 μM) or gabazine (10 μM), demonstrating that indeed they were $GABA_A$-mediated (Safiulina et al., 2006; Sivakumaran et al., 2009; **Figures 1C,D**). As expected for GABA-mediated events, MF-GPSCs were potentiated by NO-711, a blocker of the GABA transporter GAT-1 and by flurazepam, an allosteric modulator of $GABA_A$ receptors (Safiulina et al., 2006). Moreover, pressure application of glutamate to granule cells dendrites in *stratum moleculare* (in the presence of DNQX to prevent the recruitment of GABAergic interneurons by glutamate) induced in targeted cells barrages of L-AP4 sensitive currents that were completely abolished by picrotoxin. It is therefore likely that NMDA-induced depolarization of granule cells dendrites causes

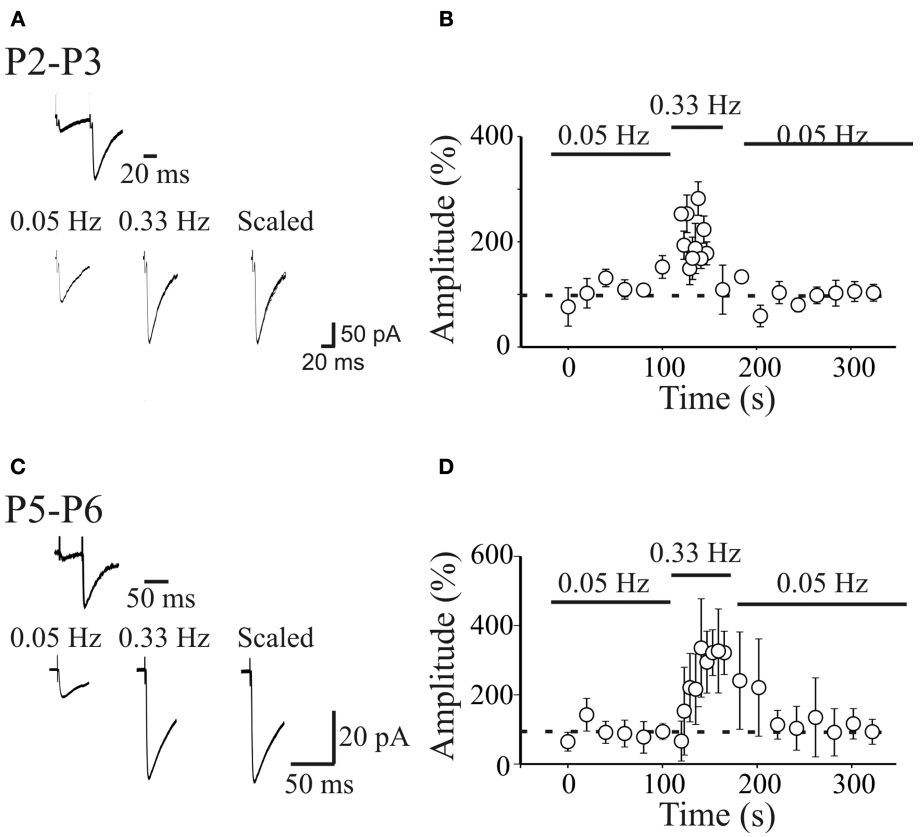

FIGURE 2 | Short-term frequency-dependent facilitation of MF-GPSCs. **(A)** Upper trace: MF-GPSCs exhibiting strong paired-pulse facilitation in response to two stimuli (50 ms apart) delivered to the granule cells in the dentate gyrus (P3). Lower traces represent averaged responses (including failures) obtained at 0.05 and 0.33 Hz from the same neuron. **(B)** The mean amplitude of synaptic currents evoked in 6 pyramidal cells at 0.05 and 0.33 Hz (bars) is plotted against time. Note the slow build-up of facilitation of synaptic responses at 0.33 Hz that completely reversed to control values after returning to 0.05-Hz stimulation. Bars represent SEM. **(C,D)** as in **(A,B)** but for neurons recorded at P5–P6. **(C,D)** modified from Safiulina et al., 2006.

the release of GABA from MF terminals. Blocking NMDA receptors with D-APV prevented the effects of chemical stimulation of granule cell dendrites on CA3 principal cells. Moving the pressure pipette few μm away towards the hilus to activate hilar interneurons caused barrage of synaptic currents that were insensitive to L-AP4 but were blocked by picrotoxin, implying that they were mediated by the release of GABA from GABAergic interneurons (Safiulina et al., 2006).

In contrast to MF-GPSCs, synaptic currents mediated by GABA released from GABAergic interneurons were insensitive to L-AP4 but were blocked by bicuculline or picrotoxin. They occurred with high probability and exhibited a strong paired-pulse depression in response to two closely spaced stimuli.

Towards P5–P6, additional fibres releasing both glutamate and GABA into CA3 principal cells could be recruited. These responses intermingled with failures and fluctuating between outwards, biphasic and inwards were sensitive to L-AP4 and were abolished by the respective receptor antagonists (Safiulina et al., 2006). The possibility of releasing different neurotransmitters from the same fibre has been well documented in the retina (O'Malley and Masland, 1989) and the spinal cord (Jonas et al., 1998). In particular, GABA has been reported to be released with glutamate from MF terminals of juvenile rats (Gutierrez et al., 2003) or guinea pigs (Walker et al., 2001) and glutamate from inhibitory terminals in the lateral superior olive of the developing auditory system (Gillespie et al., 2005).

GABA RELEASED FROM MF TERMINALS DEPOLARIZES PRINCIPAL CELLS

To evaluate whether GABA released from MFs exerts a depolarizing or a hyperpolarizing action in principal cells, $GABA_A$-mediated postsynaptic potentials (GPSPs) were recorded with gramicidin-perforated patch to preserve the anionic conditions of the recorded cells. Using this method, we found that on average GPSPs reversed at a potential more positive that the resting membrane potential (E_{GPSP}: −47.6 ± 3.3 mV; V_R: −56 ± 2.2 mV) indicating that GABA exerts a depolarizing action (Sivakumaran et al., 2009; see also Sipilä et al., 2006; Tyzio et al., 2007). To see whether accumulation of chloride inside the cell *via* the cation-chloride co-transporter NKCC1 was responsible for the depolarizing action of GABA, slices were incubated with bumetanide, a selective inhibitor of NKCC1 (Dzhala et al., 2005; Sipilä et al., 2006). Bumetanide caused a slight membrane hyperpolarization and a shift of E_{GPSP} towards values more negative than V_R (E_{GPSP}: −71.5 ± 3.09 mV; V_R: −63.6 ± 2.3 mV). These data demonstrate that at birth, due to the low expression of the KCC2 extruder, Cl^- accumulates inside the cell via NKCC1 leading to a membrane depolarization.

Interestingly, the value of E_{GPSP} was very close to the threshold for action potential generation (−49.3 ± 2.4 mV) suggesting that GABA may also be excitatory. To see whether synaptic stimulation of granule cells in the dentate gyrus could initiate action potentials in CA3 pyramidal neurons, experiments were performed in cell-attach mode to maintain the intracellular milieu of principal cells intact. Synaptic stimulation of MF in stratum lucidum elicited in 2/9 cases action potentials in CA3 principal cells (**Figure 3A**), an effect that was blocked by picrotoxin (100 μM) indicating that was mediated through the activation of $GABA_A$ receptors (**Figure 3A**). Furthermore, bath application of the GABA agonist isoguvacine (3 μM) to whole cell patched CA3 principal cells under current clamp conditions, increased the frequency of GDPs in seven out of ten CA3 principal cells (from 0.08 ± 0.02 Hz to 0.15 ± 0.04 Hz; $n = 7/10$; $p < 0.01$; **Figure 3B**). These results indicate that GABA released from MF terminals not only depolarizes CA3 principal cells but in some cases also excites them, thus overwhelming the concomitant shunting effect of this neurotransmitter (see also Tyzio et al., 2007).

Whether GABA is released from low threshold GABAergic cells (Uchigashima et al., 2007) which early in development are supposed to migrate to the upper and middle portions of the granule cell layer (Dupuy and Houser, 1997) or by glutamatergic cells synthesising GABA which would be downregulated in adulthood (Frahm and Draguhn, 2001) it remains to be elucidated. It is clear however, that in spite of their origin, synaptic responses elicited at birth by granule cells stimulation in the dentate gyrus maintain most of the functional properties of later appearing glutamatergic MF responses.

GABA RELEASE FROM MF TERMINALS IS REGULATED BY PRESYNAPTIC $GABA_B$ AND KAINATE RECEPTORS

In adulthood, MFs are endowed with a number of receptor types whose activation regulates glutamate release. These include metabotropic glutamate receptors (Kamiya et al., 1996),

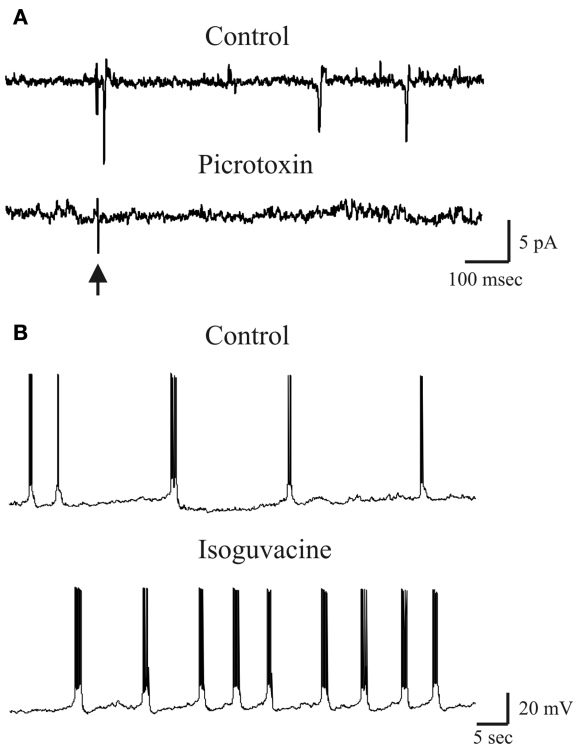

FIGURE 3 | Excitatory action of GABA exerted on a P3 CA3 pyramidal cells. (A) Stimulation of MF in stratum lucidum (arrow) evoked in a P3 CA3 pyramidal cell (recorded in cell-attached) an action potential that was abolished by picrotoxin (100 μM). **(B)** GDPs recorded from a P3 CA3 pyramidal neuron (whole cell under current-clamp conditions) before (control) and after bath application of Isoguvacine (3 μM). Note the increase in GDPs frequency in isoguvacine.

kainate receptors (Castillo et al., 1997; Vignes and Collingridge, 1997), adenosine receptors (Thompson et al., 1992), peptides (Weisskopf et al., 1993), $GABA_A$ (Trigo et al., 2008) and $GABA_B$ receptors (Min et al., 1998; Vogt and Nicoll, 1999). As already mentioned immature MF-GPSCs were reduced in amplitude by L-AP4, a group III mGluR agonist, and enhanced by DNQX (applied in the presence of the AMPA receptor antagonist GYKI) suggesting the involvement of mGlu and kainate receptors in their regulation.

The presence of ionotropic $GABA_A$ receptors on MF terminals has been unambiguously demonstrated by Alle and Geiger (2007) who, using patch clamp recordings from single MF boutons, detected $GABA_A$-activated currents following local application of GABA from a puff pipette. While in 3 to 4-weeks-old guinea pigs activation of presynaptic MF $GABA_A$ receptors by ambient GABA or GABA spillover from neighboring interneurons led to a reduction of MFs excitability (Ruiz et al., 2003), in juvenile rats led to a depolarization of MF terminals and to an increase in cell firing (Nakamura et al., 2007). Whether a similar effect may occur during the first week of postnatal life when MFs release GABA is unknown. However, experiments from immature glutamatergic Schaffer collateral-CA1 synapses have unveiled a decreased in cell excitability after bath application of picrotoxin, suggesting that tonic activation of presynaptic $GABA_A$ receptors localized on glutamatergic terminals may account for the observed effects (Marchionni et al., 2007).

In neonates, spillover of GABA from MF terminals down regulates its own release also *via* presynaptic $GABA_B$ receptors. In addition, evidence has been recently provided that GABA released can be down regulated by presynaptic kainate receptors activated by glutamate present in the extracellular space. Activation of both $GABA_B$ and kainate receptors may often lead to synapse silencing.

Synapses can be silent *via* both post and presynaptic mechanisms. "Postsynaptically" silent synapses are unable to detect the neurotransmitter release because they lack the respective receptors on the subsynaptic membrane (Malenka and Nicoll, 1997; Malinow et al., 2000; Kerchner and Nicoll, 2008). "Presynaptically" silent connections are those in which the probability of transmitter release or the concentration of the transmitter in the cleft is too low to produce a detectable quantal response (Voronin and Cherubini, 2004). Silent synapses are common during postnatal development in the hippocampus. However, while at glutamatergic synapses both post and presynaptically silent synapses have been observed (Durand et al., 1996; Gasparini et al., 2000; Montgomery et al., 2001), at GABAergic connections mainly presynaptically silent ones have been detected (Kannenberg et al., 1999; Kasyanov et al., 2004; Bekkers, 2005; Safiulina and Cherubini, 2009).

PRESYNAPTIC $GABA_B$ RECEPTORS

While postsynaptic $GABA_B$ receptors are poorly expressed at birth and start appearing towards the end of the first postnatal week, presynaptic $GABA_B$ receptors are already present and functional (Gaiarsa et al., 1995; Lei and McBain, 2003). As shown in **Figures 4A,B**, application of the selective $GABA_B$ receptor antagonist CGP55845 (1 μM) to a presynaptically silent neuron induced the appearance of synaptic responses. Overall, in ten neurons CGP55845 significantly increased the amplitude of GPSCs (from 6.6 ± 2.5 pA to 13.4 ± 3.5 pA; $p < 0.05$) and the successes rate (from 14.5 ± 5.7% to 41.5 ± 11.2%, $p < 0.05$). In addition, in seven non-silent cells, CGP55845 reduced the PPR (the ratio between the amplitude of the second response over the first, from 6.4 ± 1.9 to 3.1 ± 1.3 and increased the CV^{-2} from 0.3 ± 0.1 to 1.5 ± 0.4) in the absence of any change in holding current or membrane input resistance (**Figure 4E**, white columns). This indicates that activation of $GABA_B$ autoreceptors by spillover of GABA from MF terminals down regulates GABA release and contributes to switching off conductive synapses making them silent.

In contrast, the $GABA_B$ receptor agonist baclofen induced a powerful depression of MF-GPSCs, an effect that was associated with a significant increase in transmitter failures and in the paired-pulse ratio. Interestingly, the IC_{50} value for baclofen (400 nM) was at least one order of magnitude lower than that found at GABAergic synapses in CA3 stratum radiatum interneurons (Lei and McBain, 2003). This may be related to differences in $GABA_B$ receptors expression, distribution, and/or affinity among different cell types and suggests a target-specific modulation of GABAergic signaling by $GABA_B$ autoreceptors.

Furthermore, by increasing the concentration of GABA in the extracellular space by stimulating the MFs at 3–10 Hz or by blocking GABA uptake with NO711, a selective blocker of the GABA transporter GAT-1, resulted, in the majority of cases in synaptic depression that was rescued by CGP55845, further suggesting the involvement of $GABA_B$ receptors in reducing the probability of GABA release leading sometimes to synapses silencing (Safiulina and Cherubini, 2009).

PRESYNAPTIC KAINATE RECEPTORS

A recent study from our laboratory has provided evidence that at immature MF-CA3 synapses GABAergic signalling is modulated by presynaptic kainate receptors (Caiati et al., 2010). Among the kainate receptor subtypes expressed in the immature hippocampus GluK1 seems to exert a powerful control on transmitter release (Bettler et al., 1990; Bahn et al., 1994; Ritter et al., 2002; Lauri et al., 2006). In a previous work performed on hippocampal slices from neonatal animals, Lauri et al. (2005) have demonstrated that GluK1 contributes to modulate spontaneous, network-driven giant depolarizing potentials or GDPs, which are generated by the synergistic action of glutamate and GABA, that at this developmental stage is depolarizing and excitatory (Ben-Ari et al., 2007). Thus, we tested whether GluK1 can modulate GABA release from MF terminals. The selective GluK1 kainate receptor antagonist UBP 302 (10 μM), applied in the bath in the presence of GYKI (30 μM), reversibly enhanced the amplitude of GPSCs (from 48 ± 8 pA to 84 ± 15 pA; $n = 19$; $p = 0.001$) and the successes rate (from 0.4 ± 0.04 to 0.7 ± 0.03; $p = 0.001$). These effects were associated with a significant decrease in PPR (from 1.4 ± 0.2 to 0.6 ± 0.1; $p = 0.001$) and a significant increase in CV^{-2} (from 0.97 ± 0.2 to 3.3 ± 0.9; $p = 0.001$) indicating a presynaptic type of action (**Figure 4E**, black columns). In addition, in six "presynaptically" silent cases, UBP 302 induced the appearance of synaptic responses to the first stimulus suggesting that

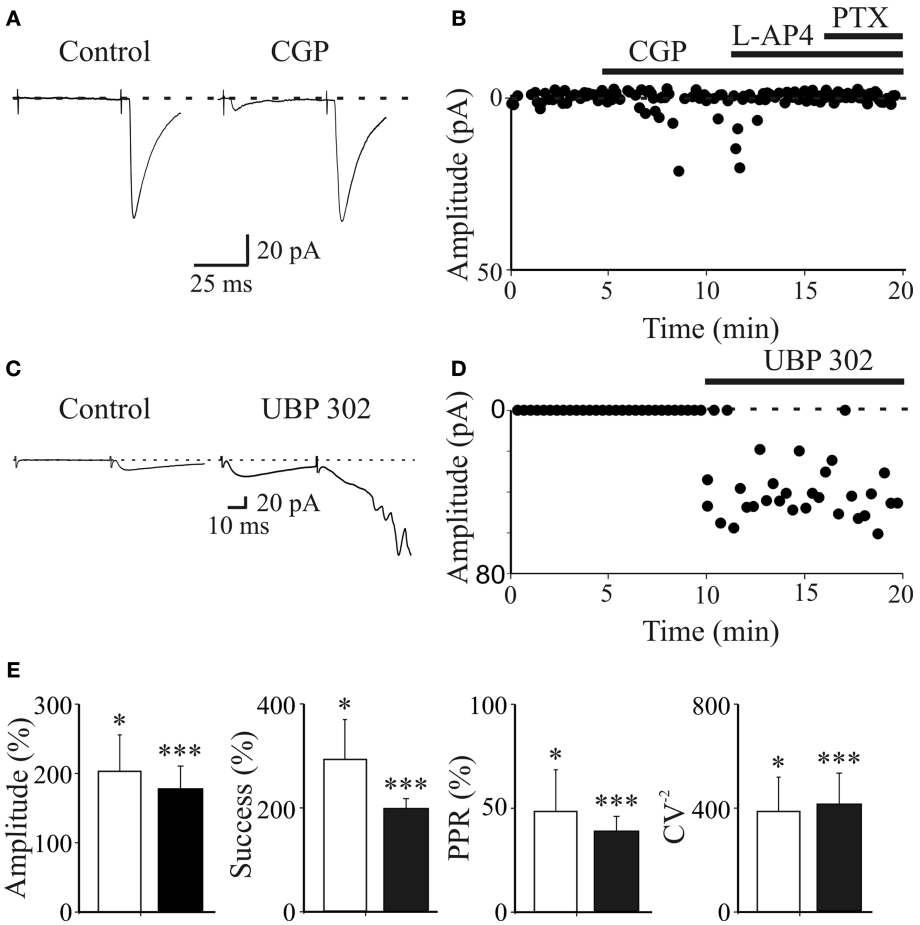

FIGURE 4 | Presynaptic $GABA_B$ and kainate receptors localized on MF terminals down regulate GABA release contributing to synapse silencing. **(A)** A "presynaptically" silent neuron before (left) and during bath application of CGP55845 (1 μM, right). Each trace is the average of 20 responses. Note the appearance of a synaptic current in the presence of CGP55845 (each trace is the average of 20 individual samples). **(B)** The peak amplitude of MF-GPSCs shown in A, before and during bath application of CGP55845, L-AP4 and PTX (closed bars) is plotted against time. **(C,D)** as in **(A,B)** but before and during bath application of UBP 302 (10 μM). Note that UBP 302 induced the appearance of the first response and enhanced the amplitude of the second one giving rise to an evoked GDP. **(E)** Each column in the graph represents the summary plot of peak amplitude, successes rate, PPR and inverse squared of CV of MF-GPSCs (expressed as percentage of controls) during CGP55845 (white columns; $n = 10$) or UBP 302 (black columns; $n = 19$). While amplitude and successes refer to both silent and non-silent neurons, PPR and CV^{-2} only to non-silent cells. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. **(A,B)** modified from Safiulina and Cherubini, 2009.

endogenous activation of GluK1 contributes to switching off conductive synapses (**Figures 4C,D**). The depression of MF-GPSCs by kainate receptors was not indirectly mediated *via* other signalling molecules known to inhibit GABA release, since the potentiating effect of the kainate antagonist on GPSCs persisted in the presence of $GABA_B$, nicotinic, muscarinic, P2Y and mGluR antagonists (Caiati et al., 2010).

One obvious question is how could presynaptic GluK1 receptors be activated if the main neurotransmitter released from MF is GABA? One possibility is that GluK1 receptors are activated by endogenous glutamate present in the extracellular space. A less efficient glutamate transport mechanism and a poorly developed diffusional barrier in neonates (Danbolt, 2001) would facilitate the maintenance of a relatively high glutamate concentration in the extracellular space. To test whether this was the case, we used an enzymatic glutamate scavenger system (Overstreet et al., 1997; Min et al., 1998) which catalyzes the conversion of glutamate and pyruvate to α-ketoglutarate and alanine. By enhancing the clearance of glutamate from the extracellular space, the scavenger prevented the activation of presynaptic kainate receptors and similarly to UBP 302 induced an increase in amplitude (from 44 ± 8 pA and 73 ± 10 pA) and in successes rate (from 0.5 ± 0.1 to 0.8 ± 0.1; $p = 0.01$) of GPSCs. Like UBP 302, this effect was associated with a significant decrease in PPR (from 1.5 ± 0.2 to 0.7 ± 0.1; $n = 7$; $p = 0.008$), and a significant increase in CV^{-2} (from 1.02 ± 0.2 to 3.4 ± 0.94; $n = 7$; $p = 0.01$), further suggesting a presynaptic site of action. In addition, the facilitating effect of the scavenger on GPSCs was fully occluded by subsequent application of UBP 302.

These data indicate that early in postnatal life, depression of GABA release from MF terminals occurs *via* activation of GluK1 receptors by endogenous glutamate present in the extracellular space.

CALCIUM TRANSIENTS ASSOCIATED WITH GDPs ACT AS A COINCIDENT DETECTOR SIGNALS FOR ENHANCING SYNAPTIC EFFICACY AT MF-CA3 SYNAPSES

Correlated network activity such as GDPs constitutes a hallmark of developmental networks, well preserved during evolution (Ben-Ari et al., 1989). GDPs which have been proposed to be the *in vitro* counterpart of "sharp waves" recorded in rat pups during immobility periods, sleep and feeding (Leinekugel et al., 2002) can be considered a primordial form of synchrony between neurons, which precedes more organized forms of activity such as the theta and the gamma rhythms (Buzsaki and Draguhn, 2004). GDPs and associated calcium signalling are crucial for several developmental processes including synaptogenesis (Kasyanov et al., 2004; Ben-Ari et al., 2007; Mohajerani et al., 2007).

To assess whether GDPs are able to modify MF-CA3 connections in an associative type of manner, we have developed a "pairing" procedure consisting in correlating calcium transients generated by GDPs with MF activation (Kasyanov et al., 2004). To this purpose, after a control period of 5–10 min, the patch was switched from voltage-clamp to current-clamp mode and MF-evoked GPSCs were paired for 5 min with GDPs.

"Pairing" consisted in triggering MF stimulation with the rising phases of GDPs (**Figures 5A,B**). After this period the patch was switched back to voltage-clamp mode and synaptic currents were recorded as in control for a prolonged period of time (at least 30 min). As illustrated in **Figures 5C,D**, in the case of presynaptically silent synapses (Gasparini et al., 2000), the pairing protocol caused the appearance of responses to the first stimulus and increased the number of successes to the second one. In the case of non-silent low probability synapses, the pairing procedure produced a strong and persistent potentiation of MF responses, which was associated with a significant increase in the number of successes and a significant reduction in the paired-pulse ratio, suggesting that an increased probability of transmitter release accounts for the persistent increase in synaptic efficacy.

When the interval between GDPs and MF stimulation was progressively increased, synaptic potentiation declined and reached control level 2–3 s after GDPs onset. In the absence of pairing, no significant changes in synaptic efficacy could be detected. Pairing-induced long-lasting changes in synaptic efficacy involved elevation of calcium *via* voltage-gated calcium channels because it was suppressed by nifedipine, that blocks L-type of voltage-dependent calcium channels or by loading the cell with BAPTA that chelates calcium ions (Kasyanov et al., 2004).

In conclusions, during development, coincident detection signals provided by GDPs could be crucial for enhancing synaptic transmission at emerging MF-CA3 connections. How this contributes to wiring assembly and to the establishment of appropriate synaptic connections remains to be elucidated.

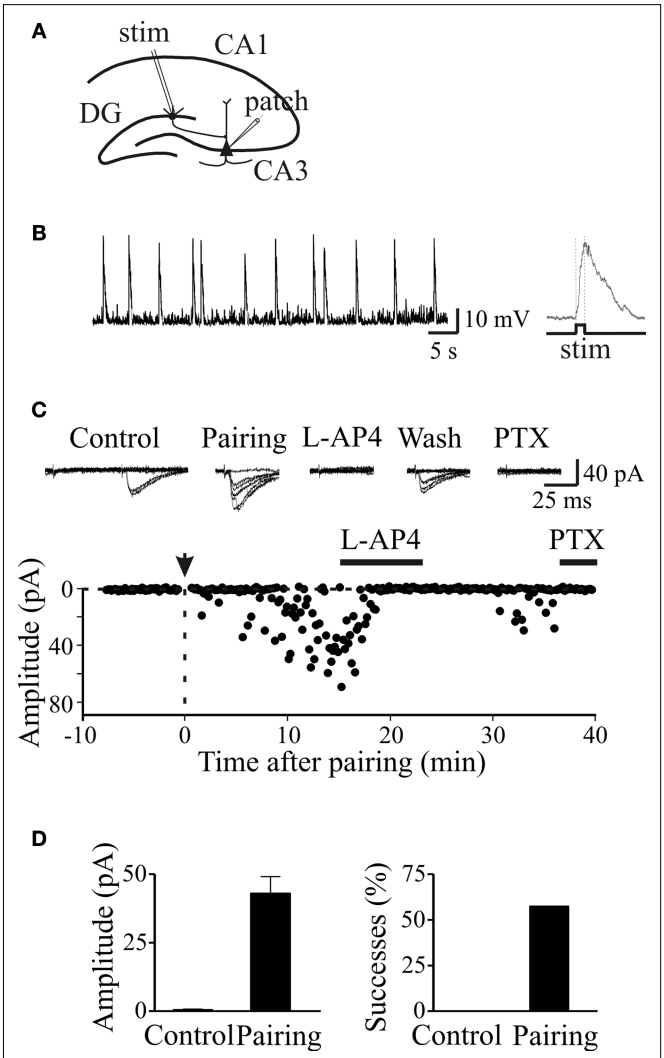

FIGURE 5 | Pairing switched on apparent "presynaptically" silent neurons. (A) Diagram of the hippocampus showing a CA3 pyramidal cell receiving a MF input. The stimulating electrode (stim) is in granule cell layer. **(B)** GDPs recorded from a CA3 pyramidal cell in current-clamp mode from the hippocampus at P2. On the right, a single GDP is shown on an expanded time scale (the intracellular solution contained QX 314 to block action potentials). The rising phase of GDPs (between the dotted lines) was used to trigger synaptic stimulation. **(C)** Peak amplitudes of MF-GPSCs before and after pairing (arrow at time 0) are plotted against time. The traces above the graph represent individual responses evoked in different experimental conditions. This synapse was considered "presynaptically" silent since it did not exhibit any response to the first stimulus over 48 trials (at 0.1 Hz) but occasional (two) responses to the second one. **(D)** Mean GPSCs amplitude and mean percentage of successes before (Control) and after pairing (n = 5). Modified from Kasyanov et al. (2004).

WHY GABA AND NOT GLUTAMATE?

In the adult hippocampus, MFs provide a strong excitatory input to CA3 pyramidal cells. Under certain conditions they may act as "conditional detonators" able to discharge principal cells in a temporally and spatially precise way, their relative strength being dynamically regulated by the activity of granule cells, CA3 pyramidal neurons and cells in the entorhinal cortex (Henze et al., 2000; Urban et al., 2001). Excitation may be limited by the inhibitory influence of GABA released by a large population of interneurons innervated by MF. However, it is unclear how early in postnatal life MF signalling *via* $GABA_A$ receptors may influence the CA3 associative network since AMPA and $GABA_A$ receptors have

different biophysical properties. We have thus compared the relative strength of CA3 pyramidal cells output in relation to their MF GABAergic or glutamatergic inputs using a realistic model which takes into account different parameters, including unitary conductance of MF-GPSCs or MF-EPSCs, quantal content, and kinetics of GABA- or AMPA-mediated responses (see **Table 1**). The model has been simplified, implementing the macroscopic overall conductance changes observed during the experiments. It did not include the expression of postsynaptic NMDA receptors or more detailed mechanisms such as postsynaptic receptor density, neurotransmitter diffusion, reuptake, etc. The presence of equidistant peaks in peak amplitude distribution histograms of MF-GPSCs evoked 0.1 Hz by minimal stimulation of granule cells in the *dentate gyrus*, under conditions of low quantal output (by reducing the extracellular Ca^{2+}/Mg^{2+} ratio from 1.53 to 0.16) was used to estimate the unitary conductance. The representative traces of **Figure 6A** show selected events (excluding failures) recorded at −60 mV, each of them multiple of the quantal size as obtained from the peak amplitude distribution of the same cell (**Figure 6B**). The unbinned peak amplitude data were directly fitted with the sum of four Gaussians functions using the expectation-maximization algorithm for parameters estimation (Redman, 1990). The separation of the peaks was 21.7 pA and the SD 6.6 pA (see Jonas et al., 1993).

In three different cells (out of eight) in which amplitude histograms were judged as quantal according to the criteria of the equidistance of peaks and the consistency of the location of the peaks through the data set, the peak separation was 24.1 ± 4.7 pA. Assuming a reversal potential for GABA of −44 mV (Sivakumaran et al., 2009) the corresponding apparent quantal conductance was 1.5 nS. Interestingly, the quantal size value obtained in the present experiments was similar to that obtained for synaptically-evoked IPSCs in neurons of the granule cell layer in juvenile animals (Edwards et al., 1990). In seven cells recorded under control conditions (Ca^{2+}/Mg^{2+} ratio of 1.53) the peak GPSC conductance varied from a minimum 1.2 nS to a maximum 13.8 nS, suggesting an apparent maximum quantal content of ~ 10. In **Table 1**, these and other parameters for MF-GPSCs and MF-EPSCs have been reported. Some of MF-EPSCs parameters have been taken from Jonas et al. (1993). It is worth mentioning that the firing frequency of granule cells was estimated from the firing rate of spikes occurring at the top of spontaneous GDPs. In addition, we estimated the number of MF inputs impinging into CA3 principal cells equal to 30 on the assumption that during the first week of postnatal life more than one third of MF reach their targets (Gaarskjaer, 1985). In adulthood, each principal cell receives ~50 MF inputs from granule cells.

Thirty synapses were randomly distributed on the proximal apical dendrites (within 100 μm from the soma) of a realistic CA3 model neuron (Hemond et al., 2008). The average number of spikes generated by a train of 10 random (poissonian) activations of GABAergic or glutamatergic inputs, at an average frequency of 20 or 40 Hz, was then calculated from 10 simulations. A few typical somatic recordings are shown in **Figure 7**, and the average (±SEM) spikes using GABA or glutamatergic synapses were 12.6 ± 0.5 and 7 ± 0.3 (at 20 Hz), or 8.7 ± 0.8 and 5 ± 0.5 (at 40 Hz), respectively. The model predicts that the more positive reversal potential of glutamatergic synapses can easily cause a strong depolarizing envelope that could transiently hinder the membrane repolarization process, inactivating Na channels and saturating the cell. In addition to elicit less spikes, with obvious consequences for frequency-dependent synaptic plasticity mechanisms, this effect could generate a number of other problems, ranging from excitotoxicity caused by a much higher elevation in intracellular calcium concentration to receptor desensitization.

Overall, these data strongly suggest that early in postnatal development, in spite of its shunting action, GABA released from MF terminals is able to elicit action potentials in targeted neurons and to convey information into the auto-associative CA3 network. GABA-induced excitation would provide the basis for enhancing synaptic efficacy at emerging synapses in a Hebbian type of way (spike time-dependent plasticity), thus contributing to synaptogenesis and to the establishment of the adult neuronal circuit. The down regulation of GABA

Table 1| Comparison of the relative strength of CA3 pyramidal cells output in relation to their MF GABAergic or glutamatergic inputs.

	MF-EPSCs	MF-GPSCs
E_{rev}	0 mV	–44 mV
Mean (20–80%) rise time	0.4 ms	1.4 ms
Mean (20–80%) decay time	4.8 ms	27 ms
Firing frequency	20–50 Hz	20–50 Hz
Unitary conductance	133 pS	1.5 nS
Maximum peak conductance	5.5 nS	13.8 nS
Estimated quantal content	up to 50	up to 10
MF inputs (randomly activated)	30	30
Spike threshold	–45 mV	–45 mV

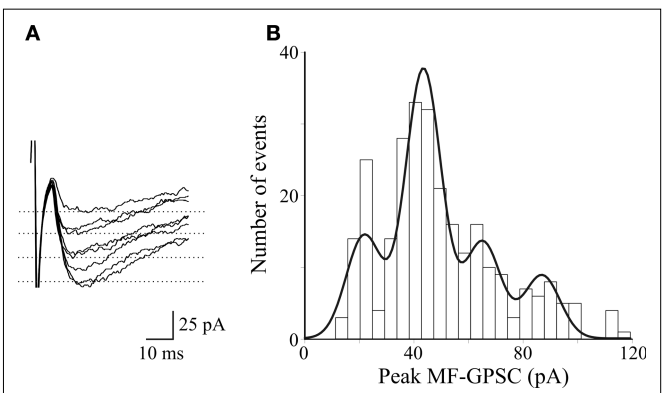

FIGURE 6 | Amplitude distribution of stimulus evoked MF-GPSCs under conditions of low quantal content (extracellular Ca^{2+}/Mg^{2+} ratio 0.16). (A) Selected events, excluding failures, recorded from a P3 CA3 pyramidal neuron at –60 mV. **(B)** Histogram of GPSCs peak amplitude (289 events excluding failures) for the cell shown in A was fitted with the sum of four Gaussian functions with means of 21.7, 43.5, 65.2, 86.9 pA, respectively and a fixed SD of 6.5 pA; bin size = 5 pA.

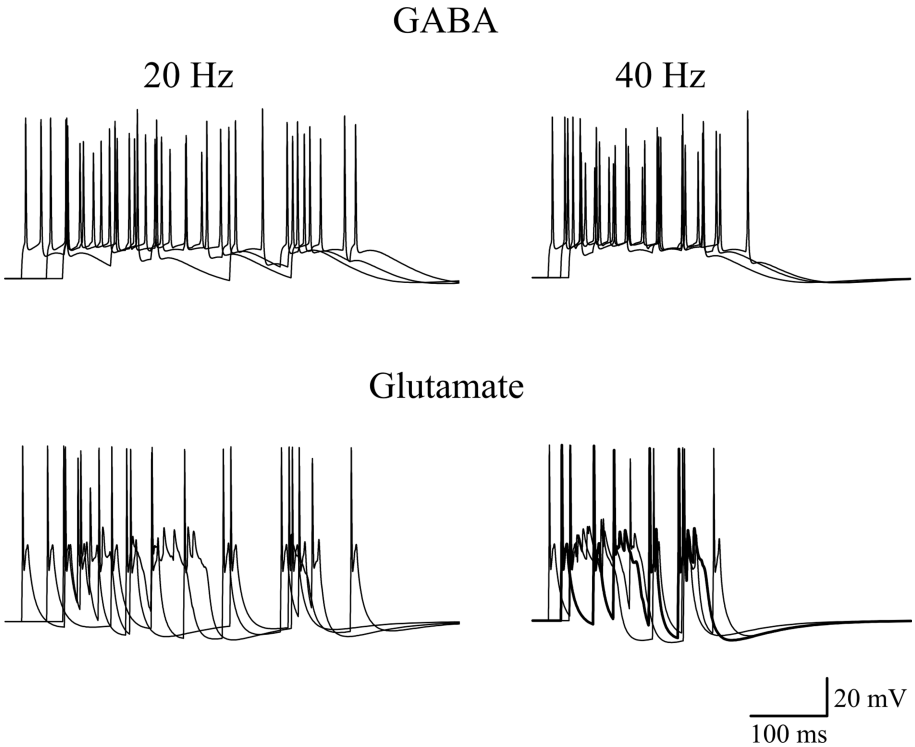

FIGURE 7 | Effects of GABAergic or glutamatergic synapses on a model neuron. Typical traces of somatic membrane potential are plotted during three simulations activating 30 synaptic inputs at an average frequency of 20 Hz (*left*) or 40 Hz (*right*), using GABAergic (*top*) or glutamatergic (*bottom*) synapses. The same stimulation pattern was used in both cases. The passive and active properties of the neuron were those used in a previous work, in a configuration that reproduced the experimental properties of CA3 cells showing firing adaptation (see Figure 9E in Hemond et al., 2008), adjusted to take into account the lower spike threshold (–45 mV) observed in early development in these neurons (Sivakumaran et al., 2009). The model files are available for download from the ModelDB section of the Senselab database (http://senselab.med.yale.edu).

release exerted by activation of $GABA_B$ and kainate receptors localized on MF terminals would in turn limit the excessive activation of the CA3 network and would prevent the development of seizures.

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REFERENCES

Acsády, L., Kamondi, A., Sik, A., Freund, T., and Buzsaki, G. (1998). GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. *J. Neurosci.* 18, 3386–3403.

Alle, H., and Geiger, J. R. (2007). GABAergic spill-over transmission onto hippocampal mossy fiber boutons. *J, Neurosci*. 27, 942–950.

Allen, C., and Stevens, C. F. (1994). An evaluation of causes for unreliability of synaptic transmission. *Proc. Natl. Acad. Sci. U.S.A.* 91, 10380–10383.

Amaral, D. G., and Dent, J. A. (1981). Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J. Comp. Neurol.* 195, 51–86.

Bahn, S., Volk, B., and Wisden, W. (1994). Kainate receptor gene expression in the developing rat brain. *J. Neurosci.* 14, 5525–5547.

Bekkers, J. M. (2005). Presynaptically silent GABA synapses in hippocampus. *J. Neurosci.* 25, 4031–4039.

Ben-Ari, Y., Cherubini, E., Corradetti, R., and Gaiarsa, J. L. (1989). Giant synaptic potentials in immature rat CA3 hippocampal neurones. *J. Physiol.* 416, 303–325.

Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R., and Khazipov, R. (2007). GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol. Rev.* 87, 1215–1284.

Bergersen, L., Ruiz, A., Bjaalie, J. G, Kullmann, D. M., and Gundersen, V. (2003). GABA and GABAA receptors at hippocampal mossy fibre synapses. *Eur. J. Neurosci.* 18, 931–941.

Bettler, B., Boulter, J., Hermans-Borgmeyer, I., O'Shea-Greenfield, A., Deneris, E. S., Moll, C., Borgmeyer, U., Hollmann, M., and Heinemann, S. (1990). Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. *Neuron* 5, 583–595.

Bischofberger, J., Engel, D., Frotscher, M., and Jonas, P. (2006). Timing and efficacy of transmitter release at mossy fiber synapses in the hippocampal network. *Pflugers Arch.* 453, 361–372.

Buzsaki, G., and Draguhn, A. (2004). Neuronal oscillations in cortical networks. *Science* 304, 1926–1929.

Caiati, M. D., Sivakumaran, S., and Cherubini, E. (2010). In the developing rat hippocampus, endogenous activation of presynaptic kainate receptors reduces GABA release from mossy fiber terminals. *J. Neurosci.* 30, 1750–1759.

Castillo, P. E., Malenka, R. C., and Nicoll, R. A. (1997). Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. *Nature* 388, 182–186.

Danbolt, N. C. (2001). Glutamate uptake. *Prog. Neurobiol.* 65, 1–105.

Dupuy, S. T., and Houser, C. R. (1997). Developmental changes in GABA neurons of the rat dentate gyrus: an *in situ* hybridization and birthdating study. *J. Comp. Neurol.* 389, 402–418.

Durand, G. M., Kovalchuk, Y., and Konnerth, A. (1996). Long-term potentiation and functional synapse

induction in developing hippocampus. *Nature* 381, 71–75.

Dzhala, V. I., Talos, D. M., Sdrulla, D. A., Brumback, A. C., Mathews, G. C., Benke, T. A., Delpire, E., Jensen, F. E., and Staley, K. J. (2005). NKCC1 transporter facilitates seizures in the developing brain. *Nat. Med*. 11, 1205–1213.

Edwards, F. A., Konnerth, A. and Sakmann, B. (1990). Quantal analysis of inhibitory synaptic transmission in the dentate gyrus of rat hippocampal slices: a patch-clamp study. *J. Physiol*. 430, 213–249.

Frahm, C., and Draguhn, A. (2001). GAD and GABA transporter (GAT-1) mRNA expression in the developing rat hippocampus. *Brain Res. Dev. Brain Res*. 132, 1–13.

Frotscher, M., Jonas, P., and Sloviter, R. S. (2006). Synapses formed by normal and abnormal hippocampal mossy fibers. *Cell Tissue Res*. 326, 361–367.

Gaarskjaer, F. B. (1985). The development of the dentate area and the hippocampal mossy fiber projection of the rat. *J. Comp. Neurol*. 241, 154–170.

Gaiarsa, J. L., Tseeb, V., and Ben-Ari, Y. (1995). Postnatal development of pre- and postsynaptic GABAB-mediated inhibitions in the CA3 hippocampal region of the rat. *J. Neurophysiol.* 73, 246–255.

Gasparini, S., Saviane, C., Voronin, L. L., and Cherubini, E. (2000). Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? *Proc. Natl. Acad. Sci. U.S.A*. 97, 9741–9746.

Gillespie, D. C., Kim, G., and Kandler, K. (2005). Inhibitory synapses in the developing auditory system are glutamatergic. *Nat. Neurosci.* 8, 332–338.

Gómez-Lira, G., Lamas, M., Romo-Parra, H., and Gutiérrez, R. (2005). Programmed and induced phenotype of the hippocampal granule cells. *J. Neurosci*. 25:6939–6946.

Gutierrez, R., and Heinemann, U. (2001). Kindling induces transient fast inhibition in the dentate gyrus–CA3 projection. *Eur. J. Neurosci*. 13, 1371–1379.

Gutierrez, R., Romo-Parra, H., Maqueda, J., Vivar, C., Ramirez, M., Morales, M. A., and Lamas, M. (2003). Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. *J. Neurosci*. 23, 5594–5598.

Hemond, P., Epstein, D., Boley, A., Migliore, M., Ascoli, G. A., and Jaffe, D. B. (2008). Distinct classes of pyramidal cells exhibit mutually exclusive firing patterns in hippocampal area CA3b. *Hippocampus* 18, 411–24.

Henze, D. A., Urban, N. N., and Barrionuevo, G. (2000). The multifarious hippocampal mossy fiber pathway: a review. *Neuroscience* 98, 407–427.

Jaffe, D. B., and Gutiérrez, R. (2007). Mossy fiber synaptic transmission: communication from the dentate gyrus to area CA3. *Prog. Brain. Res*. 163, 109–132.

Jonas, P., Bischofberger, J., and Sandkuhler, J. (1998). Corelease of two fast neurotransmitters at a central synapse. *Science* 281, 419–424.

Jonas, P., Major, G., and Sakmann, B. (1993). Quantal components of unitary EPSCs at the mossy fibre synapse on CA3 pyramidal cells of rat hippocampus. *J. Physiol*. 472, 615–663.

Kamiya, H., Shinozaki, H., and Yamamoto, C. (1996). Activation of metabotropic glutamate receptor type 2/3 suppresses transmission at rat hippocampal mossy fibre synapses. *J. Physiol.* 493, 447–455.

Kannenberg, K., Sieghart, W., and Reuter, H. (1999). Clusters of GABAA receptors on cultured hippocampal cells correlate only partially with functional synapses. *Eur. J. Neurosci.* 11, 1256–1264.

Kasyanov, A. M., Safiulina, V. F., Voronin, L. L., and Cherubini, E. (2004). GABA-mediated giant depolarizing potentials as coincidence detectors for enhancing synaptic efficacy in the developing hippocampus. *Proc. Natl. Acad. Sci. U.S.A*. 101, 3967–3972.

Kerchner, G. A., and Nicoll, R. A. (2008). Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat. Rev. Neurosci.* 9, 813–825.

Lamas, M., Gomez-Lira, G., and Gutierrez, R. (2001). Vesicular GABA transporter mRNA expression in the dentate gyrus and in mossy fiber synaptosomes. *Brain Res. Mol. Brain Res.* 93, 209–214.

Lanthorn, T. H., Ganong, A. H., and Cotman, C. W. (1984). 2-Amino-4-phosphonobutyrate selectively blocks mossy fiber-CA3 responses in guinea pig but not rat hippocampus. *Brain Res.* 290, 174–178.

Lauri, S. E., Segerstråle, M., Vesikansa, A., Maingret, F., Mulle, C., Collingridge, G. L., Isaac, J. T., and Taira, T. (2005). Endogenous activation of kainate receptors regulates glutamate release and network activity in the developing hippocampus. *J. Neurosci*. 25, 4473–4484.

Lauri, S. E., Vesikansa, A., Segerstråle, M., Collingridge, G. L., Isaac, J. T., and Taira, T. (2006). Functional maturation of CA1 synapses involves activity-dependent loss of tonic kainate receptor-mediated inhibition of glutamate release. *Neuron* 50, 415–429.

Lei, S., and McBain, C. J. (2003). GABA B receptor modulation of excitatory and inhibitory synaptic transmission onto rat CA3 hippocampal interneurons. *J. Physiol*. 546, 439–453.

Leinekugel, X., Khazipov, R., Cannon, R., Hirase, H., Ben-Ari, Y., and Buzsaki, G (2002). Correlated bursts of activity in the neonatal hippocampus *in vivo*. *Science* 296, 2049–2052.

Malenka, R. C., and Nicoll, R. A. (1997). Silent synapses speak up. *Neuron* 19, 473–476.

Malinow, R., Mainen, Z. F., and Hayashi, Y. (2000). LTP mechanisms: from silence to four-lane traffic. *Curr. Opin. Neurobiol*. 10, 352–357.

Marchionni, I., Omrani, A., and Cherubini, E. (2007). In the developing rat hippocampus a tonic $GABA_A$-mediated conductance selectively enhances the glutamatergic drive of principal cells. *J. Physiol*. 581, 515–528.

McBain, C. J. (2008). Differential mechanisms of transmission and plasticity at mossy fiber synapses. *Prog. Brain Res*. 169, 225–240.

Min, M. Y., Rusakov, D. A., and Kullmann, D. M. (1998). Activation of AMPA, kainate, and metabotropic receptors at hippocampal mossy fiber synapses: role of glutamate diffusion. *Neuron* 21, 561–570.

Mohajerani, M. H., Sivakumaran, S., Zacchi, P., Aguilera, P., and Cherubini, E. (2007). Correlated network activity enhances synaptic efficacy *via* BDNF and the ERK pathway at immature CA3–CA1 connections in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 104, 13176–13181.

Montgomery, J. M., Pavlidis, P., and Madison, D. V. (2001). Pair recordings reveal all-silent synaptic connections and the postsynaptic expression of long-term potentiation. *Neuron* 29, 691–701.

Nakamura, M., Sekino, Y., and Manabe, T. (2007). GABAergic interneurons facilitate mossy fiber excitability in the developing hippocampus. *J. Neurosci*. 27, 1365–1373.

Nicoll, R. A., and Schmitz, D. (2005). Synaptic plasticity at hippocampal mossy fibre synapses. *Nat. Rev. Neurosci.* 6, 863–876.

O'Malley, D. M., and Masland, R. H. (1989). Co-release of acetylcholine and gamma-aminobutyric acid by a retinal neuron. *Proc. Natl. Acad. Sci. U.S.A*. 86, 3414–3418.

Ottersen, O. P., and Storm-Mathisen, J. (1984). Neurons containing or accumulating transmitter amino acids. In Handbook of Chemical Neuroanatomy, A. Bjorklund, T. Hokfelt and M. J. Kuhar, eds (Amsterdam, Elsevier). pp. 141–246.

Overstreet, L. S., Pasternak, J. F., Colley, P. A., Slater, N. T., and Trommer, B. L. (1997). Metabotropic glutamate receptor mediated long-term depression in developing hippocampus. *Neuropharmacology* 36, 831–844.

Rao, A., Cha, E. M., and Craig, A. M. (2000). Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. *J. Neurosci*. 20, 8344–8353.

Ramon y Cajal, S. R. (1911). Histologie du Système Nerveux de l'Homme et des Vertébrés, Vol. II. Maloine, Paris.

Redman, S. (1990). Quantal analysis of synaptic potentials in neurons of the central nervous system. *Physiol. Rev.* 70, 165–198.

Ritter, L. M., Vazquez, D. M., and Meador-Woodruff, J. H. (2002). Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. *Brain Res. Dev. Brain Res.* 139, 227–236.

Romo-Parra, H., Vivar, C., Maqueda, J., Morales, M. A., and Gutierrez, R. (2003). Activity-dependent induction of multitransmitter signaling onto pyramidal cells and interneurons of hippocampal area CA3. *J. Neurophysiol*. 89, 3155–3167.

Ruiz, A., Fabian-Fine, R., Scott, R., Walker, M. C., Rusakov, D. A., and Kullmann, D. M. (2003). GABAA receptors at hippocampal mossy fibers. *Neuron* 39, 961–973.

Safiulina, V. F., and Cherubini, E. (2009). At immature mossy fibers-CA3 connections, activation of presynaptic GABA(B) receptors by endogenously released GABA contributes to synapses silencing. *Front. Cell. Neurosci*. 3, 1–11. doi: 10.3389/neuro.03.001.2009.

Safiulina, V. F., Fattorini, G., Conti, F., and Cherubini, E. (2006). GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. *J. Neurosci.* 26, 597–608.

Salin, P. A., Scanziani, M., Malenka, R. C., and Nicoll, R. A. (1996). Distinct short-term plasticity at two excitatory synapses in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A*. 93, 13304–13309.

Schwarzer, C., and Sperk, G. (1995). Hippocampal granule cells express glutamic acid decarboxylase-67 after limbic seizures in the rat. *Neuroscience* 69, 705–709.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P. J., Neki, A., Abe, T., Nakanishi, S., and Mizuno, N. (1997). Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J. Neurosci*. 17, 7503–7522.

Sipilä, S. T., Schuchmann, S., Voipio, J., Yamada, J., and Kaila, K. (2006).

The cation-chloride cotransporter NKCC1 promotes sharp waves in the neonatal rat hippocampus. *J. Physiol.* 573, 765–773.

Sivakumaran, S., Mohajerani, M. H., and Cherubini, E. (2009). At immature mossy-fiber-CA3 synapses, correlated presynaptic and postsynaptic activity persistently enhances GABA release and network excitability via BDNF and cAMP-dependent PKA. *J. Neurosci.* 29, 2637–2647.

Sloviter, R. S., Dichter, M. A., Rachinsky, T. L., Dean, E., Goodman, J. H., Sollas, A. L., and Martin, D. L. (1996). Basal expression and induction of glutamate decarboxylase and GABA in excitatory granule cells of the rat and monkey hippocampal dentate gyrus. *J. Comp. Neurol.* 373, 593–618.

Stirling, R. V., and Bliss, T. V. (1978). Hippocampal mossy fiber development at the ultrastructural level. *Prog. Brain Res.* 48, 191–198.

Thompson, S. M., Haas, H. L., and Gähwiler, B. H. (1992). Comparison of the actions of adenosine at pre- and postsynaptic receptors in the rat hippocampus *in vitro*. *J. Physiol.* 451, 347–363.

Trigo, F. F., Marty, A., and Stell, B. M. (2008). Axonal $GABA_A$ receptors. *Eur. J. Neurosci.* 28:841–848.

Tyzio, R., Holmes, G. L., Ben-Ari, Y., and Khazipov, R. (2007). Timing of the developmental switch in GABA(A) mediated signaling from excitation to inhibition in CA3 rat hippocampus using gramicidin perforated patch and extracellular recordings. *Epilepsia* 48, 96–105.

Uchigashima, M., Fukaya, M., Watanabe, M., and Kamiya, H. (2007). Evidence against GABA release from glutamatergic mossy fiber terminals in the developing hippocampus. *J. Neurosci.* 27, 8088–8100.

Urban, N. N., Henze, D. A. and Barrionuevo, G. (2001). Revisiting the role of the hippocampal mossy fiber synapse. *Hippocampus* 11, 408–417.

Vignes, M., and Collingridge, G. L. (1997). The synaptic activation of kainate receptors. *Nature* 388, 179–182.

Vogt, K. E., and Nicoll, R. A. (1999). Glutamate and gamma-aminobutyric acid mediate a heterosynaptic depression at mossy fiber synapses in the hippocampus. *Proc. Natl. Acad. Sci. U.S.A.* 96, 1118–1122.

Voronin, L. L., and Cherubini, E. (2004). 'Deaf, mute and whispering' silent synapses: their role in synaptic plasticity. *J. Physiol.* 557, 3–12.

Walker, M. C., Ruiz, A., and Kullmann, D. M. (2001). Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. *Neuron* 29, 703–715.

Weisskopf, M. G., Zalutsky, R. A., and Nicoll, R. A. (1993). The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. *Nature* 362, 423–427.

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Cellular/Molecular

Depolarizing Actions of GABA in Immature Neurons Depend Neither on Ketone Bodies Nor on Pyruvate

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GABA depolarizes immature neurons because of a high $[Cl^-]_i$ and orchestrates giant depolarizing potential (GDP) generation. Zilberter and coworkers (Rheims et al., 2009; Holmgren et al., 2010) showed recently that the ketone body metabolite DL-3-hydroxybutyrate (DL-BHB) (4 mM), lactate (4 mM), or pyruvate (5 mM) shifted GABA actions to hyperpolarizing, suggesting that the depolarizing effects of GABA are attributable to inadequate energy supply when glucose is the sole energy source. We now report that, in rat pups (postnatal days 4–7), plasma D-BHB, lactate, and pyruvate levels are 0.9, 1.5, and 0.12 mM, respectively. Then, we show that DL-BHB (4 mM) and pyruvate (200 μM) do not affect (i) the driving force for $GABA_A$ receptor-mediated currents (DF_{GABA}) in cell-attached single-channel recordings, (2) the resting membrane potential and reversal potential of synaptic $GABA_A$ receptor-mediated responses in perforated patch recordings, (3) the action potentials triggered by focal GABA applications, or (4) the GDPs determined with electrophysiological recordings and dynamic two-photon calcium imaging. Only very high nonphysiological concentrations of pyruvate (5 mM) reduced DF_{GABA} and blocked GDPs. Therefore, DL-BHB does not alter GABA signals even at the high concentrations used by Zilberter and colleagues, whereas pyruvate requires exceedingly high nonphysiological concentrations to exert an effect. There is no need to alter conventional glucose enriched artificial CSF to investigate GABA signals in the developing brain.

Introduction

GABA depolarizes and excites immature neurons in many animal species because of their higher $[Cl^-]_i$ compared with mature neurons. This developmental change reflects the sequential operation of the chloride cotransporters NKCC1 and KCC2 (Ben-Ari et al., 1989; Owens et al., 1996; Rivera et al., 1999; Ganguly et al., 2001; Akerman and Cline, 2006) (for review, see Ben-Ari, 2002; Owens and Kriegstein, 2002; Ben-Ari et al., 2007). GABAergic synapses are expressed before glutamatergic synapses, and GABA provides most of the early activity and orchestrates the generation of the first synaptic network-driven giant depolarizing potentials (GDPs) (Ben-Ari et al., 1989; Garaschuk et al., 1998; Tyzio et al., 1999; Ben-Ari, 2001; Sipila et al., 2006; Crépel et al., 2007; Bonifazi et al., 2009).

Rodent maternal milk is enriched in fatty acids that are transformed in the liver to aceto-acetate and DL-3-hydroxybutyrate (DL-BHB). On the basis of this information, Zilberter and colleagues have recently challenged the developmental sequence of GABA action (Rheims et al., 2009) and reported that adding DL-BHB (4 mM) to artificial CSF (ACSF) almost completely eliminated depolarizing and excitatory actions of GABA. This was suggested to reflect the actions of the Cl^-/HCO_3^- exchanger and not KCC2, reported to be inactive in the neocortex at the ages investigated [postnatal day 1 (P1) to P8] (Rheims et al., 2009). In a subsequent paper, the same group (Holmgren et al., 2010) extended these observations to hippocampal CA3 pyramidal neurons and reported that, in addition to DL-BHB, lactate or pyruvate at exceedingly high concentrations (5 mM) also shifted reversal potential of synaptic $GABA_A$ receptor ($GABA_AR$)-mediated responses (E_{GABA}) and blocked GDPs, suggesting that the depolarizing actions of GABA are attributable to energy deprivation when glucose is the sole energetic source. Because this suggestion has important implications for neonatal slice studies, we have now reexamined the effects of DL-BHB and pyruvate on GABA actions on neonatal deep layers neocortical neurons and CA3 pyramidal neurons.

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We report that physiological plasma levels of D-BHB, lactate, and pyruvate are in pups (P5–P7) 0.9, 1.5, and 0.12 mM, respectively. Then, using a wide range of techniques that include extracellular field potential, cell-attached single-channel, and perforated patch-clamp recordings and calcium imaging we report that neither DL-BHB (Sigma-Aldrich) nor physiological concentrations of pyruvate alter GABA actions or spontaneous network dynamics, notably GDPs on rat neocortical and hippocampal neurons. Only very high nonphysiological concentrations of pyruvate altered GABA signaling and GDPs. Our results suggest that depolarizing GABA and GDPs are attributable to neither the absence of BHB/pyruvate nor the metabolic state of neurons in glucose-containing ACSF.

Materials and Methods

All investigations were analyzed in a double-blind manner with results obtained by an investigator analyzed by another researcher. In addition, this study was supervised by an independent group of Institut de Neurobiologie de la Méditerranée (INMED) principle investigators that were not involved in research on GABA in the developing brain (see Acknowledgments). They critically reviewed the results that were discussed in internal meetings of all INMED researchers.

Endogenous plasma D-BHB, lactate, and pyruvate

For D-BHB determination, plasma of pups was first deproteinized using 6% (w/v) perchloric acid and centrifuged, and the supernatant was neutralized with KOH before enzymatic determination. D-BHB was determined enzymatically using the spectrophotometric procedure as described previously (Ferré et al., 1983). Dosage of serum lactate and pyruvate were done in the metabolic biochemistry laboratory of Timone Hospital (Marseille, France). Enzymatic technique was used for lactate (RAPIDLAB 1265). Enzymatic dosage based on the reduction of pyruvate to lactate by the lactate dehydrogenase at pH 7.5 with excess nicotinamide adenine dinucleotide was used for the dosage of pyruvate (Vassault, 1991).

Brain slices

Brain slices were prepared from P4–P8 Wistar rats of both sexes. All animal use protocols conformed to the national guidelines on the use of laboratory animals and were approved by the Animal Care and Use Committees of Inserm and International School for Advanced Studies. Animals were rapidly decapitated, and brains were removed. Coronal slices (300–500 μm) were cut using a tissue slicer (Leica-VT1200S; Microm International) in ice-cold oxygenated modified ACSF with 0.5 mM $CaCl_2$ and 7 mM $MgSO_4$, in which Na^+ was replaced by an equimolar concentration of choline. Slices were then transferred to oxygenated (95% O_2/5% CO_2) standard ACSF containing the following (in mM): 126 NaCl, 3.5 KCl, 2.0 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 10 glucose, pH 7.4, at room temperature (20–22°C) for at least 1 h before use. For recordings, slices were placed into a conventional, fully submerged chamber superfused with ACSF (32–34°C) at a rate of 2–3 ml/min.

Perforated patch-clamp and whole-cell recordings

Patch-clamp recordings were performed from neocortical pyramidal and CA3 pyramidal neurons using EPC-10 Double (HEKA Elektronik Dr. Schulze GmbH) and Axopatch 200A (Molecular Devices) amplifiers. Patch electrodes were made from borosilicate glass capillaries (GC150F-15; Clark Electromedical Instruments). Patch pipette solution for gramicidin perforated patch-clamp recording contained the following (in mM): 150 KCl and 10 HEPES, buffered to pH 7.2 with Tris-OH. Gramicidin was first dissolved in DMSO to prepare a stock solution of 10–40 mg/ml and then diluted in the pipette solution to a final concentration of 80 μg/ml. The gramicidin-containing solution was prepared and sonicated <1 h before the experiment. To facilitate cell-attached formation (4–10 GΩ), patch pipettes were backfilled with a gramicidin-containing solution. Between 20 and 30 min after formation of the cell-attached seal, the series resistance (R_s) stabilized at 8–60 MΩ. Series resistance was monitored during all recording sessions. At the end of each recording, negative pressure was applied to break the membrane and establish whole-cell configuration. This was associated with a shift of the reversal potential of the GABA-mediated responses to near 0 mV. The membrane potential values (E_m) were corrected for series resistance offline as V(corrected) = V(holding) − IR_s. For whole-cell recordings, we used the pipette solution containing the following (in mM): 135 K-gluconate, 20 KCl, 10 HEPES, 4 MgATP, 0.3 GTP, and 0.5 EGTA. A picospritzer (General Valve Corporation) was used to puff apply GABA (100 μM in ACSF) from a glass pipette in stratum radiatum at a distance of ~100 μm from the soma in gramicidin perforated patch recordings. The pressure varied from 10 to 20 kPa, and the duration of the puff varied from 50 to 200 ms.

Cell-attached recordings of $GABA_A$ and NMDA receptor channels

Patch-clamp recordings from visually identified pyramidal cells in a cell-attached configuration were performed using an EPC-10 double amplifier or Axopatch 200B amplifier. For recordings of single GABA channels, the following patch pipette solution was added on the day of the experiment from a 1 mM frozen stock solution (in mM): 120 NaCl, 5 KCl, 20 tetraethylammonium-Cl, 5 4-aminopyridine, 0.1 $CaCl_2$, 10 $MgCl_2$, 10 glucose, and 10 HEPES–NaOH, buffered to pH 7.2–7.3 (with GABA at 1–5 μM). E_m was estimated using cell-attached recordings of single NMDA receptor (NMDAR) channels as described previously (Tyzio et al., 2003). For recordings of single NMDAR channels, pipette solution contained nominally Mg^{2+}-free ACSF with NMDA (10 μM), glycine (1 μM), and strychnine (1 μM). Pipettes (resistance of 3.5–8 MΩ) were pulled from borosilicate glass capillaries (GC150F-15; Clark Electromedical Instruments). Recordings were digitized (10 kHz) online with Digidata 1200 or 1440 interface cards (Molecular Devices), filtered (2.9 kHz), and analyzed offline with Axon package (Molecular Devices) and Origin (Microcal Software) as described previously (Tyzio et al., 2003, 2006). Group measures are expressed as means ± SEM; error bars also indicate SEM. The statistical significance of differences was assessed with Student's t test. The level of significance was set at $p < 0.05$.

Calcium imaging

Slice preparation for calcium imaging. Horizontal slices of neocortex and hippocampus (400 μm thick) were prepared from P7 rats using a Vibratome tissue slicer (Leica VT 1200S) in ice-cold oxygenated modified ACSF (mACSF) (with 0.5 mM $CaCl_2$ and 7 mM $MgSO_4$; NaCl replaced by an equimolar concentration of choline). Slices were then transferred for rest (~1 h) in oxygenated normal ACSF containing the following (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 1.3 $MgCl_2$, 2.0 $CaCl_2$, and 10 D-glucose, pH 7.4. For AM loading, slices were incubated in a small vial containing 2.5 ml of oxygenated ACSF with 25 μl of a 1 mM fura-2 AM solution (in 100% DMSO; Invitrogen) for 20–30 min. Slices were incubated in the dark, and the incubation solution was maintained at 35–37°C. Slices were perfused at a rate of 4 ml/min with continuously aerated (95% O_2/5% CO_2) normal ACSF at 35–37°C. Imaging was performed with a multibeam two-photon laser scanning system (Trimscope-LaVision Biotec) coupled to an Olympus microscope as described previously (Crépel et al., 2007). Images were acquired through a CCD camera (La Vision Imager 3QE), which typically resulted in a time resolution of ~100 ms (2 × 2 binning; pixel size, 600 nm). Slices were imaged using a low-magnification, high numerical aperture objective (20×, numerical aperture 0.95; Olympus). The size of the imaged field was typically 430 × 380 μm^2. Imaging depth was on average 80 μm below the surface (range, 50–100 μm).

Analysis of multineuron calcium activity. Analysis of the calcium activity was performed using a previously designed software for neocortical and hippocampal slice analysis (Allène et al., 2008). To summarize briefly, this allowed (1) automatic identification of loaded cells, (2) measuring the average fluorescence transients from each cell as a function of time, and (3) detecting the onsets and offsets of calcium signals. To quantify synchronous activity patterns, we used two parameters: frequency and amplitude and duration of synchronous events. The frequency of a network pattern was derived from the average time interval between two peaks of synchronous activity. The amplitude of a network pattern in a given movie was the average of the maximum of cells coactive in each peak of synchrony across the movie. To identify peaks of synchro-

nous activity that included more cells than expected by chance, we used interval reshuffling (randomly reordering of intervals between events for each cell) to create set of surrogate event sequences. Reshuffling was performed 1000 times for each movie, and a surrogate histogram was constructed for each reshuffling. The threshold corresponding to a significance level of $p < 0.05$ was estimated as the number of coactive cells exceeded in a single frame in only 5% of these histograms. This threshold was used to calculate the duration of a synchronous activity pattern that is the number of successive frames for which the number of coactive cells was superior to threshold. Experimental values are given as means ± SEMs. Student's *t* test and χ^2 test were used for statistical comparisons.

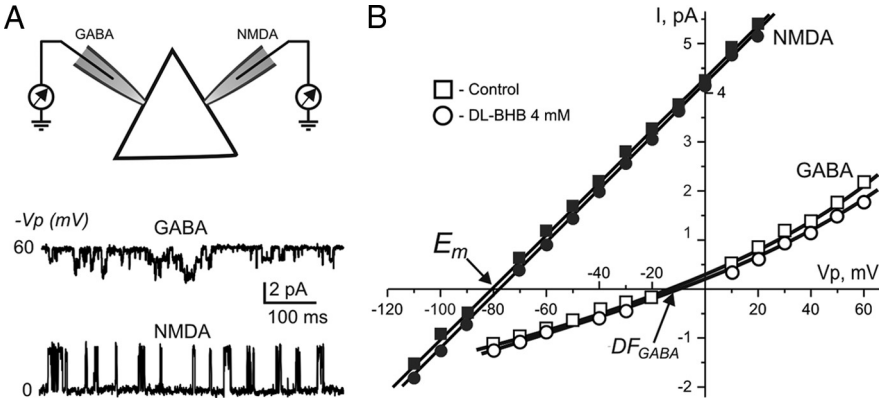

Figure 1. Estimation of E_{GABA} by double recordings of DF_{GABA} and E_m from the same neocortical cell. ***A***, Scheme of consecutive cell-attached recordings of single NMDAR and $GABA_AR$ channels. First we recorded from the same neuron in control conditions for NMDAR channels (for E_m) and then $GABA_AR$ channels (for DF_{GABA}). These two measurements allow us to determine E_{GABA} for this neuron. Then, we applied DL-BHB for 40 min and repeated the recordings with the same sequence (NMDAR and then $GABA_AR$ channels). Thus, every cell studied was patched four times. Representative traces of recordings of the single-channel openings are shown below. ***B***, Representative plot of *I–V* relationships of single $GABA_AR$ and NMDAR channels used for estimation of E_{GABA} in neocortical pyramidal cell ($E_{GABA} = DF_{GABA} + E_m$). Each point is mean amplitude of ~30 openings at a given pipette potential (V_p). The reversal potential that corresponds to DF_{GABA} was estimated by the exponential growth fit of the *I–V* curve. The current–voltage relationships of NMDAR channels were best fitted with linear function (Tyzio et al., 2003, 2008). Note that application of DL-BHB does not change significantly DF_{GABA} and E_m.

Chemical identification of contaminants

One milligram of DL-BHB was dissolved in a mixture of ethyl acetate and pentafluoropropionic anhydride (PFPA) (Aldrich) and composed of 80 μl each. After 30 min at 80°C, 1 μl of the mixture was directly injected into a gas chromatography–mass spectrometry (GC–MS) instrument (Agilent Technologies) equipped with a 6890N GC and a 5973 MSD system. Electron impact mass spectrum of dibenzylamine (DBA) as PFPA derivative was identified in the DL-BHB acid standard from Acros Organics but not in DL-BHB from Sigma-Aldrich. A PFPA was identified as its molecular ion at a mass-to-charge ratio (*m*/*z*) of 383 and a typical fragmentation with the predicted M-91 at *m*/*z* of 252 and the benzyl core at *m*/*z* of 91. Chromatographic conditions were as follows: DB-1MS capillary column (Agilent Technologies), 30 m × 0.25 mm inner diameter, film thickness of 1.0 μm; column temperature, 100–260°C at a rate of 4°C/min; injection port, 280°C; gaz vector helium at 1 ml/min (10.4 psi); electron impact mode at an ionization energy of 70 eV. Comparisons of mass spectra were done by using a standard of DBA treated in the same conditions.

Pharmacology

Bicuculline, 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline, D-APV, DNQX, and CNQX were purchased from Tocris Bioscience; DL-BHB was from Sigma-Aldrich (catalog #54965, batch #1316259 31908044) and from Acros Organics. Isoguvacine, dybenzylamine, GABA, lactate, and pyruvate were from Sigma. TTX was from Ascent Scientific Ltd.

Results

Blood concentrations of D-BHB, lactate, and pyruvate in neonatal rodent

In newborn rodents, the blood levels of active D-BHB are in the submillimolar range (see Discussion). We reinvestigated this issue, using an assay that determines D-BHB levels, and found that, in P7 rats, the D-BHB plasma concentration was 0.91 ± 0.15 mM ($n = 14$). We measured D-BHB levels after administration of insulin (0.33 U/kg, i.m.) to inhibit fatty acid oxidation and 2-mercaptoacetate (100 mg/kg, i.p.) to inhibit mitochondrial acyl-CoA dehydrogenase with 5 mM glucose to prevent hypoglycemia. This treatment strongly and rapidly (within 1 h) reduced the plasma level of the D-BHB to 0.14 ± 0.03 mM ($n = 14$). Conversely, injections of exogenous 4 mM DL-BHB increased the blood D-BHB concentration to 1.74 ± 0.42 mM ($n = 7$). Using conventional hospitals kits, plasma lactate and pyruvate levels at P4 were of 1.5 ± 0.25 mM and 123 ± 16 μM, respectively ($n = 5$). Therefore, plasma D-BHB, pyruvate, and lactate concentrations are much lower than those used by Zilberter and colleagues (4 mM DL-BHB, 5 mM pyruvate, and 5 mM lactate). The difference is particularly high for pyruvate (40-fold) (see Discussion).

DL-BHB (from Sigma-Aldrich) does not alter the driving force for somatic $GABA_AR$-mediated currents

The binding of GABA to $GABA_A$ receptors opens channels permeable notably to chloride. The resulting trans-membrane chloride current can either depolarize or hyperpolarize the membrane according to its E_{GABA} and E_m of the cell. As indicated in previous studies, measurement of E_m using NMDAR channels as the voltage sensor has an advantage over the other microelectrode techniques especially in immature cells with high input resistance (Tyzio et al., 2003). Single NMDAR and $GABA_AR$ channels were recorded from either the same neurons or from different neurons to determine E_m and driving force for somatic $GABA_AR$-mediated currents (DF_{GABA}) and thereby allow a precise determination of E_{GABA} (Tyzio et al., 2008). As in the studies by Rheims et al. (2009) and Holmgren et al. (2010), slices were incubated in 4 mM DL-BHB (Sigma-Aldrich) for at least 40 min and then transferred to a recording chamber in which they were continuously superfused with the same concentration of DL-BHB.

We first determined E_{GABA} by alternate cell-attached recordings (in sum, four attempts for every cell) of NMDAR and $GABA_AR$ single channels from the soma of the same neuron in the neocortex (Fig. 1). As shown in Table 1, DL-BHB altered neither E_m nor E_{GABA} in neocortical neurons (P4–P5 rats) of both superficial and deep layers (all $p > 0.05$). Similar results were obtained when single NMDAR and $GABA_AR$ channels were recorded in different neurons (to avoid the potential local membrane alterations): DF_{GABA} in hippocampal CA3 pyramidal cells was not significantly different in control and 4 mM DL-BHB (Table 2) ($p > 0.05$); E_m in the same population of cells also did not change significantly in DL-BHB ($p > 0.05$). Similarly, in both superficial and deep layers of the neocortex, DF_{GABA} was strongly depolarizing in control conditions and in the presence of DL-BHB; the values of E_m were similar in control conditions and in the presence of DL-BHB (all $p > 0.05$) (Table 2). Therefore, DL-

Table 1. Double measurement of DF_{GABA} and E_m from the same neocortical cell reveal that DL-BHB does not alter E_{GABA} in pyramidal neocortical cells

	Regular ACSF	ACSF + DL-BHB at 4 mM
n	6	6
E_m (mV)	−81.7 ± 2.6	−79.2 ± 2.9
DF_{GABA} (mV)	14.8 ± 3.3	13.7 ± 3.1
E_{GABA} (mV)	−66.9 ± 3.9	−65.5 ± 3.1

DF_{GABA} inferred from *I–V* curves of single $GABA_AR$ channels, E_m inferred from *I–V* curves of single NMDAR, and the $GABA_AR$ channels reversal potential.

BHB alters neither E_m nor E_{GABA} in neocortical and hippocampal neurons.

DL-BHB does not alter the polarity of synaptic GABAergic responses

We then used perforated patch recordings to determine the effects of DL-BHB on synapse-driven $GABA_AR$-mediated postsynaptic potentials (GPSPs). In immature hippocampal slices, GABA released from MF terminals exerts a depolarizing action on CA3 principal cells in gramicidin perforated patch recordings (Sivakumaran et al., 2009). With gramicidin patch recordings from CA3 pyramidal cells, the resting membrane potential was −56 ± 2.2 mV ($n = 10$) (Fig. 2*A–C*). The more depolarized E_m value found with these experiments (with respect to that observed in cell-attached recordings) could be attributed to the leakage via gigaseal contact introduced in perforated patch recordings (Barry and Lynch, 1991; Tyzio et al., 2003). In the presence of 20 μM DNQX and 50 μM D-AP-5 to block AMPA and NMDA receptors, respectively, local stimulation of GABAergic interneurons in stratum radiatum generated PSPs with reversed polarity (E_{GPSPs}) at −47.6 ± 3.3 mV ($n = 10$). The driving force for GABA was ~9 mV positive to E_m, indicating that GABA is depolarizing from the resting potential as a result of the activity of chloride accumulating NKCC1 cotransporter (Tyzio et al., 2003, 2007; Sipila et al., 2006). Addition of DL-BHB (4 mM for at least 40 min) altered neither E_m (−60 ± 1 mV) nor E_{GPSPs} (−48 ± 2 mV; $n = 11$), with an average DF_{GABA} of ~12 mV. The values of E_m and E_{GPSPs} obtained in the presence of DL-BHB were not significantly different from those obtained in the absence of DL-BHB ($p > 0.05$ for both) (Fig. 2*A–C*). Therefore, DL-BHB does not alter E_{GPSPs}.

We also used gramicidin perforated patch recordings to determine whether DL-BHB alters the response evoked by $GABA_A$ receptor agonists. The reversal of isoguvacine-evoked responses (E_{iso}) was determined in control and during bath application of 4 mM DL-BHB for at least 40 min. Isoguvacine (40 μM, from a holding potential of −60 mV) generated inward currents that reversed at −48 ± 3 mV in control ($n = 6$) and −47 ± 3 mV in the presence of DL-BHB ($n = 6$). These values were not significantly different ($p > 0.05$) (Fig. 2*D*).

Furthermore, in three cells kept in current-clamp conditions in the presence of blockers of fast synaptic transmission (DNQX at 50 μM, D-APV at 50 μM, and bicuculline at 10 μM), bath application of 4 mM DL-BHB altered neither the resting membrane potential nor the input resistance [values for E_m and R_{in} were −60 ± 3 and −61 ± 2 mV ($n = 6$) and 625 ± 37 and 575 ± 42 MΩ ($n = 6$, all $p > 0.05$), before and 40 min after DL-BHB application, respectively] (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

DL-BHB does not alter the excitatory effects of GABA

To determine whether DL-BHB alters the excitatory actions of GABA, we focally applied GABA on neurons recorded in cell-attached configuration in the presence of CNQX (10 μM), APV (40 μM), and CGP 55845 ((2*S*)-3-[[(1*S*)-1-(3,4-dichlorophenyl)ethyl] amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride) (2 μM) to block ionotropic glutamate and metabotropic $GABA_B$ receptors.

In these conditions, GABA triggered spikes and this action was reversibly blocked by the $GABA_A$ receptor antagonists (data not shown), indicating that they were generated by the activation of $GABA_A$ receptors. As shown in Figure 3*A*, focal applications of GABA, in the hippocampus in the presence of DL-BHB generated a similar number of spikes as in controls (1.29 ± 0.09 spikes in control and 1.44 ± 0.1 in DL-BHB, $n = 7$, $p > 0.05$). Similar results were obtained with neocortical layer V pyramidal neurons (1.39 ± 0.06 spikes in control and 1.23 ± 0.05 in the presence of DL-BHB, $n = 6$, $p > 0.05$). The specific NKCC1 antagonist bumetanide (10 μM) prevented GABA from inducing spikes generated by focal applications of GABA in the presence of the same mixture and DL-BHB, confirming that they were generated by depolarizing actions of GABA (Fig. 3*B*) ($n = 3$). Therefore, DL-BHB applied in conditions similar to those used by Rheims et al. (2009) and Holmgren et al. (2010) does not alter the driving force and reversal potential of somatic (extrasynaptic) and synaptic GABA activated responses, the resting membrane potential, and excitatory actions of GABA on neocortical or hippocampal neurons.

DL-BHB does not alter GDPs

We next examined whether DL-BHB affects GDPs, which are dependent on depolarizing actions of GABA and are very sensitive to alterations of neuronal excitability and to insufficient energy supply (Ben-Ari et al., 1989; Dzhala et al., 1999; Allène et al., 2008). As shown in Figure 4, GDPs occurred synchronously with extracellular field potentials and were readily identified in whole-cell recordings by their characteristic shape and kinetics. DL-BHB altered neither the frequency [0.054 ± 0.005 and 0.046 ± 0.004 Hz in control ($n = 7$) and DL-BHB ($n = 8$), respectively; $p > 0.05$] nor the integrated area under GDPs [26.6 ± 3.1 mV/s ($n = 6$) and 24.3 ± 3.2 mV/s ($n = 7$) in control and DL-BHB, respectively; $p > 0.05$].

We next used fast multineuron calcium imaging to measure the actions of DL-BHB on neuronal activity synchronization in cortical networks. This approach enables investigating the spatiotemporal dynamics of spontaneous neuronal activity in large neocortical and hippocampal networks with single-cell resolution. With this approach, immature hippocampal and neocortical networks display similar correlated activity patterns composed of synchronous plateau assemblies (SPAs) and GDPs that are both strongly modulated by the excitatory action of GABA (Crépel et al., 2007; Allène et al., 2008; Bonifazi et al., 2009). These patterns can be easily identified in single cells based on their characteristic calcium dynamics because SPAs are associated with long-lasting (~10–20 s on average) calcium plateau potentials synchronized across small groups of neurons, whereas the intracellular calcium correlate of GDPs are fast calcium transients (~250 ms) decay (Crépel et al., 2007; Allène et al., 2008) synchronizing larger neuronal populations. To determine whether DL-BHB affected neuronal calcium dynamics, multibeam two-photon imaging in neocortical slices loaded with a calcium indicator (fura-2 AM) was performed. Slices were incubated in 4 mM DL-BHB for at least 40 min. Custom software was used to measure fluorescence changes in each cell and to mark the onset and offset of individual calcium transients (see Materials

Table 2. DL-BHB does not alter DF_{GABA} and E_m in principal cells of rat hippocampus and neocortex: changes of DF_{GABA} determined with cell-attached recording of $GABA_A$R channels in hippocampal CA3 cells and both superficial and deep layers of neocortex

	Hippocampus		Neocortex	
	regular ACSF	ACSF + DL-BHB at 4 mM	regular ACSF	ACSF + DL-BHB at 4 mM
E_m (mV)	−77.8 ± 3.5 ($n = 9$)	−76.2 ± 4.2 ($n = 12$)	−72.2 ± 5.4 ($n = 5$)	−70.6 ± 3.2 ($n = 7$)
DF_{GABA} (mV)	19.8 ± 6.5 ($n = 12$)	16.5 ± 3.5 ($n = 14$)	22.9 ± 4.2 ($n = 10$)	18.2 ± 3.6 ($n = 11$)

Note that, in this set of experiments, DF_{GABA} and E_m were examined in different cells.

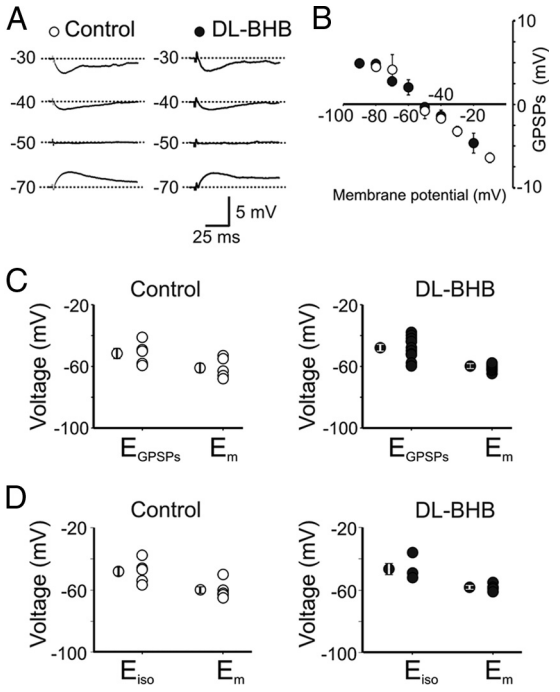

Figure 2. DL-BHB does not alter the polarity of GABAergic responses in CA3 pyramidal cells. ***A***, Example of $GABA_A$-mediated postsynaptic potentials evoked in control and in the presence of 4 mM DL-BHB at four different holding potentials (to the left of the traces) by local stimulation of GABAergic interneurons in stratum radiatum. ***B***, The mean GPSP amplitudes obtained in 11 cells are plotted against membrane potentials (E_m). Vertical bars represent the SEM. ***C***, Each symbol represents the E_{GPSPs} and the E_m of individual cells. Average values are shown on the left of each group (control: $E_{GPSPs} = -47.6 \pm 3.3$ mV, $E_m = -56 \pm 2.2$ mV, $n = 10$; in DL-BHB: $E_{GPSPs} = -48.2 \pm 2$ mV, $E_m = -60 \pm 1$ mV, $n = 11$). ***D***, The E_{iso} was determined in control and during bath application of DL-BHB (4 mM, for at least 40 min). The isoguvacine application (40 μM) generated the responses, which reversed at −48 ± 3 mV in control ($n = 6$) and −47 ± 3 mV in DL-BHB ($n = 6$), respectively. These values were not significantly different ($p > 0.05$).

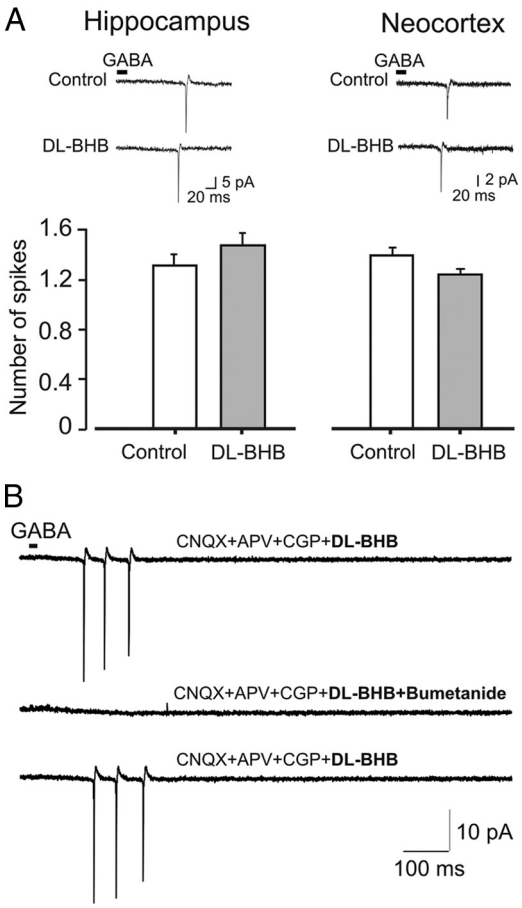

Figure 3. DL-BHB does not alter the excitation produced by focal application of GABA in both hippocampus and neocortical pyramidal neurons. ***A***, Each column represents number of spikes induced by focal application of GABA (top traces) recorded in cell-attached mode from hippocampus and neocortex in control and in presence of 4 mM DL-BHB. ***B***, Bumetanide (10 μM) fully blocked the spikes generated by focal applications of GABA in the presence of the same mixture and DL-BHB ($n = 3$).

and Methods). In these conditions, we did not find any significant decrease in either the fraction of neurons producing SPAs or the frequency and amplitude of GDPs in neocortical slices ($p > 0.05$, $n = 5$ slices) (Fig. 5, Table 3). As a positive control for excitatory GABA actions, we applied the NKCC1 antagonist bumetanide (10 μM). Bumetanide significantly reduced the occurrence of GDPs both in regular ACSF and in the presence of DL-BHB (Fig. 5). Frequency and amplitude of GDPs were reduced to 26 ± 21 and 38 ± 10%, respectively, in regular ACSF ($n = 7$, $p < 0,05$) and to 12 ± 7 and 56 ± 34%, respectively, in DL-BHB conditions ($n = 5$, $p < 0.05$). As reported previously in hippocampal slices (Crépel et al., 2007), the fraction of SPA cells was significantly increased in the presence of bumetanide (10 μM, to 535 ± 192 and 349 ± 104% in regular ACSF and DL-BHB, respectively, $n = 5$, $p < 0.05$). The effects of bumetanide cannot be reconciled with a reduction of the depolarizing action of GABA in the presence of DL-BHB (Rheims et al., 2009) (see Discussion).

A contamination in DL-BHB (from Acros Organics) reduces DF_{GABA}

While conducting our experiments, we found that DL-BHB from another source (Acros Organics) gave different results. In slices incubated in 4 mM DL-BHB (Acros Organics for at least 40 min), DF_{GABA} measured using cell-attached recordings of $GABA_A$R channels was significantly reduced in CA3 pyramidal cells from 12.2 ± 3.9 mV ($n = 6$) to 0.47 ± 3.04 mV ($n = 10$, $p < 0.05$; data not shown). Similar effects of DL-BHB (Acros Organics) were found with perforated patch recordings of GPSPs evoked by local stimulation of GABAergic interneurons in stratum radiatum in the presence of DNQX (20 μM) and D-APV (50 μM). As shown in supplemental Figure 2 (available at www.jneurosci.org as supplemental material), DL-BHB from Acros Organics (applied for at least 45 min) hyperpolarized the membrane and shifted E_{GPSPs} toward more negative values (on average, on seven neurons E_m

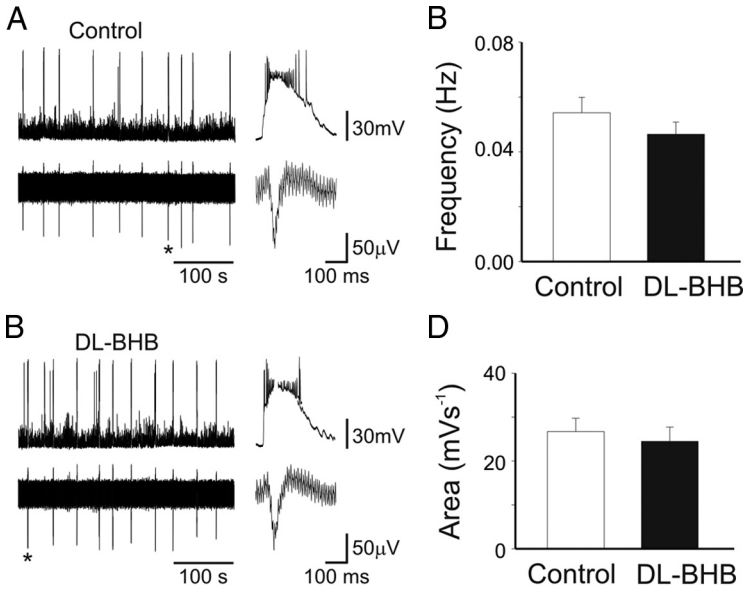

Figure 4. DL-BHB does not alter spontaneous neuronal activity patterns at early postnatal stages in hippocampal slices. ***A***, ***B***, Individual traces of spontaneous GDPs recorded at −70 mV from CA3 pyramidal cell (top traces) and field potentials (bottom traces) in slice exposed to ACSF (control, ***A***) or ACSF plus DL-BHB at 4 mM. ***B***, DL-BHB did not alter the frequency or the shape of GDPs (shown on the right in an expanded timescale). ***C***, ***D***, Each column represent the mean GDPs frequency (***C***) or area (***D***) in control (white; $n = 6$) or during bath application of DL-BHB (black; $n = 7$). *$p < 0.01$.

and E_{GPSPs} were −80 ± 4 and −67 ± 4 mV, respectively). In additional experiments, we tested the actions of DL-BHB (Acros Organics) on GDPs using calcium imaging. As shown in supplemental Figure 3 (available at www.jneurosci.org as supplemental material), DL-BHB (Acros Organics) reduced the frequency and amplitude of GDPs in neocortical slices (from 0.12 ± 0.02 to 0.02 ± 0.01 Hz and from 22 ± 3 to 5 ± 4% of active cells, $n = 5$, $p < 0.05$). Therefore, DL-BHB (Acros Organics) reduces DF_{GABA} and alters GDPs.

Because previous studies reported the presence of a contaminant in L-BHB (Donevan et al., 2003) (see Discussion), we decided to test whether a similar contaminant was also present in DL-BHB obtained from this source (Acros Organics). Using a GC–MS instrument, several contaminants were found in this source of DL-BHB, notably DBA as PFPA derivative identified in the DL-BHB from Acros Organics (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) but not in DL-BHB (Sigma-Aldrich; data not shown). We therefore tested the actions of DBA on DF_{GABA} and found that 50 μM DBA switched DF_{GABA}, determined with single-channel recordings, from depolarizing 12.8 ± 2.8 mV ($n = 10$) to hyperpolarizing −2.9 ± 2.2 mV ($n = 10$, $p < 0.001$; data not shown). With whole-cell recordings, DBA also blocked GDPs in a concentration-dependent way with an EC_{50} of 57 μM (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). The dose–response curve was steep: whereas at 60 μM DBA severely reduced the frequency of GDPs from 0.046 ± 0.007 to 0.01 ± 0.002 Hz, at 80 μM it completely abolished them ($n = 10$). These observations suggest that DL-BHB does not alter GABA signals, but care must be taken when using BHB compounds to ensure absence of contaminants. We next investigated the actions of pyruvate on the same parameters.

High but not physiological concentrations of pyruvate affect GABA signaling

We first tested the effect of pyruvate at 5 mM on DF_{GABA} in single-channel recordings of CA3 pyramidal cells from P7 rat hippocampus. DF_{GABA} shifted from 12.2 ± 5.7 mV in control ($n = 9$) to 0.4 ± 3 mV in pyruvate ($n = 9$, $p < 0.01$; data not shown). In contrast, more relevant physiological concentrations (200 μM) did not significantly change DF_{GABA} (7.1 ± 3.2 mV in control, $n = 7$ and 6.7 ± 2.5 mV in pyruvate, $n = 8$, $p > 0.05$; data not shown). Therefore, pyruvate does not affect DF_{GABA} at physiological levels and alters DF_{GABA} only at excessively high concentrations.

Gramicidin perforated patch experiments were performed to assess whether pyruvate alters E_m and E_{GPSPs}. Like DL-BHB, the addition of a physiological concentration of 200 μM pyruvate to the ACSF did not modify E_m and E_{GPSPs} (Fig. 6). E_m values were −56.8 ± 0.9 and −57.1 ± 1.02 mV in control and in the presence of pyruvate, respectively ($p > 0.05$, $n = 7$). In contrast, 5 mM pyruvate caused a negative shift of E_{GPSPs} (from −48.3 ± 1.9 to −55.7 ± 3 mV, $n = 7$) without altering E_m (E_m values were −56.8 ± 0.9 and −56.6 ± 1.8 mV in control and in the presence of 5 mM pyruvate, respectively, $n = 7$). The driving force for GABA was ~0.9 mV positive to E_m, indicating that GABA does not exert a depolarizing action. The E_{GPSPs} value observed in the presence of 5 mM pyruvate was significantly different from that obtained in control ($p = 0.003$) or in the presence of 200 μM pyruvate ($p = 0.005$). E_m and E_{GPSPs} were unaffected by the further addition of DL-BHB to 5 mM pyruvate. In the presence of 4 mM DL-BHB and 5 mM pyruvate, E_m and E_{GPSPs} were −57.4 ± 2.5 and −59.6 ± 2.9 mV, respectively ($n = 6$). These values are similar to those obtained when cells were exposed only to pyruvate (data not shown).

Furthermore, with extracellular field potential recordings from the CA3 region (P4–P7), pyruvate at a physiological concentration did not affect the frequency of GDPs (0.06 ± 0.008 and 0.06 ± 0.01 Hz in control and in the presence of 200 μM pyruvate, respectively; $p > 0.05$, $n = 6$) (Fig. 7). In contrast, in the presence of 5 mM pyruvate, the frequency of GDPs severely depressed (from 0.06 ± 0.008 to 0.02 ± 0.007 Hz, $p = 0.005$, $n = 6$).

Similar observations were made with imaging techniques. We monitored spontaneous neuronal activity in neocortical and hippocampal slices bathed in mACSF that mimics physiological conditions (lactate at 1.5 mM, pyruvate at 150 μM, and DL-BHB at 2 mM; see Discussion). There was no change in GDPs or SPA patterns of activity (Fig. 8, Table 4) ($p > 0.05$, five neocortical slices and three hippocampal slices). Indeed, the frequency of neocortical GDPs in mACSF was 0.1 ± 0.02 Hz compared with 0.12 ± 0.02 Hz in control conditions, and the amplitude of neocortical GDPs was 24 ± 4 compared with 22 ± 3% of active cells in regular ACSF. The frequency of hippocampal GDPs in mACSF was not affected either (0.07 ± 0.03 vs 0.1 ± 0.03 Hz in control conditions). In contrast, pyruvate (5 mM), added to regular ACSF, significantly reduced the frequency and amplitude of hippocampal GDPs to 8 ± 7 and 53 ± 33% of control conditions, respectively (supplemental Fig. 6) ($n = 5$, $p < 0.05$). Therefore, pyruvate alters GABA postsynaptic currents and GDPs only at high nonphysiological concentrations.

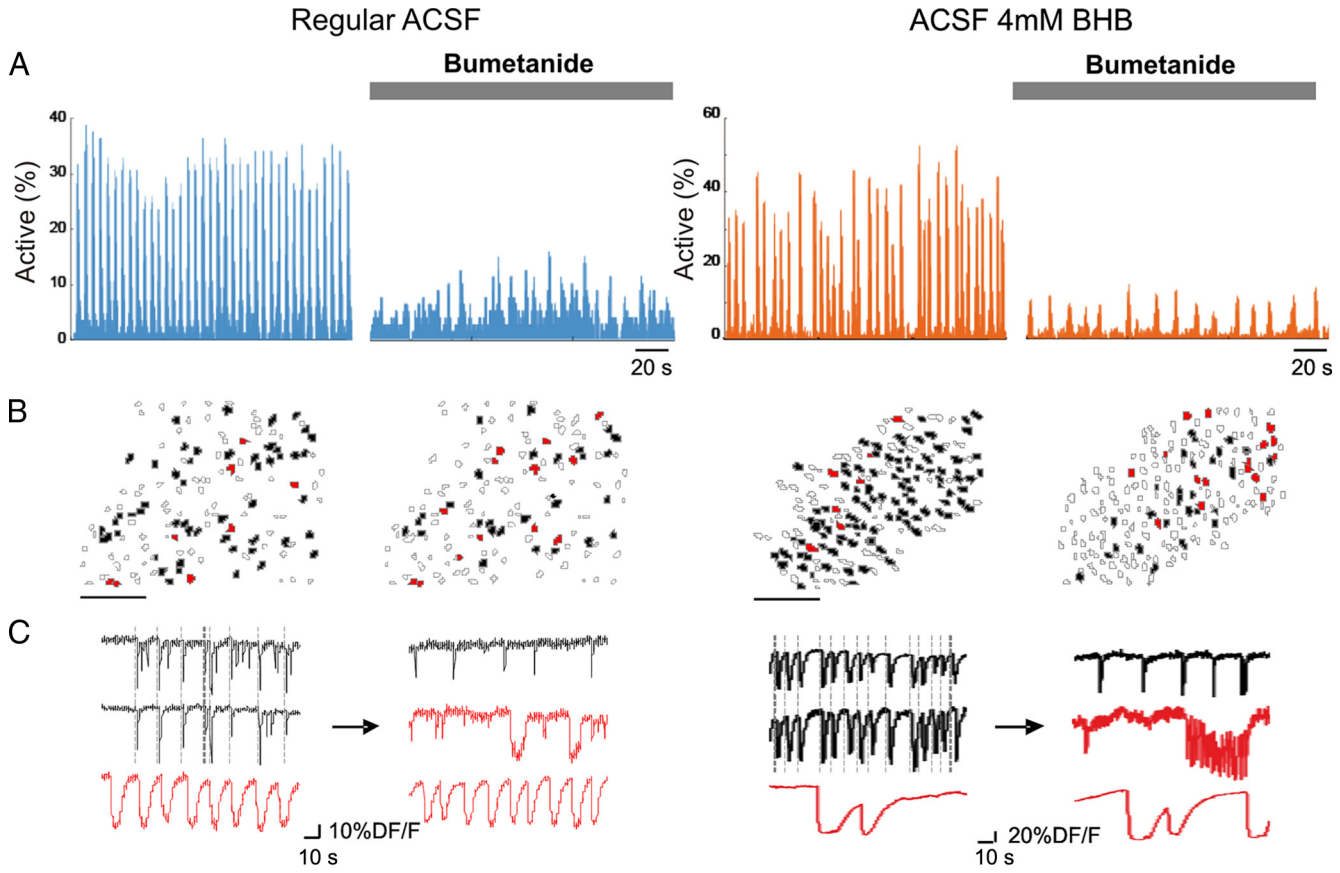

Figure 5. DL-BHB does not alter spontaneous neuronal activity patterns in neocortical slices. ***A***, Histograms indicating the fraction of active cells as a function of time in calcium movies in regular ACSF and in the presence of 4 mM DL-BHB (Sigma-Aldrich). Each peak of synchronous neuronal activity in the histograms corresponds to a GDP. GDPs were strongly reduced in the presence of the NKCC1 antagonist bumetanide (10 μM) in neocortical slices from P7 rats. ***B***, Automatically detected contours of the imaged cells: open contours indicate silent cells, black filled contours indicate cells involved in GDPs, and red filled contours are SPA cells. Note that the number of SPA cells relative to the number of active cells increased in the presence of 10 μM bumetanide in the neocortex and hippocampus (scale bar, 100 μm). ***C***, Calcium fluorescence traces of representative cells implicated in GDPs (black) and SPAs (red). Note that some GDP cells display an SPA pattern of activity after adding bumetanide (middle traces).

Table 3. Dynamics of cortical GDPs and SPAs in regular ACSF and in the presence of DL-BHB (Sigma-Aldrich)

	Regular ACSF	ACSF + DL-BHB at 4 mM
n	7	5
GDP_{freq} (Hz)	0.12 ± 0.02	0.14 ± 0.05
GDP_{amp} (%)	22 ± 3	22 ± 4
SPA cells (%)	8 ± 2	17 ± 11

GDP_{freq}, Frequency of occurrence of GDPs (in hertz); GDP_{amp}, fraction of active cells involved in GDPs (percentage); SPA cells, fraction of active cells involved in SPAs (percentage).

Discussion

We show that neither DL-BHB, used in similar concentrations as Zilberter and colleagues, nor pyruvate at physiologically relevant concentrations alters GABA depolarizing actions and GDPs, suggesting that depolarizing GABA and GDPs in immature neurons are not attributable "to energy deprived conditions when relying only on glucose" (Rheims et al., 2009; Holmgren et al., 2010).

Possible reasons for the discrepancies

DL-BHB at 4 mM (Sigma-Aldrich) had no effects on (1) E_m, E_{GABA}, and DF_{GABA} in somatic recordings of single GABA and NMDA channels, (2) E_m, synaptic GABAergic potentials and the responses evoked by GABA application in gramicidin perforated patch recordings, (3) spikes generated by GABA in cell-attached recordings, and (4) frequency of GDPs or SPAs relying on calcium imaging and electrophysiological recordings.

The wide range of E_{GABA} values reported by Holmgren et al. (2010) suggests a heterogeneity possibly attributable to pooling different ages particularly between P1 and P4 in which major shifts occur in E_{GABA} (Tyzio et al., 2006, 2007) in superficial and deep neocortical layers neurons or CA1 and CA3 pyramidal neurons with their different age (Ben-Ari et al., 2007; Rheims et al., 2008). Additional explanations to the discrepancy between the present study and the work of Zilberter and colleagues include the experimental approaches to measure E_{GABA}. (1) Cell-attached recordings of GABA channels without potassium channel blockers in the pipette could be contaminated by potassium channels and shift the measures to potassium reversal. (2) Prolonged activation of GABA receptors by long (2 s) isoguvacine applications and voltage ramps at the peak of the response used to determine E_{GABA} can be associated with profound changes in ionic distribution. Here, we used brief (50–200 ms) isoguvacine applications and synaptic stimulations at different holding potentials to minimize the error. (3) The "random" burst protocol used to record the spikes evoked by synaptic activation of GABA receptors may introduce activity-dependent disturbance in ionic gradients (the same applies for repetitive puff application of isoguvacine). This was controlled in the present study by wash in

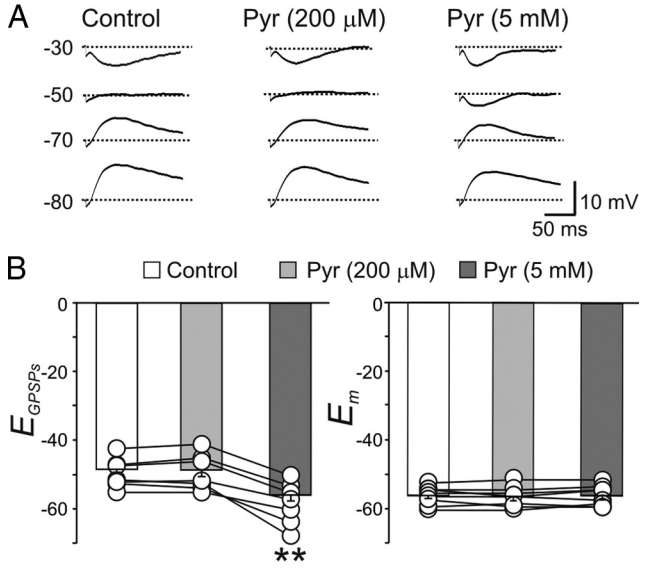

Figure 6. E_{GPSPs} shift in the presence of high but not low concentrations of pyruvate. ***A***, Examples of $GABA_A$-mediated postsynaptic potentials evoked in control (white, left), in the presence of 200 μM pyruvate (Pyr) (light gray, middle), and in the presence of 5 mM pyruvate (dark gray, right). ***B***, Open circles represent E_{GPSP} (graph on the left) and E_m (graph on the right) obtained in individual cells recorded in normal ACSF (white columns; left), in ACSF containing 200 μM (light gray; middle) or 5 mM (dark gray; right) pyruvate ($n = 7$). Bars at the top of the columns represent the SEM. Note the negative shift of E_{GPSPs} but not in E_m of cells exposed to 5 mM pyruvate. The mean E_{GPSP} value obtained in 5 mM pyruvate was significantly different from that obtained in control ($p = 0.003$) and in 200 μM pyruvate ($p = 0.005$). $^{**}p < 0.01$.

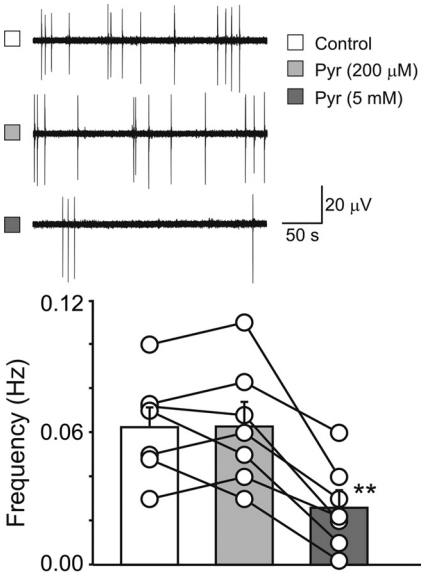

Figure 7. High but not low concentrations of pyruvate reduce GDPs frequency. On the left, sample traces of spontaneous GDPs recorded from the same slice before (white symbol; top trace) or during exposure to 200 μM pyruvate (Pyr) (light gray; middle trace) and 5 mM pyruvate (dark gray; bottom trace). Note the reduction in GDPs frequency with 5 mM but not 200 μM pyruvate. On the right, each symbol represents the mean frequency value of GDPs obtained in individual slices before (white column) or during exposure to 200 μM (light gray) and 5 mM (dark gray; right) pyruvate ($n = 7$). Bars at the top of the columns represent the SEM. The mean GDPs frequency value obtained in 5 mM pyruvate was significantly different from that obtained in control ($p = 0.005$) and in 200 μM pyruvate ($p = 0.005$). $^{*}p < 0.05$.

and washout of bicuculline or bumetanide. Finally, the non-invasive quantitative determination of the percentage of neurons active during SPAs and GDPs suggests that both DL-BHB and pyruvate have no effects on these patterns.

The presence of a contaminant dibenzylamine in DL-BHB (Acros Organics) that changes DF_{GABA} and E_{GABA} is important for future studies. DBA mediates effects thought previously to be attributable to DL-BHB blockade of cardiac K^+ channels and anticonvulsive actions of ketone bodies (Doepner et al., 1997, 2001; Rho et al., 2002; Donevan et al., 2003). Investigations using DL-BHB must take this parameter into account.

Physiologically relevant concentrations of D-BHB, lactate, and pyruvate

Neonatal plasma concentrations of D-BHB (present results; see also Nehlig and Pereira de Vasconcelos, 1993; Lust et al., 2003; Vannucci and Simpson, 2003; Erecinska et al., 2004; Nehlig, 2004) are significantly lower than those used by Rheims et al. (2009) and Holmgren et al. (2010), particularly because only a fraction (40%) of plasma D-BHB is found in neonatal cortex (Lust et al., 2003). In addition, the argument that 4 mM DL-BHB is equivalent to 2 mM active D-BHB (Rheims et al., 2009) is invalidated by the observations that L-BHB exerts complex biological actions (Moore et al., 1976; Webber and Edmond, 1977; Herzberg and Gad, 1984; Eaton et al., 2003; Tsai et al., 2006; Chou et al., 2008). Also, ketogenic diet reduces glucose utilization by 10% per millimolar plasma ketone bodies (Robinson and Williamson, 1980; Harding and Charlton, 1990; LaManna et al., 2009), hampering the interpretation and relevance of observations made using ACSF with glucose and DL-BHB.

The lactate (5 mM) and pyruvate (5 mM) concentrations used by Holmgren et al. (2010) are never observed in postnatal physiological conditions. Plasma lactate levels are high *in utero* (10 mM), shift to 3 mM during the presuckling period, and remain thereafter close to 1 mM (Medina, 1985), and even then glucose supplies most of the energy (Burd et al., 1975; Jones et al., 1975; Pégorier et al., 1977). The plasma lactate/pyruvate ratio is close to 10 (with 100 μM pyruvate), and higher lactate and (or) lactate/pyruvate ratios are only observed in dystonia, subarachnoid hemorrhage, brain traumas, epilepsies, pyruvate dehydrogenase mutations, and other severe pathological conditions (Owen et al., 1967; Medina, 1985; Fernandez et al., 1986; Mintun et al., 2004; Bjerring et al., 2008; Brody et al., 2008; Rex et al., 2009). In keeping with this, we found pyruvate levels ~120 μM that are identical to human levels and 40-fold lower than the concentrations used by Zilberter and colleagues. At physiological levels, pyruvate and lactate had no effects on excitatory actions of GABA, DF_{GABA}, and GDPs.

The reduction of DF_{GABA} and GDPs by exceedingly high concentrations of pyruvate (5 mM) is most likely attributable to the acidosis produced at these concentrations. Agents that alter tissue pH alter intracellular chloride (Kaila and Voipio, 1987; Kaila et al., 1993; Chesler, 2003; Glykys et al., 2009; Kim and Trussell, 2009), neuronal excitability, and seizures (Roos and Boron, 1981; Aram and Lodge, 1987; Balestrino and Somjen, 1988; Jarolimek et al., 1989; Kaila, 1994; Bonnet et al., 2000; Dulla et al., 2005, 2009; Ziemann et al., 2008). Propionate, D- and L-lactate reduce GDPs, although only L-lactate is metabolically active (Roos and Boron, 1981; Dulla et al., 2005; Ruussuvori et al., 2010). Therefore, depolarizing actions of GABA are not attributable to the absence of DL-BHB or lactate/pyruvate in the ACSF.

Ketone bodies and GABA signaling

Ketosis (Nehlig and Pereira de Vasconcelos, 1993), like transporters that import D-BHB (Pellerin et al., 1998; Magistretti et al., 1999; Bergersen et al., 2002; Pierre et al., 2002; Rafiki et al., 2003; Vannucci and Simpson, 2003; Erecinska et al., 2004) or BHB

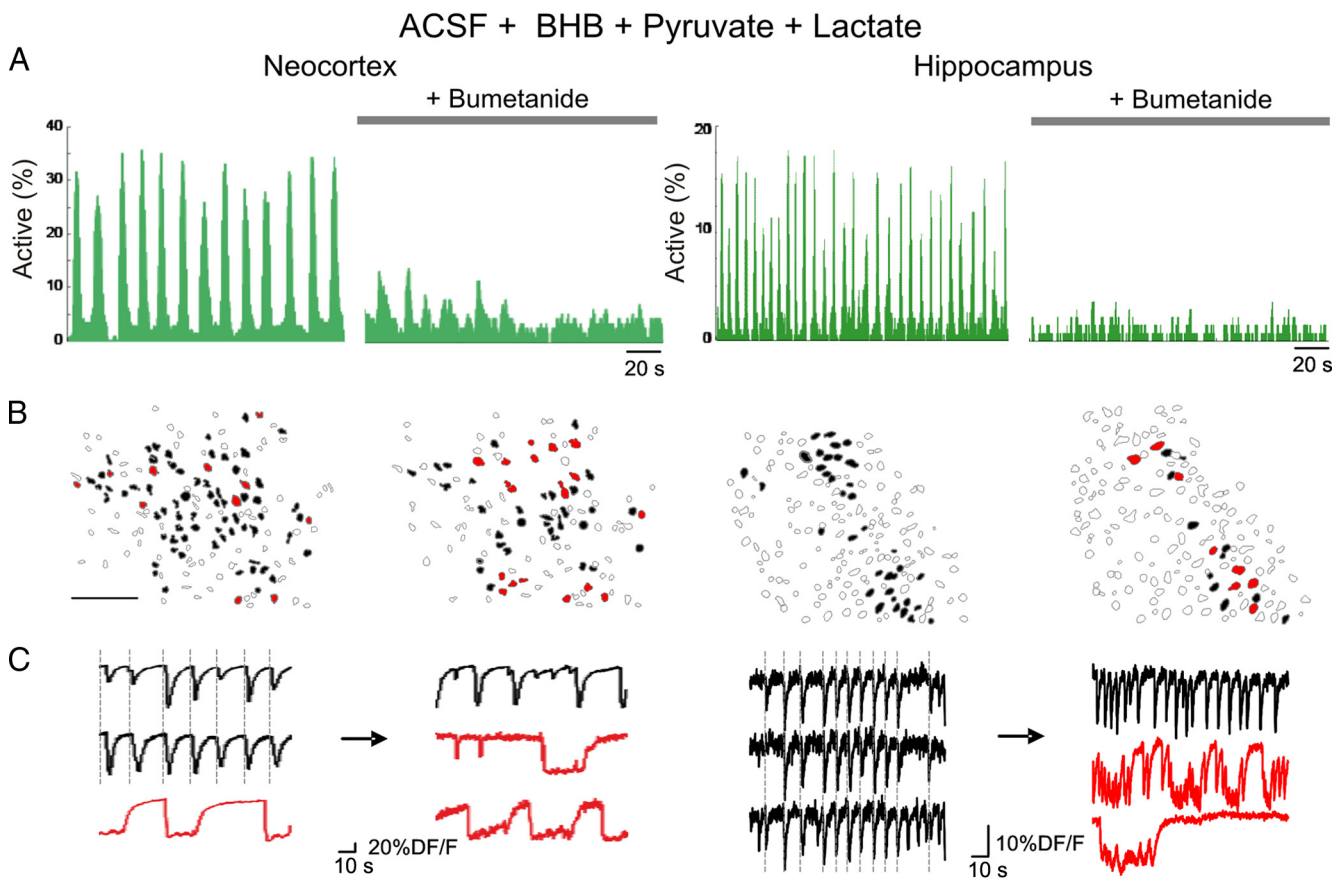

Figure 8. GDPs recorded in the presence of physiological concentrations of lactate, pyruvate, and DL-BHB are similar to those recorded in regular ACSF and are reduced by bumetanide. ***A***, Histograms indicating the fraction of active cells as a function of time in calcium movies in slices incubated with 1.5 mM lactate, 150 μM pyruvate, 5 mM glucose, and 4 mM DL-BHB (Sigma-Aldrich) for at least 1 h. Each peak of synchronous neuronal activity in the histograms corresponds to a GDP. GDPs were strongly reduced in the presence of the NKCC1 antagonist bumetanide (10 μM) in neocortical and hippocampal slices from P7 rats. ***B***, Automatically detected contours of the imaged cells: open contours indicate silent cells, black filled contours indicate cells involved in GDPs, and red filled contours are SPA cells. Note that the number of SPA cells relative to the number of active cells increased in the presence of 10 μM bumetanide in both the neocortex and hippocampus (scale bar, 100 μm). ***C***, Calcium fluorescence traces of representative cells implicated in GDPs (black) and SPAs (red). Note that some GDP cells display an SPA pattern of activity after adding bumetanide (middle traces).

Table 4. Dynamics of cortical GDPs and SPAs in the neocortex and the hippocampus in the presence of physiological concentrations of lactate, pyruvate, and DL-BHB

ACSF and DL-BHB + Pyruvate + Lactate	Neocortex	Hippocampus
n	5	3
GDP_{freq} (Hz)	0.1 ± 0.02	0.07 ± 0.03
GDP_{amp} (%)	60 ± 10	13 ± 1
SPA cells (%)	16 ± 4	3 ± 0.003

GDP_{freq}, Frequency of occurrence of GDPs (in hertz); GDP_{amp}, fraction of active cells involved in GDPs (percentage); SPA cells, fraction of active cells involved in SPAs (percentage).

dehydrogenase that metabolize it (Page et al., 1971; De Vivo et al., 1975; Leong and Clark, 1984; Bilger and Nehlig, 1991; Clark et al., 1993), peak during development well after the GABA shift. Fatty acid oxidation supports gluconeogenesis (Pégorier et al., 1977), and ketosis acts to "spare glucose for the emergence of audition, vision and more integrated behavior whose appearance during brain maturation seems to critically relate upon active glucose supply" (Nehlig, 2004). GABA currents are not affected by DL-BHB and ketogenic diet reduces seizures generated by GABA receptor antagonists (Appleton and De Vivo, 1973, 1974; Bough and Eagles, 1999; Bough et al., 2000; Thio et al., 2000; Sullivan et al., 2003; Hartman et al., 2007; Yellen, 2008; Maalouf et al., 2009), suggesting, contrary to Zilberter and colleagues, that the antiepileptic actions of ketone bodies are not mediated by GABA signaling. GABA depolarizes immature neurons in nonmammalian animal species and *in utero* in rodents, suggesting that maternal milk and ketone bodies are not required for that effect (Akerman and Cline, 2006; Ben-Ari et al., 2007).

The NKCC1/KCC2 sequence in brain maturation

Contrary to the suggestions of Zilberter and colleagues (Rheims et al., 2009; Holmgren et al., 2010), extensive pharmacological, anatomical, and genetic observations suggest that the removal of chloride in neonatal pups heavily depends on KCC2 (Li et al., 2002; Rheims et al., 2008; Riekki et al., 2008; Zhu et al., 2008; Takayama and Inoue, 2010). The parallel alterations of KCC2 and GABA polarity have been confirmed in a large variety of animal species from invertebrates to humans (Rivera et al., 1999, 2005; Delpire, 2000; Payne et al., 2003; Sernagor et al., 2003; Dzhala et al., 2005; Akerman and Cline, 2006; Liu et al., 2006; Ben-Ari et al., 2007; Howard et al., 2007; Kahle et al., 2008; Reynolds et al., 2008; Blaesse et al., 2009; Glykys et al., 2009; Stil et al., 2009; Tanis et al., 2009; Boulenguez et al., 2010). KCC2 and E_{GABA} developmental sequences are unlikely to depend on global metabolic diets because they are cell and sex specific (Kandler and Friauf, 1995; Kandler et al., 2002; Balakrishnan et al., 2003; Gulacsi et al., 2003; Lee et al., 2005; Lohrke et al., 2005; Banke and McBain, 2006; Blaesse et al., 2006; Kim and Trussell, 2009; Belenky et al., 2010). Neurons in which GABA remains depolar-

izing do not express KCC2 (Price et al., 2005; Gilbert et al., 2007; Pozas et al., 2008), and early overexpression of KCC2 in zebra fish embryos (Reynolds et al., 2008) or cortical neurons (Chudotvorova et al., 2005; Lee et al., 2005; Ben-Ari et al., 2007; Cancedda et al., 2007; Wang and Kriegstein, 2008) alter GABA polarity, GABA synapse formation, and neuronal development *in vivo*. KCC2 is downregulated by activity and E_{GABA} shifts accordingly (Woodin et al., 2003; Fiumelli et al., 2005).

Neonatal slices are not energy deprived in 10 mM glucose because they have a low rate of oxygen and glucose consumption and are less susceptible to energy deprivation than adult slices (Cherubini et al., 1989; Novotny et al., 2001; Tyzio et al., 2006). Lowering glucose reduces GDPs, ATP levels, and mitochondrial pH, confirming the sensitivity of GDPs to energy deprivation, but these are not restored by lactate (Takata and Okada, 1995; Wada et al., 1998; Takata et al., 2001) (Ruussuvori et al., unpublished report). Slices and intact hippocampi sustain GDPs for hours (Ben-Ari et al., 1989; Cherubini et al., 1989; Khalilov et al., 1997, 2003; Leinekugel et al., 1997; Safiulina et al., 2006) and are replaced by glutamate-driven early network oscillations when energy is deprived (Allène et al., 2008).

We conclude that the depolarizing action of GABA and related network-driven GDPs in immature cortical slices are not attributable to metabolic insufficiency. Therefore, conventional glucose containing ACSF provides adequate energy supply for cortical slices *in vitro*.

References

Akerman CJ, Cline HT (2006) Depolarizing GABAergic conductances regulate the balance of excitation to inhibition in the developing retinotectal circuit *in vivo*. J Neurosci 26:5117–5130.

Allène C, Cattani A, Ackman JB, Bonifazi P, Aniksztejn L, Ben-Ari Y, Cossart R (2008) Sequential generation of two distinct synapse-driven network patterns in developing neocortex. J Neurosci 28:12851–12863.

Appleton DB, De Vivo DC (1973) An experimental animal model for the effect of ketogenic diet on epilepsy. Proc Aust Assoc Neurol 10:75–80.

Appleton DB, DeVivo DC (1974) An animal model for the ketogenic diet. Epilepsia 15:211–227.

Aram JA, Lodge D (1987) Epileptiform activity induced by alkalosis in rat neocortical slices: block by antagonists of *N*-methyl-D-aspartate. Neurosci Lett 83:345–350.

Balakrishnan V, Becker M, Löhrke S, Nothwang HG, Güresir E, Friauf E (2003) Expression and function of chloride transporters during development of inhibitory neurotransmission in the auditory brainstem. J Neurosci 23:4134–4145.

Balestrino M, Somjen GG (1988) Concentration of carbon dioxide, interstitial pH and synaptic transmission in hippocampal formation of the rat. J Physiol 396:247–266.

Banke TG, McBain CJ (2006) GABAergic input onto CA3 hippocampal interneurons remains shunting throughout development. J Neurosci 26:11720–11725.

Barry PH, Lynch JW (1991) Liquid junction potentials and small cell effects in patch-clamp analysis. J Membr Biol 121:101–117.

Belenky MA, Sollars PJ, Mount DB, Alper SL, Yarom Y, Pickard GE (2010) Cell-type specific distribution of chloride transporters in the rat suprachiasmatic nucleus. Neuroscience 165:1519–1537.

Ben-Ari Y (2001) Developing networks play similar melody. Trends Neurosci 24:354–360.

Ben-Ari Y (2002) Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 3:728–739.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303–325.

Ben-Ari Y, Gaiarsa JL, Tyzio R, Khazipov R (2007) GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 87:1215–1284.

Bergersen L, Rafiki A, Ottersen OP (2002) Immunogold cytochemistry identifies specialized membrane domains for monocarboxylate transport in the central nervous system. Neurochem Res 27:89–96.

Bilger A, Nehlig A (1991) Quantitative histochemical changes in enzymes involved in energy metabolism in the rat brain during postnatal development. I. Cytochrome oxidase and lactate dehydrogenase. Int J Dev Neurosci 9:545–553.

Bjerring PN, Hauerberg J, Frederiksen HJ, Jorgensen L, Hansen BA, Tofteng F, Larsen FS (2008) Cerebral glutamine concentration and lactate-pyruvate ratio in patients with acute liver failure. Neurocrit Care 9:3–7.

Blaesse P, Guillemin I, Schindler J, Schweizer M, Delpire E, Khiroug L, Friauf E, Nothwang HG (2006) Oligomerization of KCC2 correlates with development of inhibitory neurotransmission. J Neurosci 26:10407–10419.

Blaesse P, Airaksinen MS, Rivera C, Kaila K (2009) Cation-chloride cotransporters and neuronal function. Neuron 61:820–838.

Bonifazi P, Goldin M, Picardo MA, Jorquera I, Cattani A, Bianconi G, Represa A, Ben-Ari Y, Cossart R (2009) GABAergic hub neurons orchestrate synchrony in developing hippocampal networks. Science 326:1419–1424.

Bonnet U, Leniger T, Wiemann M (2000) Alteration of intracellular pH and activity of CA3-pyramidal cells in guinea pig hippocampal slices by inhibition of transmembrane acid extrusion. Brain Res 872:116–124.

Bough KJ, Eagles DA (1999) A ketogenic diet increases the resistance to pentylenetetrazole-induced seizures in the rat. Epilepsia 40:138–143.

Bough KJ, Yao SG, Eagles DA (2000) Higher ketogenic diet ratios confer protection from seizures without neurotoxicity. Epilepsy Res 38:15–25.

Boulenguez P, Liabeuf S, Bos R, Bras H, Jean-Xavier C, Brocard C, Stil A, Darbon P, Cattaert D, Delpire E, Marsala M, Vinay L (2010) Down-regulation of the potassium-chloride cotransporter KCC2 contributes to spasticity after spinal cord injury. Nat Med 16:302–307.

Brody DL, Magnoni S, Schwetye KE, Spinner ML, Esparza TJ, Stocchetti N, Zipfel GJ, Holtzman DM (2008) Amyloid-beta dynamics correlate with neurological status in the injured human brain. Science 321:1221–1224.

Burd LI, Jones MD Jr, Simmons MA, Makowski EL, Meschia G, Battaglia FC (1975) Placental production and foetal utilisation of lactate and pyruvate. Nature 254:710–711.

Cancedda L, Fiumelli H, Chen K, Poo MM (2007) Excitatory GABA action is essential for morphological maturation of cortical neurons *in vivo*. J Neurosci 27:5224–5235.

Cherubini E, Ben-Ari Y, Krnjević K (1989) Anoxia produces smaller changes in synaptic transmission, membrane potential, and input resistance in immature rat hippocampus. J Neurophysiol 62:882–895.

Chesler M (2003) Regulation and modulation of pH in the brain. Physiol Rev 83:1183–1221.

Chou YC, Tsai YC, Chen CM, Chen SM, Lee JA (2008) Determination of lipoprotein lipase activity in post heparin plasma of streptozotocin-induced diabetic rats by high-performance liquid chromatography with fluorescence detection. Biomed Chromatogr 22:502–510.

Chudotvorova I, Ivanov A, Rama S, Hübner CA, Pellegrino C, Ben-Ari Y, Medina I (2005) Early expression of KCC2 in rat hippocampal cultures augments expression of functional GABA synapses. J Physiol 566:671–679.

Clark JB, Bates TE, Cullingford T, Land JM (1993) Development of enzymes of energy metabolism in the neonatal mammalian brain. Dev Neurosci 15:174–180.

Crépel V, Aronov D, Jorquera I, Represa A, Ben-Ari Y, Cossart R (2007) A parturition-associated nonsynaptic coherent activity pattern in the developing hippocampus. Neuron 54:105–120.

Delpire E (2000) Cation-chloride cotransporters in neuronal communication. News Physiol Sci 15:309–312.

DeVivo DC, Leckie MP, Agrawal HC (1975) D-beta-Hydrozybutyrate: a major precursor of amino acids in developing rat brain. J Neurochem 25:161–170.

Doepner B, Thierfelder S, Hirche H, Benndorf K (1997) 3-hydroxybutyrate blocks the transient K^+ outward current in myocardial mouse cells in a stereoselective fashion. J Physiol 500:85–94 [retraction in J Physiol (1998) 508:956].

Doepner B, Koopmann R, Knopp A, Hirche H, Benndorf K (2001) Dibenzylamine: a novel blocker of the voltage-dependent K^+ current in myocardial mouse cells. Naunyn Schmiedebergs Arch Pharmacol 364:9–13.

Donevan SD, White HS, Anderson GD, Rho JM (2003) Voltage-dependent block of *N*-methyl-D-aspartate receptors by the novel anticonvulsant dibenzylamine, a bioactive constituent of L-(+)-beta-hydroxybutyrate. Epilepsia 44:1274–1279.

Dulla CG, Dobelis P, Pearson T, Frenguelli BG, Staley KJ, Masino SA (2005)

Adenosine and ATP link PCO2 to cortical excitability via pH. Neuron 48:1011–1023.
Dulla CG, Frenguelli BG, Staley KJ, Masino SA (2009) Intracellular acidification causes adenosine release during states of hyperexcitability in the hippocampus. J Neurophysiol 102:1984–1993.
Dzhala V, Desfreres L, Melyan Z, Ben-Ari Y, Khazipov R (1999) Epileptogenic action of caffeine during anoxia in the neonatal rat hippocampus. Ann Neurol 46:95–102.
Dzhala VI, Talos DM, Sdrulla DA, Brumback AC, Mathews GC, Benke TA, Delpire E, Jensen FE, Staley KJ (2005) NKCC1 transporter facilitates seizures in the developing brain. Nat Med 11:1205–1213.
Eaton S, Chatziandreou I, Krywawych S, Pen S, Clayton PT, Hussain K (2003) Short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency associated with hyperinsulinism: a novel glucose-fatty acid cycle? Biochem Soc Trans 31:1137–1139.
Erecinska M, Cherian S, Silver IA (2004) Energy metabolism in mammalian brain during development. Prog Neurobiol 73:397–445.
Fernandez F, Verdu A, Quero J, Ferreiros MC, Daimiel E, Roche MC, Lopez-Martin V (1986) Cerebrospinal fluid lactate levels in term infants with perinatal hypoxia. Pediatr Neurol 2:39–42.
Ferré P, Satabin P, Decaux JF, Escriva F, Girard J (1983) Development and regulation of ketogenesis in hepatocytes isolated from newborn rats. Biochem J 214:937–942.
Fiumelli H, Cancedda L, Poo MM (2005) Modulation of GABAergic transmission by activity via postsynaptic Ca^{2+}-dependent regulation of KCC2 function. Neuron 48:773–786.
Ganguly K, Schinder AF, Wong ST, Poo M (2001) GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105:521–532.
Garaschuk O, Hanse E, Konnerth A (1998) Developmental profile and synaptic origin of early network oscillations in the CA1 region of rat neonatal hippocampus. J Physiol 507:219–236.
Gilbert D, Franjic-Würtz C, Funk K, Gensch T, Frings S, Möhrlen F (2007) Differential maturation of chloride homeostasis in primary afferent neurons of the somatosensory system. Int J Dev Neurosci 25:479–489.
Glykys J, Dzhala VI, Kuchibhotla KV, Feng G, Kuner T, Augustine G, Bacskai BJ, Staley KJ (2009) Differences in cortical versus subcortical GABAergic signaling: a candidate mechanism of electroclinical uncoupling of neonatal seizures. Neuron 63:657–672.
Gulácsi A, Lee CR, Sík A, Viitanen T, Kaila K, Tepper JM, Freund TF (2003) Cell type-specific differences in chloride-regulatory mechanisms and $GABA_A$ receptor-mediated inhibition in rat substantia nigra. J Neurosci 23:8237–8246.
Harding JE, Charlton VE (1990) Effect of lactate and beta-hydroxybutyrate infusions on brain metabolism in the fetal sheep. J Dev Physiol 14:139–146.
Hartman AL, Gasior M, Vining EP, Rogawski MA (2007) The neuropharmacology of the ketogenic diet. Pediatr Neurol 36:281–292.
Herzberg GR, Gad M (1984) Evidence that the cytosolic activity of 3-hydroxybutyrate dehydrogenase in chicken liver is L-3-hydroxyacid dehydrogenase. Biochim Biophys Acta 802:67–70.
Holmgren CD, Mukhtarov M, Malkov AE, Popova IY, Bregestovski P, Zilberter Y (2010) Energy substrate availability as a determinant of neuronal resting potential, GABA signaling and spontaneous network activity in the neonatal cortex in vitro. J Neurochem 112:900–912.
Howard MA, Burger RM, Rubel EW (2007) A developmental switch to GABAergic inhibition dependent on increases in Kv1-type K^+ currents. J Neurosci 27:2112–2123.
Jarolimek W, Misgeld U, Lux HD (1989) Activity dependent alkaline and acid transients in guinea pig hippocampal slices. Brain Res 505:225–232.
Jones MD Jr, Burd LI, Makowski EL, Meschia G, Battaglia FC (1975) Cerebral metabolism in sheep: a comparative study of the adult, the lamb, and the fetus. Am J Physiol 229:235–239.
Kahle KT, Staley KJ, Nahed BV, Gamba G, Hebert SC, Lifton RP, Mount DB (2008) Roles of the cation-chloride cotransporters in neurological disease. Nat Clin Pract Neurol 4:490–503.
Kaila K (1994) Ionic basis of GABAA receptor channel function in the nervous system. Prog Neurobiol 42:489–537.
Kaila K, Voipio J (1987) Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. Nature 330:163–165.
Kaila K, Voipio J, Paalasmaa P, Pasternack M, Deisz RA (1993) The role of bicarbonate in GABAA receptor-mediated IPSPs of rat neocortical neurones. J Physiol 464:273–289.
Kandler K, Friauf E (1995) Development of glycinergic and glutamatergic synaptic transmission in the auditory brainstem of perinatal rats. J Neurosci 15:6890–6904.
Kandler K, Kullmann PH, Ene FA, Kim G (2002) Excitatory action of an immature glycinergic/GABAergic sound localization pathway. Physiol Behav 77:583–587.
Khalilov I, Esclapez M, Medina I, Aggoun D, Lamsa K, Leinekugel X, Khazipov R, Ben-Ari Y (1997) A novel in vitro preparation: the intact hippocampal formation. Neuron 19:743–749.
Khalilov I, Holmes GL, Ben-Ari Y (2003) In vitro formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures. Nat Neurosci 6:1079–1085.
Kim Y, Trussell LO (2009) Negative shift in the glycine reversal potential mediated by a Ca^{2+}- and pH-dependent mechanism in interneurons. J Neurosci 29:11495–11510.
LaManna JC, Salem N, Puchowicz M, Erokwu B, Koppaka S, Flask C, Lee Z (2009) Ketones suppress brain glucose consumption. Adv Exp Med Biol 645:301–306.
Lee H, Chen CX, Liu YJ, Aizenman E, Kandler K (2005) KCC2 expression in immature rat cortical neurons is sufficient to switch the polarity of GABA responses. Eur J Neurosci 21:2593–2599.
Leinekugel X, Medina I, Khalilov I, Ben-Ari Y, Khazipov R (1997) Ca^{2+} oscillations mediated by the synergistic excitatory actions of $GABA_A$ and NMDA receptors in the neonatal hippocampus. Neuron 18:243–255.
Leong SF, Clark JB (1984) Regional enzyme development in rat brain. Enzymes of energy metabolism. Biochem J 218:139–145.
Li H, Tornberg J, Kaila K, Airaksinen MS, Rivera C (2002) Patterns of cation-chloride cotransporter expression during embryonic rodent CNS development. Eur J Neurosci 16:2358–2370.
Liu Z, Neff RA, Berg DK (2006) Sequential interplay of nicotinic and GABAergic signaling guides neuronal development. Science 314:1610–1613.
Löhrke S, Srinivasan G, Oberhofer M, Doncheva E, Friauf E (2005) Shift from depolarizing to hyperpolarizing glycine action occurs at different perinatal ages in superior olivary complex nuclei. Eur J Neurosci 22:2708–2722.
Lust WD, Pundik S, Zechel J, Zhou Y, Buczek M, Selman WR (2003) Changing metabolic and energy profiles in fetal, neonatal, and adult rat brain. Metab Brain Dis 18:195–206.
Maalouf M, Rho JM, Mattson MP (2009) The neuroprotective properties of calorie restriction, the ketogenic diet, and ketone bodies. Brain Res Rev 59:293–315.
Magistretti PJ, Pellerin L, Rothman DL, Shulman RG (1999) Energy on demand. Science 283:496–497.
Medina JM (1985) The role of lactate as an energy substrate for the brain during the early neonatal period. Biol Neonate 48:237–244.
Mintun MA, Vlassenko AG, Rundle MM, Raichle ME (2004) Increased lactate/pyruvate ratio augments blood flow in physiologically activated human brain. Proc Natl Acad Sci U S A 101:659–664.
Moore TJ, Lione AP, Sugden MC, Regen DM (1976) Beta-hydroxybutyrate transport in rat brain: developmental and dietary modulations. Am J Physiol 230:619–630.
Nehlig A (2004) Brain uptake and metabolism of ketone bodies in animal models. Prostaglandins Leukot Essent Fatty Acids 70:265–275.
Nehlig A, Pereira de Vasconcelos A (1993) Glucose and ketone body utilization by the brain of neonatal rats. Prog Neurobiol 40:163–221.
Novotny EJ Jr, Ariyan C, Mason GF, O'Reilly J, Haddad GG, Behar KL (2001) Differential increase in cerebral cortical glucose oxidative metabolism during rat postnatal development is greater in vivo than in vitro. Brain Res 888:193–202.
Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG, Cahill GF Jr (1967) Brain metabolism during fasting. J Clin Invest 46:1589–1595.
Owens DF, Kriegstein AR (2002) Is there more to gaba than synaptic inhibition? Nat Rev Neurosci 3:715–727.
Owens DF, Boyce LH, Davis MB, Kriegstein AR (1996) Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci 16:6414–6423.
Page MA, Krebs HA, Williamson DH (1971) Activities of enzymes of

ketone-body utilization in brain and other tissues of suckling rats. Biochem J 121:49–53.
Payne JA, Rivera C, Voipio J, Kaila K (2003) Cation-chloride co-transporters in neuronal communication, development and trauma. Trends Neurosci 26:199–206.
Pégorier JP, Ferré P, Girard J (1977) The effects of inhibition of fatty acid oxidation in suckling newborn rats. Biochem J 166:631–634.
Pellerin L, Pellegri G, Martin JL, Magistretti PJ (1998) Expression of monocarboxylate transporter mRNAs in mouse brain: support for a distinct role of lactate as an energy substrate for the neonatal vs. adult brain. Proc Natl Acad Sci U S A 95:3990–3995.
Pierre K, Magistretti PJ, Pellerin L (2002) MCT2 is a major neuronal monocarboxylate transporter in the adult mouse brain. J Cereb Blood Flow Metab 22:586–595.
Pozas E, Paco S, Soriano E, Aguado F (2008) Cajal-Retzius cells fail to trigger the developmental expression of the Cl^- extruding co-transporter KCC2. Brain Res 1239:85–91.
Price TJ, Cervero F, de Koninck Y (2005) Role of cation-chloride-cotransporters (CCC) in pain and hyperalgesia. Curr Top Med Chem 5:547–555.
Rafiki A, Boulland JL, Halestrap AP, Ottersen OP, Bergersen L (2003) Highly differential expression of the monocarboxylate transporters MCT2 and MCT4 in the developing rat brain. Neuroscience 122: 677–688.
Rex A, Bert B, Fink H, Voigt JP (2009) Stimulus-dependent changes of extracellular glucose in the rat hippocampus determined by in vivo microdialysis. Physiol Behav 98:467–473.
Reynolds A, Brustein E, Liao M, Mercado A, Babilonia E, Mount DB, Drapeau P (2008) Neurogenic role of the depolarizing chloride gradient revealed by global overexpression of KCC2 from the onset of development. J Neurosci 28:1588–1597.
Rheims S, Minlebaev M, Ivanov A, Represa A, Khazipov R, Holmes GL, Ben-Ari Y, Zilberter Y (2008) Excitatory GABA in rodent developing neocortex in vitro. J Neurophysiol 100:609–619.
Rheims S, Holmgren CD, Chazal G, Mulder J, Harkany T, Zilberter T, Zilberter Y (2009) GABA action in immature neocortical neurons directly depends on the availability of ketone bodies. J Neurochem 110: 1330–1338.
Rho JM, Anderson GD, Donevan SD, White HS (2002) Acetoacetate, acetone, and dibenzylamine (a contaminant in l-(+)-beta-hydroxybutyrate) exhibit direct anticonvulsant actions in vivo. Epilepsia 43:358–361.
Riekki R, Pavlov I, Tornberg J, Lauri SE, Airaksinen MS, Taira T (2008) Altered synaptic dynamics and hippocampal excitability but normal long-term plasticity in mice lacking hyperpolarizing $GABA_A$ receptor-mediated inhibition in CA1 pyramidal neurons. J Neurophysiol 99: 3075–3089.
Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K (1999) The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251–255.
Rivera C, Voipio J, Kaila K (2005) Two developmental switches in GABAergic signalling: the K^+-Cl^- cotransporter KCC2 and carbonic anhydrase CAVII. J Physiol 562:27–36.
Robinson AM, Williamson DH (1980) Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiol Rev 60:143–187.
Roos A, Boron WF (1981) Regulation of intracellular pH in barnacle muscle. Kroc Found Ser 15:205–219.
Ruusuvuori E, Kirilkin I, Pandya N, Kaila K (2010) Spontaneous network events driven by depolarizing GABA action in neonatal hippocampal slices are not attributable to deficient mitochondrial energy metabolism. J Neurosci 30:15638–15642.
Safiulina VF, Fattorini G, Conti F, Cherubini E (2006) GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. J Neurosci 26:597–608.
Sernagor E, Young C, Eglen SJ (2003) Developmental modulation of retinal wave dynamics: shedding light on the GABA saga. J Neurosci 23:7621–7629.
Sipilä ST, Huttu K, Voipio J, Kaila K (2006) Intrinsic bursting of immature CA3 pyramidal neurons and consequent giant depolarizing potentials are driven by a persistent Na current and terminated by a slow Ca-activated K current. Eur J Neurosci 23:2330–2338.
Sivakumaran S, Mohajerani MH, Cherubini E (2009) At immature mossy-fiber–CA3 synapses, correlated presynaptic and postsynaptic activity persistently enhances GABA release and network excitability via BDNF and cAMP-dependent PKA. J Neurosci 29:2637–2647.
Stil A, Liabeuf S, Jean-Xavier C, Brocard C, Viemari JC, Vinay L (2009) Developmental up-regulation of the potassium-chloride cotransporter type 2 in the rat lumbar spinal cord. Neuroscience 164:809–821.
Sullivan PG, Dubé C, Dorenbos K, Steward O, Baram TZ (2003) Mitochondrial uncoupling protein-2 protects the immature brain from excitotoxic neuronal death. Ann Neurol 53:711–717.
Takata T, Okada Y (1995) Effects of deprivation of oxygen or glucose on the neural activity in the guinea pig hippocampal slice: intracellular recording study of pyramidal neurons. Brain Res 683:109–116.
Takata T, Sakurai T, Yang B, Yokono K, Okada Y (2001) Effect of lactate on the synaptic potential, energy metabolism, calcium homeostasis and extracellular glutamate concentration in the dentate gyrus of the hippocampus from guinea-pig. Neuroscience 104:371–378.
Takayama C, Inoue Y (2010) Developmental localization of potassium chloride co-transporter 2 (KCC2), GABA and vesicular GABA transporter (VGAT) in the postnatal mouse somatosensory cortex. Neurosci Res 67:137–148.
Tanis JE, Bellemer A, Moresco JJ, Forbush B, Koelle MR (2009) The potassium chloride cotransporter KCC-2 coordinates development of inhibitory neurotransmission and synapse structure in *Caenorhabditis elegans*. J Neurosci 29:9943–9954.
Thio LL, Wong M, Yamada KA (2000) Ketone bodies do not directly alter excitatory or inhibitory hippocampal synaptic transmission. Neurology 54:325–331.
Tsai YC, Chou YC, Wu AB, Hu CM, Chen CY, Chen FA, Lee JA (2006) Stereoselective effects of 3-hydroxybutyrate on glucose utilization of rat cardiomyocytes. Life Sci 78:1385–1391.
Tyzio R, Represa A, Jorquera I, Ben-Ari Y, Gozlan H, Aniksztejn L (1999) The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. J Neurosci 19:10372–10382.
Tyzio R, Ivanov A, Bernard C, Holmes GL, Ben-Ari Y, Khazipov R (2003) Membrane potential of CA3 hippocampal pyramidal cells during postnatal development. J Neurophysiol 90:2964–2972.
Tyzio R, Cossart R, Khalilov I, Minlebaev M, Hübner CA, Represa A, Ben-Ari Y, Khazipov R (2006) Maternal oxytocin triggers a transient inhibitory switch in GABA signaling in the fetal brain during delivery. Science 314:1788–1792.
Tyzio R, Holmes GL, Ben-Ari Y, Khazipov R (2007) Timing of the developmental switch in $GABA_A$ mediated signalling from excitation to inhibition in CA3 rat hippocampus using gramicidin perforated patch and extracellular recordings. Epilepsia 48:96–105.
Tyzio R, Minlebaev M, Rheims S, Ivanov A, Jorquera I, Holmes GL, Zilberter Y, Ben-Ari Y, Khazipov R (2008) Postnatal changes in somatic gamma-aminobutyric acid signalling in the rat hippocampus. Eur J Neurosci 27:2515–2528.
Vannucci SJ, Simpson IA (2003) Developmental switch in brain nutrient transporter expression in the rat. Am J Physiol Endocrinol Metab 285: E1127–E1134.
Vassault A, Bonnefont JP, Specola N, Saudubray JM (1991) Lactate, pyruvate and ketone bodies. In: Techniques in diagnostic human biochemical genetics a laboratory manual (Home FA, ed), pp 285–308. New York: Willey-Liss.
Wada H, Okada Y, Uzuo T, Nakamura H (1998) The effects of glucose, mannose, fructose and lactate on the preservation of neural activity in the hippocampal slices from the guinea pig. Brain Res 788:144–150.
Wang DD, Kriegstein AR (2008) GABA regulates excitatory synapse formation in the neocortex via NMDA receptor activation. J Neurosci 28:5547–5558.
Webber RJ, Edmond J (1977) Utilization of L(+)-3-hydroxybutyrate, D(−)-3-hydroxybutyrate, acetoacetate, and glucose for respiration and lipid synthesis in the 18-day-old rat. J Biol Chem 252:5222–5226.
Woodin MA, Ganguly K, Poo MM (2003) Coincident pre- and postsynaptic activity modifies GABAergic synapses by postsynaptic changes in Cl^- transporter activity. Neuron 39:807–820.
Yellen G (2008) Ketone bodies, glycolysis, and KATP channels in the mechanism of the ketogenic diet. Epilepsia 49 [Suppl 8]:80–82.
Zhu L, Polley N, Mathews GC, Delpire E (2008) NKCC1 and KCC2 prevent hyperexcitability in the mouse hippocampus. Epilepsy Res 79:201–212.
Ziemann AE, Schnizler MK, Albert GW, Severson MA, Howard MA 3rd, Welsh MJ, Wemmie JA (2008) Seizure termination by acidosis depends on ASIC1a. Nat Neurosci 11:816–822.

Chapter

In the Developing Hippocampus Kainate Receptors Control the Release of GABA from Mossy Fiber Terminals via a Metabotropic Type of Action

Enrico Cherubini,* Maddalena D. Caiati and Sudhir Sivakumaran

Abstract

Kainate receptors (KARs) are glutamate-gated ion channels, assembled from various combinations of GluK1-GluK5 subunits with different physiological and pharmacological properties. In the hippocampus, KARs expressed at postsynaptic site mediate a small component of excitatory postsynaptic currents while at presynaptic sites they exert a powerful control on transmitter release at both excitatory and inhibitory connections. KARs are developmentally regulated and play a key role in several developmental processes including neuronal migration, differentiation and synapse formation. Interestingly, they can signal through a canonical ionotropic pathway but also through a noncanonical modality involving pertussis toxin-sensitive G proteins and downstream signaling molecules.

In this Chapter some of our recent data concerning the functional role of presynaptic KARs in regulation of transmitter release from immature mossy fiber terminals and in synaptic plasticity processes will be reviewed. Early in postnatal development, MFs release into their targeted neurons mainly GABA which is depolarizing and excitatory. Endogenous activation of GluK1 KARs localized on MF terminals by glutamate present in the extracellular space down regulates GABA release, leading sometimes to synapse silencing. The depressant effect of GluK1 on MF responses is mediated by a metabotropic process, sensitive to pertussis toxin and phospholipase C (PLC) along the transduction pathway downstream to G protein activation. Blocking PLC with the selective antagonist U73122, unmasks the potentiating effect of GluK1 on MF-evoked GABAergic currents, which probably depend on the ionotropic type of action of these receptors.

In addition, GluK1 KARs dynamically regulate the direction of spike-time dependent plasticity, a particular form of Hebbian type of learning which consists in bidirectional modifications in synaptic strength according to the temporal order of pre and postsynaptic spiking. At immature MF-CA3 synapses pairing MF stimulation with postsynaptic spiking and vice versa induces long term depression of MF-evoked GABAergic currents. In the case of positive pairing synaptic depression can be switched into spike-time dependent potentiation by blocking GluK1 KARs with UBP 302. The depressant action exerted by GluK1 KARs on

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MF responses would prevent the excessive activation of the CA3 associative network by the excitatory action of GABA early in postnatal development.

Introduction

Glutamate receptors, which belong to the ligand-gated ion channels family, are integral membrane proteins mediating fast excitatory transmission in the brain. On the basis of their molecular, pharmacological and biophysical properties, three distinct receptor classes have been identified. They have been named after their respective agonists: *N*-methyl *D*-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. Among these, kainate receptors (KARs) have received particular attention only in recent years, due to the relatively late development of selective pharmacological tools which have allowed their specific functions to be distinguished from those of AMPA receptors.[1] Moreover, the generation of transgenic mice lacking specific KAR subunits has provided additional clues for understanding their respective role in synaptic transmission.[2]

Molecular cloning has identified five different KAR subunits, each of molecular mass of ~100 kDa, referred in accord to the new nomenclature as GluK1-GluK5,[3] which can co-assemble in various combinations to form functional receptor channels permeable to cations. In addition, RNA editing and alternative splicing further increase the likelihood of pharmacological and physiological heterogeneity in KAR family. While GluK1-GluK3 (formerly GluR5-7), when expressed in heterologous systems can form homomeric kainate-gated ion channels,[4-6] GluK4 and GluK5 (former KA1 and KA2) do not form channels on their own but only when co-expressed with other subunits.[7,8] These subunits are often termed the "high affinity" subunits because of their low nanomolar affinity for the seaweed toxin kainic acid.

KAR subunits are expressed throughout the CNS where they are distributed not only on dendrites and postsynaptic membranes but also predominantly on nerve fibers and synaptic terminals.[9] At postsynaptic site these receptors carry at least in part current charges of synaptic responses, while at presynaptic sites they exert a powerful control on transmitter release.[9]

In this Chapter we will focus mainly on KARs expressed in the hippocampus. Here, activation of KARs localized on CA3 pyramidal cells by glutamate released from mossy fiber terminals (MFs) induces slow excitatory postsynaptic currents (EPSCs) whose amplitude has been found to be strictly dependent on the pattern of stimulation.[10,11] MF-evoked synaptic currents involve GluK2 receptor subunits as demonstrated by the loss of slow kainate-mediated EPSCs in hippocampal slices from GluK2 KO mice.[12,13] In contrast, GluK2 KO mice still exhibit slow kainate-mediated EPSCs on GABAergic interneurons, suggesting the involvement of other KAR subtypes. The loss of kainate-mediated synaptic responses in GluK1 or GluK1/GluK2 KO mice suggests the involvement of GluK1 subunits.[14] The slower deactivation kinetics of kainate-mediated synaptic currents with respect to the AMPA ones, would allow integrating excitatory inputs on the targeted neuron over a larger time window.

Presynaptic Kainate Receptors Control the Release of Glutamate and GABA

In addition to their postsynaptic role, KARs play a crucial role in regulating transmitter release at both excitatory at inhibitory synapses. In particular, at excitatory synapses, kainate-induced increase in glutamate release contributes to short- and long-term synaptic plasticity processes. While in the case of excitatory synapses, KARs are activated by glutamate released from glutamatergic nerve endings in the case of inhibitory synapses KARs are stimulated by glutamate present in the extracellular medium or spilled over from adjacent excitatory synapses. At excitatory synapses, KARs have been shown to facilitate glutamate release, particularly during frequency-dependent facilitation, a form of short-term plasticity characteristic of MF-CA3 synapses[15-17] (but see Kwon and Castillo).[18] Synaptic facilitation can be mimicked by low concentrations of kainate while high concentrations of the agonist produce synaptic depression.[19] The

facilitatory effect of kainate has been attributed to kainate-induced depolarization of presynaptic boutons or axon terminals[16,20,21] and the release of calcium from local stores.[22] Similarly, KARs bi-directionally modify synaptic efficacy at inhibitory connections.[14,23-27] Here, the depressant effect of kainate on GABA release has been shown to be mediated by a different, noncanonical signaling modality, involving a metabotropic instead of a ionotropic type of action.[25] This may occur directly through a G-protein coupled KAR or indirectly through the participation of an intermediate or a "linker" protein. Whatever the case, this kind of response is usually sensitive to inhibitors of the putative metabotropic signaling pathway(s) thought to be involved. Thus, kainate-induced depression of IPSCs evoked in CA1 principal cells by stimulation of stratum oriens or stratum radiatum interneurons was found to be dependent on the activation of a pertussis toxin (PTX)-sensitive G protein and was suppressed by inhibitors of phospholipase C (PLC) and protein kinase C (PKC) but independent of presynaptic ion channel activity.[25] Furthermore, biochemical studies clearly demonstrated the involvement of $G_{i/o}$ proteins, PLC and PKC in kainate-induced depression of GABA release from hippocampal synaptosomes, which by definition are devoid of somato-dendritic compartments.[28,29] Altogether these data give direct support to the assumption that inhibition of GABA release by hetero-synaptically activated KARs localized on presynaptic GABAergic terminals occurs via a novel noncanonical metabotropic type of mechanism.

Similarly, at excitatory CA3-CA1, the depressant effect of kainate on glutamate release was demonstrated to be mediated by G-protein coupled KARs possibly through a direct interaction of βγ subunits of G proteins with presynaptic voltage-dependent calcium channels.[30] Unlike Schaffer collaterals, at mossy fibers (MF)-CA3 connections, the mechanisms involved in the depressant effect of high concentrations of kainate on glutamate release are still under debate. While early reports have attributed this effect to the ionotropic action of kainate which would induce a depolarization of presynaptic terminals with consequent inactivation of sodium, calcium channels and/or electrical shunting,[20,31,32] a more recent study has suggested a metabotropic type of mechanism.[33] In support of this hypothesis is the observation that the reduction of glutamate release by kainate was prevented by PTX, a $G_{i/o}$ inhibitor. In addition, the depressant effect of kainate on glutamate release from MF terminals was found to be dependent on the activity of adenylyl cyclase, cAMP/PKA signaling cascade.[33] Interestingly, KARs via a metabotropic type of action have been shown to contribute together with type II mGluRs to LTD induced at MF-CA3 synapses by low frequency stimulation of afferent inputs.[34,35]

Kainate Receptors Modulate Cell Excitability via a Metabotropic Type of Action

Kainate, at nanomolar concentrations, is known to increase cell excitability by inhibiting at postsynaptic level the calcium-activated potassium current (I_{sAHP}),[36,37-39] which contributes to spike frequency adaptation. This effect has been attributed to the metabotropic action of KARs since it involves a PTX-sensitive G-protein and downstream signaling pathways including PKC and PKA.[37-39] The concentrations of kainate needed to block I_{sAHP} (IC_{50} values 15 and 5 nM in CA1 and CA3 pyramidal cells, respectively) are within the range of high affinity KARs binding sites and within the range of GluK4 and GluK5 KAR subunits, suggesting the involvement of these subunits in regulating cell excitability. As expected the inhibition of I_{sAHP} by KA was lost in GluK5 knock out mice.[39] However, KA-induced inhibition of I_{sAHP} was lost also in GluK2 knock out mice,[38,39] probably due to the indirect loss of GluK5 because in the absence of GluK2 subunits, GluK5 are likely to undergo degradation.[40] In other words, GluK5 subunits assembled in heteromeric complexes with GluK2 subunits, would be critical for triggering the activation of G proteins. In contrast with this view, a recent study has shown that in double GluK4 and GluK5 KO mice low concentrations of kainate are still able to inhibit I_{sAHP} suggesting that high affinity GluK4 and GluK5 receptors, thought to be essential for the metabotropic type of action of KARs are not obligatory.[13]

The way in which these two different modalities (ionotropic and metabotropic) interact is intriguing and not fully understood. In particular, the molecular mechanisms by which KARs operate in a metabotropic modality are still unclear given that the receptors do not exhibit the classical topology of metabotropic G-protein-coupled receptors. A recent study has outlined the possibility that the dual modes of signaling interact to dynamically auto-regulate the number of KAR expressed on the cell surface.[41] It has been demonstrated that the repetitive activation of KARs, expressed in a recombinant system, is able to trigger their internalization via a G-protein dependent PKC phosphorylation mechanism, thus limiting their over-activation.[41]

Kainate Receptors in the Immature Hippocampus

Ionotropic glutamate receptors play key roles in multiple developmental mechanisms, including regulation of neuronal migration, differentiation and synapses formation. KARs are developmentally regulated, given their temporal and spatial expression is transient in some brain regions, compatible with their crucial role in controlling the maturation of neuronal networks.[42] The developmental profile of KARs gene expression is well preserved during evolution: it can be detected also in *Drosophila* during a major period of neurogenesis.[43] In an early study on the developing mouse brain, Bettler et al[44] found that gene transcripts for the GluK1 subunits are expressed in the entire CNS already between E10 and E14, a period of intense cellular differentiation. GluK1 transcript levels which are particularly pronounced in areas where synaptogenesis is in progress later in development become more spatially restricted and downregulated. In the hippocampus, high affinity kainate binding sites can be detected already at E14. GluK1-5 genes expression undergoes a peak in the late embryonic/early postnatal period (in coincidence with periods of intense synaptogenesis) and starts declining at P14.[45,46] In particular, the perinatal peak of GluK1 gene expression observed in the barrel cortex, septum, thalamus and CA1 interneurons is compatible with a role of this subunit in developmental plasticity. The disruption of barrel cortex formation after chronic blockade of ionotropic glutamate receptors (during the first 24 hours after birth) further supports this hypothesis.[47] The decline in KAR subunits expression observed at late developmental stages could be related to cell elimination, due to network remodeling. The permeability of some edited forms of GluK1 and GluK2 KAR subunits to calcium may contribute to limit neuronal number via their excitotoxic action which leads to cell death. Altogether, these observations indicate that KARs are essential for neuronal development.

In the hippocampus, mossy fibers, the axons of dentate gyrus granule cells, which are endowed with presynaptic KARs,[48] develop postnatally producing three types of connections with their targets: (i) mossy terminals which synapse with thorny excrescences of CA3 principal cells; (ii) small en passant terminals; (iii) filopodial extensions which specifically contact GABAergic interneurons.[49] In a study aimed at elucidating the motility of axonal filopodia and dendritic spines during postnatal development, a process thought to be involved in synapse formation and rearrangement, it was demonstrated that synaptic activation of KARs enhances motility at mossy fiber axons in younger hippocampal slice culture but inhibits it in more mature slices,[50] suggesting a key role for KARs for establishing synaptic contacts and for stabilizing newly formed ones.

More extensive studies relate to the functional role that KARs exert on network activity in the immature hippocampus. It is worth noting that correlated neuronal activity constitutes a hallmark of developmental networks, well preserved during evolution that has been observed not only in the hippocampus but in almost every brain structure examined, including the retina,[51] the neocortex,[52-55] the hypothalamus,[56] the cerebellum[57] and the spinal cord.[58,59] In the hippocampus, the so-called giant depolarizing potentials or GDPs are generated by the synergistic action of synaptically released glutamate and GABA, both of which are depolarizing and excitatory.[60,61] GDPs which have been proposed to be the in vitro counterpart of "sharp waves" recorded in rat pups during immobility periods, sleep and feeding[62] can be considered a primordial form of synchrony between neurons, which precedes more organized forms of activity such as the theta and the gamma rhythms crucial for information processing.[63] GDPs disappear spontaneously towards the end of the second postnatal week in concomitance with the shift of GABA

from the depolarizing to the hyperpolarizing direction. The strong developmental correlation between GDPs and KARs expression suggests that the latter are crucial for regulating early network activity which in turn contributes to sculpt neuronal circuits. In particular, signaling via presynaptic GluK1 containing KARs has been shown to be critical for regulating the number of functional glutamatergic synapses[64] and the balance between GABAergic and glutamatergic transmission which control GDPs.[27,65,66]

The hippocampal network comprises a large variety of distinct locally connected GABAergic interneurons which by pacing, timing and synchronizing principal cells give rise to coherent oscillations in both the adult[67] as well as in the immature brain.[68] Network synchronization is tightly controlled by the intrinsic properties of GABAergic interneurons[69] and by their electrical coupling via gap junctions.[70-72] Recent evidence indicates that tonic activation of GluK1 subunit-containing KARs by "ambient" glutamate can enhance, via a metabotropic action, the firing of neonatal interneurons by interfering with the calcium-dependent after hyperpolarization of medium duration (mAHP), generated by an apamin-sensitive K^+ current mediated by SK channels.[73] This effect is age-dependent since it disappears towards the end of the second postnatal week in concomitance with the uncoupling between KARs activation and mAHP. This mechanism together with the developmental shift of GABA from the depolarizing to the hyperpolarizing direction may contribute to the age-dependent disappearance of GDPs in the hippocampus.

Interestingly, at MF-CA3 connections presynaptic KARs are functional at early developmental stages,[27,66,67,74] while postsynaptic ones become operative only towards the end/beginning of the second postnatal week.[75] Thus, while in adulthood, stimulation of MF terminals evokes in CA3 principal cells excitatory postsynaptic currents (EPSCs) which carry at least in part current charges via KARs,[9,10,12,76] in neonates MFs terminals release mainly GABA (see below). A postsynaptic KAR-mediated component of the EPSC starts appearing during the second postnatal week when MFs acquire the classical glutamatergic phenotype in concomitance with the shift of GABA from the depolarizing to the hyperpolarizing direction.[75] The appearance of KAR-dependent EPSCs tightly coincides with the development of large amplitude AMPA-mediated EPSCs and with frequency-dependent facilitation. The lack of KARs-mediated postsynaptic components with summation properties which facilitate the temporal integration of synaptic inputs might change the way of synaptic signaling early in postnatal development. The lack of GluK1 and GluK2 KAR containing subunits would perturb the maturation of AMPA-mediated components of MF-EPSCs through pre and postsynaptic actions as assessed using mutant GluK1 and GluK2$^{-/-}$ mice.[75] Interestingly, GluK2 subunits of KARs interact with cell-adhesion molecules of the cadherin-catenin complex,[77] known to be involved in synapse formation together with the nectin-afadin system.[78] Therefore, the possibility that maturation of MF-CA3 synapses occurs via the mutual interaction of KARs with adhesion molecules represents an intriguing hypothesis.

GABAergic Phenotype of Immature MF Terminals

In adulthood, the primary excitatory neurotransmitter released from MF terminals is glutamate. However, in particular conditions, in addition to glutamate, MFs can release GABA. Thus, Gutiérrez and Heinemann[79] have convincingly demonstrated that in kindled rats monosynaptic bicuculline-sensitive GABAergic inhibitory postsynaptic potentials (IPSPs) occur in CA3 principal cells in response to stimulation of granule cells in the dentate gyrus. Interestingly, a high frequency input to pyramidal cells leads to IPSPs summation and consequent reduction in the probability of generating action potentials. It seems therefore likely that the release of GABA from MF terminals during seizures may act as a compensatory mechanism to counterbalance the enhanced excitability induced by the epileptic activity.[80] Interestingly, seizures have been shown to be associated with a transient up-regulation of presynaptic GABA ergic markers such as GAD_{67}, GAD_{65} and VGAT.[81-83] Moreover, hippocampal pyramidal neurons are able to express not only glutamate but also "mistargeted" $GABA_A$ receptors which, in particular conditions may become functional.[84] This suggests that MF can use GABA as a neurotransmitter since they posses all the machinery for synthesizing, storing, releasing and sensing it. Indeed, in juvenile

animals, stimulation of granule cells in the dentate gyrus induces monosynaptic GABAergic and glutamatergic responses in CA3 principal cells[85,86] and immunogold experiments have demonstrated that AMPA and $GABA_A$ receptors are colocalized on the same synapse in close apposition to MF terminals.[87]

In contrast, immediately after birth, GABA is the main neurotransmitter released from MF terminals. At this early developmental stage, MFs constitutively express GAD_{67} and its product GABA[81,82] as well as the mRNA for the vesicular GABA transporter VGAT.[88] Post embedding immunogold double labeling has revealed the coexistence in MF terminals of VGAT and VGLUT (the vesicular transporter for glutamate) further suggesting that GABA can be coreleased with glutamate.[89]

Thus, minimal stimulation of granule cells in the dentate gyrus evokes in CA3 principal cells monosynaptic currents that completely fulfill the criteria for MF identification. These currents which are insensitive to AMPA receptor antagonists[74] but sensitive to the selective group III metabotropic glutamate receptor agonist L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4), are readily blocked by picrotoxin, bicuculline or gabazine, suggesting that they are mediated by GABA acting on $GABA_A$ receptors (Fig. 1).

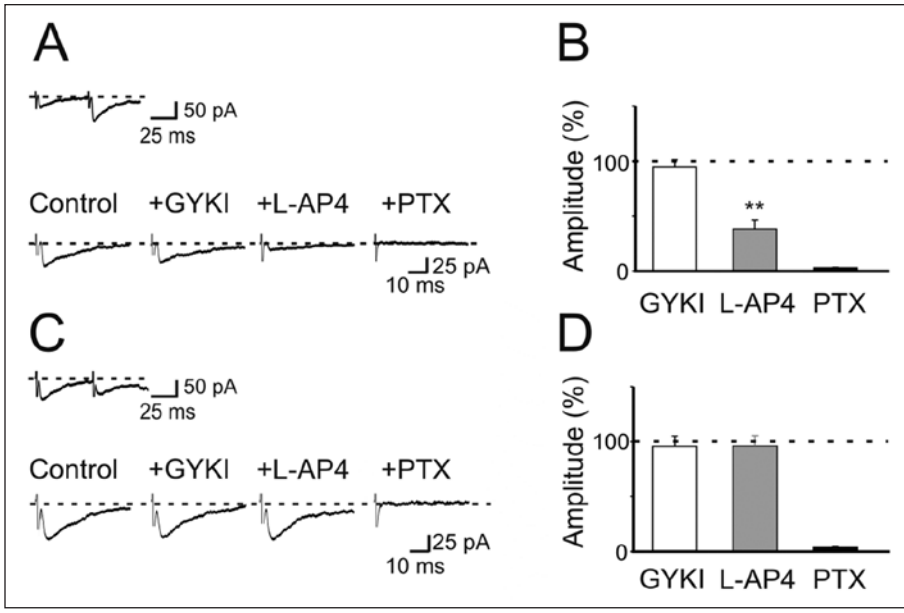

Figure 1. GABAergic origin of mossy fibers-evoked unitary postsynaptic currents in immature CA3 pyramidal cells. A) Unitary synaptic currents evoked in a CA3 pyramidal cell by minimal stimulation of granule cells in the dentate gyrus at P3 in control conditions, during application of GYKI 52466 (30 μM), GYKI plus L-AP4 (10 μM) and after addition of picrotoxin (PTX, 100 μM). Each trace is the average of 15-20 responses (including failures). Note that GYKI failed to modify synaptic currents. Synaptic currents were reduced in amplitude by L-AP4 and abolished by PTX. In the inset above the traces, MF-evoked GPSCs showing paired-pulse facilitation. B) Each column represents the mean peak amplitude current obtained in the experimental conditions shown in A and normalized to controls (dashed line; n = 6). In this and in the following figures, vertical bars refer to SEM. C) Example of GPSCs probably originated from a GABAergic interneuron impinging into a pyramidal cell exhibiting paired pulse depression. In this case, GPSCs were insensitive to L-AP4, but were blocked by PTX. D) As in B but for L-AP4 insensitive GABAergic interneurons (n = 7). $^{**}p < 0.01$.

As expected for $GABA_A$-mediated postsynaptic currents (GPSCs), synaptic responses can be potentiated by flurazepam, an allosteric modulator of $GABA_A$ receptors or by 1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO-711) a selective blocker of the GABA transporter GAT-1.[90] Additional evidence in favor of GABA as a neurotransmitter at MF-CA3 synapses is provided by the experiments in which, chemical stimulation of granule cell dendrites in stratum moleculare with glutamate (in the presence of the AMPA/kainate receptor antagonists to prevent the recruitment of GABAergic interneurons), depolarizes granule cells via activation of NMDA receptors and induces in CA3 principal cells barrages of GABAergic currents sensitive to L-AP4. Moving the pressure pipette few μm away towards the hilus to activate hilar interneurons causes barrage of $GABA_A$-mediated events insensitive to L-AP4, implying that they are mediated by the release of GABA from GABAergic interneurons.[90]

Perforated patch experiments, to preserve the anionic conditions of the recorded cells, have revealed that GABA released from MF terminals, exert a depolarizing action on targeted cells.[91] Accumulation of chloride inside the cell via the cation-chloride cotransporter NKCC1 is responsible for the depolarizing action of GABA since the positive driving force for chloride (+ 9 mV) observed in control conditions shifts towards negative value (-7 mV) when slices are exposed to the selective inhibitor of NKCC1, bumetanide,[92,93] thus confirming the general rule that GABA depolarizes immature neurons because of a reversed chloride gradient.[94]

In view of the GABAergic phenotype of immature MFs, what could the functional role of presynaptic KARs abundantly expressed on MF terminals? How could they be activated? How could they be involved in shaping neuronal networks? Some of these questions have been recently addressed.[74]

GluK1 KARs down Regulate GABA Release by from Immature MF Terminals

In the immature hippocampus, activation of GluK1 KARs subunits by tonic glutamate present in the extracellular space has been shown to downregulate the release of GABA from MF terminals via a metabotropic type of action.[74] Thus, blocking GluK1 receptors with (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxybenzyl)pyrimidine-2,4-dione (UBP 302), a specific GluK1 antagonist, in the presence of AMPA receptor blockers, enhances the amplitude of GPSCs (Fig. 2). As expected for a presynaptic type of action this effect is associated with an increase in successes rate, in CV^{-2} and a decrease in PPR (Fig. 2).

In addition, in "presynaptically" silent neurons, UBP 302 induces the appearance of synaptic responses to the first stimulus suggesting that endogenous activation of GluK1 contributes to synapses silencing (Fig. 3). Silent synapses represent a common feature of the developing brain.[95] They have been observed in a variety of different structures including the hippocampus where their number decreases significantly with age.[96]

The depressant action of GluK1 KARs on GPSCs is not indirectly mediated via other signaling molecules known to inhibit GABA release since KARs antagonists are still able to enhance the amplitude of GPSCs when applied in the presence of various receptor blockers including those for $GABA_B$, nicotinic, muscarinic, purinergic P2Y and mGlu.[74]

It is known that presynaptic receptors are usually activated by spillover of the neurotransmitter from axon terminals. One fundamental question to be addressed is how presynaptic GluK1 receptors can be activated if the main neurotransmitter released from MFs is GABA. One possibility is that these receptors are constitutively activated by ambient glutamate present in the extracellular medium which in neonates would be maintained at high levels by a less efficient glutamate transport mechanism and a poorly developed diffusional barrier.[97] Indeed, by enhancing the clearance of glutamate from the extracellular space, using an enzymatic glutamate scavenger system prevents the activation of presynaptic kainate receptors by glutamate and similarly to UBP 302 induces an increase in amplitude of GPSCs.[74]

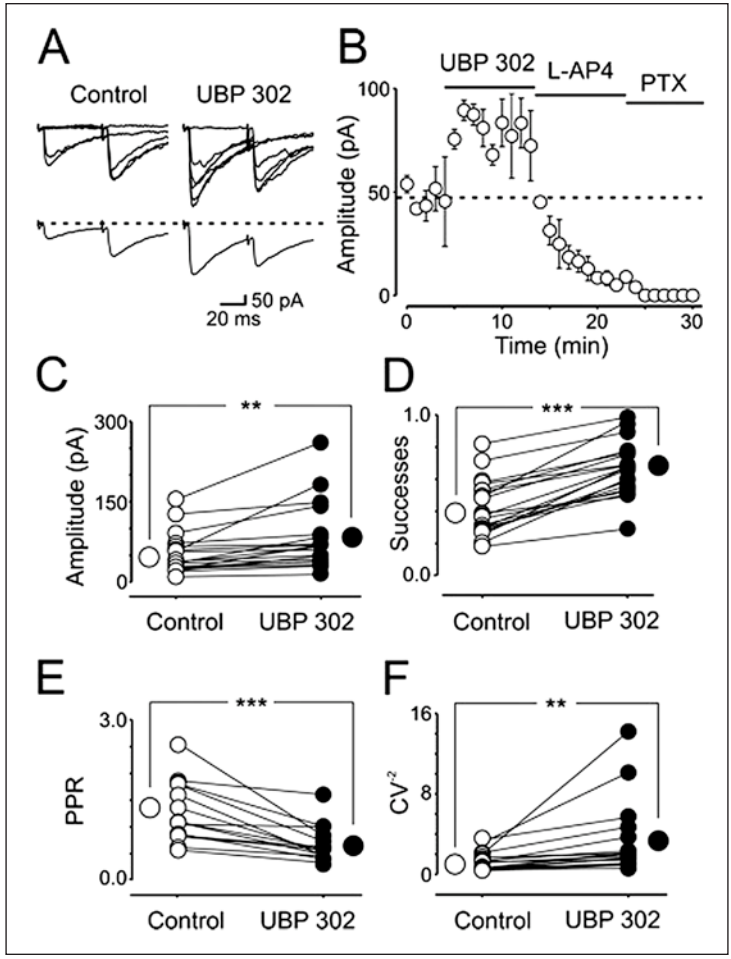

Figure 2. Endogenous activation of presynaptic kainate receptors down regulates MF-GPSCs. A) Superimposed individual traces of MF-GPSCs evoked in the presence of GYKI 52466 (30 μM, Control) and GYKI 52466 plus UBP 302 (10 μM). Below: averaged traces (successes plus failures). Note that UBP 302 enhanced the amplitude of the first response and reduced the number of synaptic failures. B) Summary plot showing the mean amplitude of GPSCs in the presence of GYKI and GYKI 52466 plus UBP 302 (n = 19). The horizontal dashed line refers to the mean amplitude value measured before UBP. C-F. Amplitude (C), Successes (D), PPR (E) and inversed square of CV (F) of MF-GPSCs measured in individual cells before and after application of UBP. Larger symbols represent averaged values. $^{**}p < 0.01$; $^{***}p < 0.001$. (Modified with permission from ref. 74.)

Since the depressant action of kainate on transmitter release seems to occur via G protein-coupled receptors,[19] to elucidate whether GluK1-induced depression of GPSCs operates through this particular modality, hippocampal slices were treated overnight with pertussis toxin (PTx) which blocks $G_{i/o}$ protein coupled receptors. In these conditions, UBP 302 fails to modify the amplitude of GPSCs. Similarly, the amplitude of GPSCs is unaltered when the glutamate scavenger is applied to slices incubated with PTx. The GluK1-induced-depression of MF-GPSCs involves G-coupled receptors localized on the presynaptic site, since blocking G

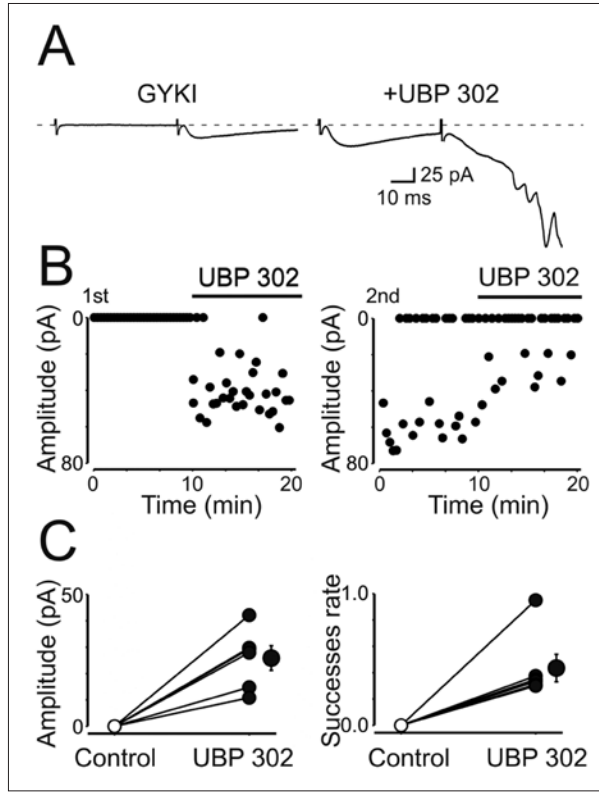

Figure 3. Blocking GluK1 receptors with UBP 302 enhances the probability of GABA release and converts "presynaptically" silent synapses into active ones. A) averaged traces of a "presynaptically" silent neuron recorded in the presence of GYKI and GYKI plus UPB 302. Note the appearance in the presence of UBP 302 of a synaptic current in response to the first stimulus. B) Time course of the peak amplitude of the first (left) and second (right) response shown in A, in control and during bath application of UBP (bars). C. Summary data (amplitude and successes rate) for 6 "presynaptically" silent cells.

proteins localized on the postsynaptic membrane using GDPβS into the patch pipette does not alter the action of UBP 302 on MF-mediated GABAergic currents, further indicating that the depression of MF-GPSCs is mediated by $G_{i/o}$ protein-coupled kainate receptors localized on MF terminals. Since the signaling pathway stimulated by G protein likely involves the release of calcium from intracellular stores by the activation of phospholipase C (PLC) and PKC stimulation, with consequent inhibition of voltage-dependent calcium channels,[25,98,99] disrupting the intracellular cascade downstream of G-protein activation with 1-[6-[[(17b)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122), a selective PLC blocker, should prevent the depressant action of GluK1 antagonist on GPSCs.

Interestingly, blocking PLC with U73122, downstream to G protein activation, unmasks the potentiating effect of GluK1 on MF GPSCs, which probably depends on the ionotropic type of action of this receptor. In keeping with this, the GluK1 antagonist UBP 302 significantly reduces the probability of evoking antidromic spikes in single granule cells by stimulation of MFs in stratum lucidum (Fig. 4).

A similar effect is produced by philanthotoxin which blocks calcium permeable AMPA/kainate receptors,[100] indicating that GluK1 increases MF excitability through the activation of

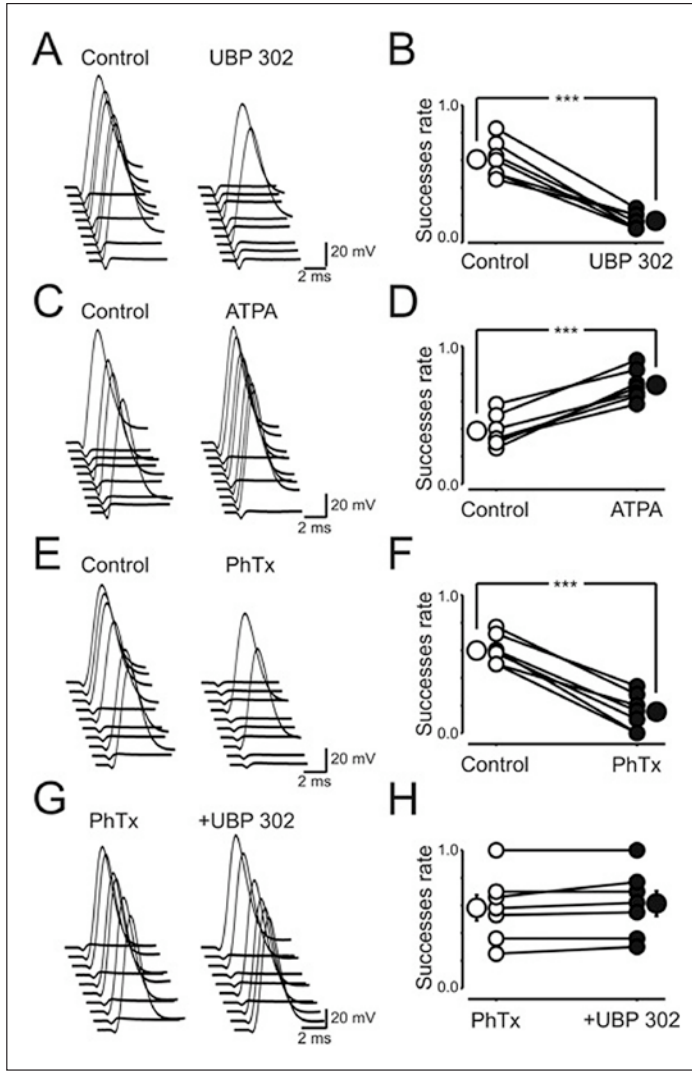

Figure 4. GluK1 receptors sensitive to philantotoxin control MF excitability. A) Consecutive traces showing antidromic spikes recorded in granule cells upon stimulation of MF in stratum lucidum before and during application of UBP 302 (note that the stimulus strength was set to obtain > than 50 % of successes. B) Summary plot of UBP 302 effects on successes rate (n = 7). C-D) In cells with < than 50% of successes, the selective GluK1 agonist ATPA enhanced MF excitability and the successes rate (n = 7). E-F) Philantotoxin (PhTx, 3 μM) mimicked the effects of UBP 302 (n = 7). G-H) In the presence of philantotoxin, UBP was not effective (n = 7). ***$p < 0.001$. (Modified with permission from ref. 74.)

calcium-permeable cationic channels and depolarization of MF terminals (Fig. 4). Consistent with a ionotropic type of action, application of UBP 302 in the presence of philanthotoxin fails to modify MFs excitability (Fig. 4). In juvenile animals, activation of presynaptic kainate receptors has been found to directly depolarize via cation channels glutamatergic MF terminals[31] or GABAergic terminals,[101] thus lowering the threshold for antidromic action potential generation.

How can an increased MF excitability be reconciled with a depression of GABA release? According to Kamiya and Ozawa,[31] a down regulation of transmitter release may occur via inactivation of Na^+/Ca^{2+} channels or electrical shunting. However, this is unlikely in view of the recent finding that KA-induced facilitation of action potential evoked calcium entry in MF boutons involves a calcium store-dependent mechanism.[22] In addition, it should be stressed that unlike adults, immature MF terminate in very small spherical expansions[102] and do not exhibit use-dependent synaptic facilitation until the second week of postnatal life.[75] It is still unclear whether the dual signaling pathways (ionotropic and metabotropic), which depend on the common ionotropic GluK1 subunit, are independent or functionally coupled. In a previous study from dorsal root ganglion cells, it has been demonstrated that GluK1 KARs induces a G protein-dependent rise in $[Ca^{2+}]_i$, favoring its release from the internal stores.[98] It is tempting to speculate that calcium entering through calcium-permeable KARs may directly or indirectly interfere with G protein-mediated signaling leading to a dominant depressant effect on MF-GPSCs. The interplay between these two different pathways has been recently shown to account for the PKC-dependent autoregulation of membrane KARs.[41]

Modulation of Spike-Time Dependent Plasticity by GluK1 KARs

Activity-dependent changes in synaptic strength such as long-term-potentiation (LTP) or long-term depression (LTD) are critical for information storage in the brain and for the development of neuronal circuits. One interesting question is whether, early in postnatal development, MF-GPSCs can undergo activity-dependent modifications in synaptic efficacy. As already mentioned, immature neurons are characterized by an elevated number of silent synapses which can be converted into active ones by activity-dependent processes and this represents the most common mechanism for LTP induction, not only during development, but also in the mature brain.[95]

A form of synaptic plasticity extensively studied at glutamatergic synapses is spike-time dependent plasticity (STDP). This is a particular form of Hebbian type of learning which consists in bi-directional modifications of synaptic strength according to the temporal order of pre and postsynaptic spiking.[103] Thus, positively correlated pre and postsynaptic spiking (pre before post) within a critical time window leads to LTP whereas a negative correlation (post before pre) to LTD.

We used the STDP protocol to verify whether activity can modify the strength of GABAergic MF-CA3 connections.[91] Unlike conventional STDP, pairing (in current clamp mode) ten postsynaptic spikes (at 0.1 Hz) with unitary MF-GPSPs, persistently downregulates synaptic efficacy in a way that is independent of the temporal order of pre and postsynaptic stimulation (Fig. 5). The decrease in amplitude of GPSCs, which persists without decrement for periods of time variable from 40 to 60 min, reaches its maximum when in the case of positive pairing antidromic spikes follow MF stimulation with a delay of 15 ms (coincident with the peak of the synaptic potentials) or when, in the case of negative pairing, it precedes MF activation by 50 ms.

These effects, which require for their induction a rise of calcium in the postsynaptic cell via voltage-dependent calcium channels, are associated with a significant decrease in successes rate, in the inverse squared value of the coefficient of variation of responses amplitude and a significant increase in paired pulse ratio, suggesting a presynaptic site of expression.

Interestingly, GluK1 KARs control the direction of STDP at immature MF-CA3 synapses. Thus, when positive pairing (15 ms delay) is delivered in the presence of the selective GluK1 antagonist UBP 302 a shift from STD-LTD to STD-LTP occurs[91] (Fig. 5). In addition, this activity-dependent form of synaptic plasticity involves the activation of a G-protein and PLC since it can be prevented by the selective PLC blocker U73122.[74] However, in the presence of KAR antagonists, negative pairing (postsynaptic spiking preceding MF stimulation with a delay of 50 ms) still induces LTD similar in all respects to that obtained in the presence of a high concentration of DNQX which blocks both AMPA and kainate receptors,[91] suggesting that KAR are not involved in this form of synaptic plasticity (Fig. 5).

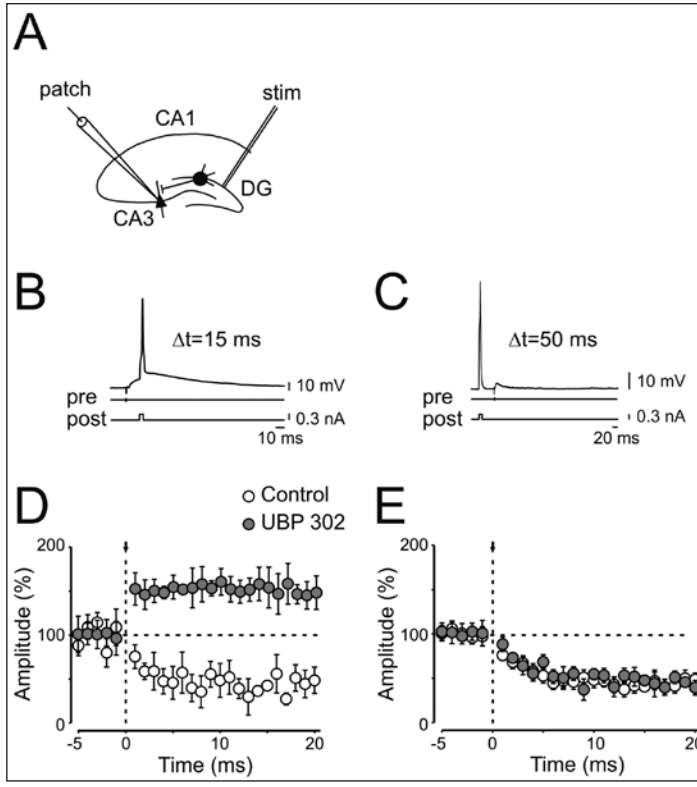

Figure 5. Presynaptic kainate receptors control the direction of spike-time dependent plasticity (STDP). A) Schematic representation of the experimental design. B) The stimulation of granule cells in the dentate gyrus (pre) preceded the postsynaptic spike (post) by 15 ms (Δt). C) the stimulation of granule cells in the dentate gyrus followed the postsynaptic spike by 50 ms. D) Summary plot of the mean peak amplitude of GPSCs recorded before and after positive pairing (arrow at time 0) in the absence (control, white symbols, n = 11) or in the presence of UBP 302 (grey symbols, n = 9). E) As in D but for negative pairing (post before pre; n = 7). Note the shift from spike-time dependent depression into spike-time dependent potentiation in the presence of UBP only in the case of positive pairing.

In summary, it is clear that tonic activation of presynaptic KAR by endogenous glutamate accounts for the persistent depression of MF-GPSCs observed after pairing presynaptic MF stimulation with postsynaptic spiking as demonstrated by the possibility to switch spike-time dependent depression into potentiation with UBP 302. Although the precise mechanisms underlying this phenomenon are still unclear, we cannot exclude the possibility that, with respect to LTD induced by negative pairing, KA-induced synaptic depression relies on a distinct calcium signal which in turn may activate a different molecular pathway, as suggested by the calcium hypothesis.[104]

Conclusion

Although much remains to be learned about the means by which KARs control GABA release at immature MF-CA3 connections and particularly the dual signaling pathways involved (ionotropic and metabotropic), it is conceivable that the depression of GABAergic transmission following tonic activation of GluK1 KARs by ambient glutamate represents a homeostatic mechanism which would limit the excessive activation of the auto-associative CA3 network by the excitatory action

of GABA, thus preventing the occurrence of seizures. These properties are likely to be critical for information processing and for the proper development of the adult hippocampal circuitry.

Acknowledgements

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References

1. Nisticò R, Dargan S, Fitzjohn SM et al. GLUK1 receptor antagonists and hippocampal mossy fiber function. Int Rev Neurobiol 2009; 85:13-27.
2. Huettner JE. Kainate receptors and synaptic transmission. Prog Neurobiol 2003; 70(5):387-407.
3. Collingridge GL, Olsen RW, Peters J et al. A nomenclature for ligand-gated ion channels. Neuropharm 2009; 56(1):2-5.
4. Egebjerg J, Bettler B, Hermans-Borgmeyer I et al. Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not by AMPA. Nature 1991; 351(6329):745-748.
5. Schiffer HH, Swanson GT, Heinemann SF. Rat GluR7 and a carboxy-terminal splice variant, GluR7b, are functional kainate receptor subunits with a low sensitivity to glutamate. Neuron 1997; 19(5):1141-1146.
6. Sommer B, Burnashev N, Verdoorn TA et al. A glutamate receptor channel with high affinity for domoate and kainate. EMBO J 1992; 11(4):1651-1656.
7. Herb A, Burnashev N, Werner P et al. The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. Neuron 1992; 8(4):775-785.
8. Werner P, Voigt M, Keinänen K et al. Cloning of a putative high-affinity kainate receptor expressed predominantly in hippocampal CA3 cells. Nature 1991; 351(6329):742-744.
9. Lerma J. Roles and rules of kainate receptors in synaptic transmission. Nat Rev Neurosci 2003; 4(6):481-495.
10. Castillo PE, Malenka RC, Nicoll RA. Kainate receptors mediate a slow postsynaptic current in hippocampal CA3 neurons. Nature 1997; 388(6642):182-186.
11. Vignes M, Collingridge GL. The synaptic activation of kainate receptors. Nature 1997; 388(6638):179-182.
12. Mulle C, Sailer A, Pérez-Otaño I et al. Altered synaptic physiology and reduced susceptibility to kainate-induced seizures in GluR6-deficient mice. Nature 1998; 392:601-615.
13. Fernandes HB, Catches JS, Petralia RS et al. High-affinity kainate receptor subunits are necessary for ionotropic but not metabotropic signaling. Neuron 2009; 63(6):818-829.
14. Mulle C, Sailer A, Swanson GT et al. Subunit composition of kainate receptors in hippocampal interneurons. Neuron 2000; 28(2):475-484.
15. Contractor A, Swanson G, Heinemann SF. Kainate receptors are involved in short- and long-term plasticity at mossy fiber synapses in the hippocampus. Neuron 2001; 29(1):209-216.
16. Lauri SE, Bortolotto ZA, Bleakman D et al. A critical role of a facilitatory presynaptic kainate receptor in mossy fiber LTP. Neuron 2001; 32(4):697–709.
17. Pinheiro PS, Perrais D, Coussen F et al. GluR7 is an essential subunit of presynaptic kainate autoreceptors at hippocampal mossy fiber synapses. Proc Natl Acad Sci USA 2007; 104(29):12181-12186.
18. Kwon HB, Castillo PE. Role of glutamate autoreceptors at hippocampal mossy fiber synapses. Neuron 2008; 60(6):1082-1094.
19. Lerma J. Kainate receptor physiology. Curr Opin Pharmacol 2006; 6(1):89-97.
20. Kamiya H, Ozawa S, Manabe T. Kainate receptor-dependent short-term plasticity of presynaptic Ca^{2+} influx at the hippocampal mossy fiber synapses. J Neurosci 2002; 22(21):9237-9243.
21. Nicoll RA, Schmitz D. Synaptic plasticity at hippocampal mossy fibre synapses. Nat Rev Neurosci 2005; 6(11):863-876.
22. Scott R, Lalic T, Kullmann DM et al. Target-cell specificity of kainate autoreceptor and Ca^{2+}-store-dependent short-term plasticity at hippocampal mossy fiber synapses. J Neurosci 2008; 28(49):13139-13149.
23. Clarke VR, Ballyk BA, Hoo KH et al. A hippocampal GluR5 kainate receptor regulating inhibitory synaptic transmission. Nature 1997; 389(6651):599-603.
24. Rodríguez-Moreno A, Herreras O, Lerma J. Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus. Neuron 1997; 19(4):893-901.
25. Rodríguez-Moreno A, Lerma J. Kainate receptor modulation of GABA release involves a metabotropic function. Neuron 1998; 20(6):1211-1218.
26. Cossart R, Tyzio R, Dinocourt C et al. Presynaptic kainate receptors that enhance the release of GABA on CA1 hippocampal interneurons. Neuron 2001; 29(2):497-508.
27. Maingret F, Lauri SE, Taira T et al. Profound regulation of neonatal CA1 rat hippocampal GABAergic transmission by functionally distinct kainate receptor populations. J Physiol 2005; 567(Pt 1):131-142.
28. Cunha RA, Constantino MD, Ribeiro JA. Inhibition of [3H] gamma-aminobutyric acid release by kainate receptor activation in rat hippocampal synaptosomes. Eur J Pharmacol 1997; 323(2-3):167-172.

29. Cunha RA, Malva JO, Ribeiro JA. Pertussis toxin prevents presynaptic inhibition by kainate receptors of rat hippocampal [(3)H]GABA release. FEBS Lett 2000; 469(2-3):159-162.
30. Frerking M, Schmitz D, Zhou Q et al. Kainate receptors depress excitatory synaptic transmission at CA3→CA1 synapses in the hippocampus via a direct presynaptic action. J Neurosci 2001; 21(9):2958-2966.
31. Kamiya H, Ozawa S. Kainate receptor-mediated presynaptic inhibition at the mouse hippocampal mossy fibre synapse. J Physiol 2000; 523(Pt 3):653-665.
32. Schmitz D, Mellor J, Frerking M et al. Presynaptic kainate receptors at hippocampal mossy fiber synapses. Proc Natl Acad Sci USA 2001; 98(20):11003-11008.
33. Negrete-Díaz JV, Sihra TS, Delgado-García JM et al. Kainate receptor-mediated inhibition of glutamate release involves protein kinase A in the mouse hippocampus. J Neurophysiol 2006; 96(4):1829-1837.
34. Bortolotto ZA, Nistico R, More JC. Kainate receptors and mossy fiber LTP. Neurotoxicology 2005; 26(5):769-777.
35. Negrete-Díaz JV, Sihra TS, Delgado-García JM. Kainate receptor-mediated presynaptic inhibition converges with presynaptic inhibition mediated by Group II mGluRs and long-term depression at the hippocampal mossy fiber-CA3 synapse. J Neural Transm 2007; 114(11):1425-1431.
36. Gho M, King AE, Ben-Ari Y et al. Kainate reduces two voltage-dependent potassium conductances in rat hippocampal neurons in vitro. Brain Res 1986; 385(2):411-414.
37. Melyan Z, Wheal HV, Lancaster B. Metabotropic-mediated kainate receptor regulation of IsAHP and excitability in pyramidal cells. Neuron 2002; 34(1):107-114.
38. Fisahn A, Heinemann SF, McBain CJ. The kainate receptor subunit GluR6 mediates metabotropic regulation of the slow and medium AHP currents in mouse hippocampal neurones. J Physiol 2005; 562(Pt 1):199-203.
39. Ruiz A, Sachidhanandam S, Utvik JK et al. Distinct subunits in heteromeric kainate receptors mediate ionotropic and metabotropic function at hippocampal mossy fiber synapses. J Neurosci 2005; 25(50):11710-11718.
40. Vincent P, Mulle C. Kainate receptors in epilepsy and excitotoxicity. Neuroscience 2009; 158(1):309-323.
41. Rivera R, Rozas JL, Lerma J. PKC-dependent autoregulation of membrane kainate receptors. EMBO J 2007; 26(20):4359-4367
42. Miller LP, Johnson AE, Gelhard RE et al. The ontogeny of excitatory amino acid receptors in the rat forebrain—II. Kainic acid receptors. Neuroscience 1990; 35(1):45-51.
43. Ultsch A, Schuster CM, Laube B et al. Glutamate receptors of Drosophila melanogaster: cloning of a kainate-selective subunit expressed in the central nervous system. Proc Natl Acad Sci USA 1992; 89(21):10484-10488.
44. Bettler B, Boulter J, Hermans-Borgmeyer I et al. Cloning of a novel glutamate receptor subunit, GluR5: expression in the nervous system during development. Neuron 1990;5(5):583-595.
45. Bahn S, Volk B, Wisden W. Kainate receptor gene expression in the developing rat brain. J Neurosci 1994; 14(9):5525-5547.
46. Ritter LM, Vazquez DM, Meador-Woodruff JH. Ontogeny of ionotropic glutamate receptor subunit expression in the rat hippocampus. Brain Res Dev Brain Res 2002; 139(2):227-236
47. Schlaggar BL, Fox K, O'Leary DD. Postsynaptic control of plasticity in developing somatosensory cortex. Nature 1993; 364(6438):623-626.
48. Represa A, Tremblay E, Ben-Ari Y. Kainate binding sites in the hippocampal mossy fibers: localization and plasticity. Neuroscience 1987; 20(3):739-748.
49. Acsády L, Kamondi A, Sík A et al. GABAergic cells are the major postsynaptic targets of mossy fibers in the rat hippocampus. J Neurosci 1998; 18(9):3386-3403.
50. Tashiro A, Dunaevsky A, Blazeski R et al. Bidirectional regulation of hippocampal mossy fiber filopodial motility by kainate receptors: a two-step model of synaptogenesis. Neuron 2003; 38(5):773-784.
51. Feller MB, Butts DA, Aaron HL et al. Dynamic processes shape spatiotemporal properties of retinal waves. Neuron 1997; 19(2):293-306.
52. Yuste R, Katz LC. Control of postsynaptic Ca^{2+} influx in developing neocortex by excitatory and inhibitory neurotransmitters. Neuron 1991; 6(3):333-344.
53. Owens DF, Boyce LH, Davis MB et al. Excitatory GABA responses in embryonic and neonatal cortical slices demonstrated by gramicidin perforated-patch recordings and calcium imaging. J Neurosci 1996; 16(20):6414-6423.
54. Dammerman RS, Flint AC, Noctor S et al. An excitatory GABAergic plexus in developing neocortical layer 1. J Neurophysiol 2000; 84(1):428-434.
55. Maric D, Liu QY, Maric I et al. GABA expression dominates neuronal lineage progression in the embryonic rat neocortex and facilitates neurite outgrowth via GABA(A) autoreceptor/Cl- channels. J Neurosci 2001; 21(7):2343-2360.

56. Chen G, Trombley PQ, van den Pol AN. Excitatory actions of GABA in developing rat hypothalamic neurones. J Physiol 1996; 494(Pt 2):451-464.
57. Eilers J, Plant TD, Marandi N et al. GABA-mediated Ca^{2+} signalling in developing rat cerebellar Purkinje neurones. J Physiol 2001; 536(Pt 2):429-437.
58. Wang J, Reichling DB, Kyrozis A et al. Developmental loss of GABA- and glycine-induced depolarization and Ca^{2+} transients in embryonic rat dorsal horn neurons in culture. Eur J Neurosci 1994; 6(8):1275-1280.
59. O'Donovan MJ. The origin of spontaneous activity in developing networks of the vertebrate nervous system. Curr Opin Neurobiol 1999; 9(1):94-104.
60. Cherubini E, Gaiarsa JL, Ben-Ari Y. GABA: an excitatory transmitter in early postnatal life. Trends Neurosci 1991; 14(12):515-519.
61. Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. Nat Rev Neurosci 2002; 3(9):728-739.
62. Leinekugel X, Khazipov R, Cannon R et al. Correlated bursts of activity in the neonatal hippocampus in vivo. Science 2002; 296(5575):2049-2052.
63. Buzsáki G, Draguhn A. Neuronal oscillations in cortical networks. Science 2004; 304(5679):1926-1929.
64. Vesikansa A, Sallert M, Taira T et al. Activation of kainate receptors controls the number of functional glutamatergic synapses in the area CA1 of rat hippocampus. J Physiol 2007; 583(Pt 1):145-157.
65. Lauri SE, Segerstråle M, Vesikansa A et al. Endogenous activation of kainate receptors regulates glutamate release and network activity in the developing hippocampus. J Neurosci 2005; 25(18):4473-4484.
66. Lauri SE, Vesikansa A, Segerstråle M et al. Functional maturation of CA1 synapses involves activity-dependent loss of tonic kainate receptor mediated inhibition of glutamate release. Neuron 2006; 50(3):415-429.
67. McBain CJ, Fisahn A. Interneurons unbound. Nat Rev Neurosci 2001; 2(1):11-23.
68. Ben-Ari Y, Khalilov I, Represa A et al. Interneurons set the tune of developing networks. Trends Neurosci 2004; 27(7):422-427.
69. Maccaferri G, McBain CJ. Long-term potentiation in distinct subtypes of hippocampal nonpyramidal neurons. J Neurosci 1996; 16(17):5334-5343.
70. Hestrin S, Galarreta M. Electrical synapses define networks of neocortical GABAergic neurons. Trends Neurosci 2005; 28(6):304-309.
71. Zsiros V, Maccaferri G. Electrical coupling between interneurons with different excitable properties in the stratum lacunosum-moleculare of the juvenile CA1 rat hippocampus. J Neurosci 2005; 25(38):8686-8695.
72. Minneci F, Janahmadi M, Migliore M et al. Signaling properties of stratum oriens interneurons in the hippocampus of transgenic mice expressing EGFP in a subset of somatostatin-containing cells. Hippocampus 2007; 17(7):538-553.
73. Segerstråle M, Juuri J, Lanore F et al. High firing rate of neonatal hippocampal interneurons is caused by attenuation of afterhyperpolarizing potassium currents by tonically active kainate receptors. J Neurosci 2010; 30(19):6507-6514.
74. Caiati MD, Sivakumaran S, Cherubini E. In the developing rat hippocampus, endogenous activation of presynaptic kainate receptors reduces GABA release from mossy fiber terminals. J Neurosci 2010; 30(5):1750-1759.
75. Marchal C, Mulle C. Postnatal maturation of mossy fibre excitatory transmission in mouse CA3 pyramidal cells: a potential role for kainate receptors. J Physiol 2004; 561(Pt 1):27-37.
76. Cossart R, Epsztein J, Tyzio R et al. Quantal release of glutamate generates pure kainate and mixed AMPA/kainate EPSCs in hippocampal neurons. Neuron 2002; 35(1):147-159.
77. Coussen F, Normand E, Marchal C et al. Recruitment of the kainate receptor subunit glutamate receptor 6 by cadherin/catenin complexes. J Neurosci 2002; 22(15):6426-6436.
78. Mizoguchi A, Nakanishi H, Kimura K et al. Nectin: an adhesion molecule involved in formation of synapses. J Cell Biol 2002; 156(3):555-565.
79. Gutiérrez R, Heinemann U. Kindling induces transient fast inhibition in the dentate gyrus-CA3 projection. Eur J Neurosci 2001; 13(7):1371-1379.
80. Jaffe DB, Gutiérrez R. Mossy fiber synaptic transmission: communication from the dentate gyrus to area CA3. Prog Brain Res 2007; 163:109-132
81. Schwarzer C, Sperk G. Hippocampal granule cells express glutamic acid decarboxylase-67 after limbic seizures in the rat. Neuroscience 1995; 69(3):705-709.
82. Sloviter RS, Dichter MA, Rachinsky TL et al. Basal expression and induction of glutamate decarboxylase and GABA in excitatory granule cells of the rat and monkey hippocampal dentate gyrus. J Comp Neurol 1996; 373(4):593-618.
83. Lamas M, Gómez-Lira G, Gutiérrez R. Vesicular GABA transporter mRNA expression in the dentate gyrus and in mossy fiber synaptosomes. Brain Res Mol Brain Res 2001; 93(2):209-214.
84. Rao A, Cha EM, Craig AM. Mismatched appositions of presynaptic and postsynaptic components in isolated hippocampal neurons. J Neurosci 2000; 20(22):8344-8353.

85. Walker MC, Ruiz A, Kullmann DM. Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. Neuron 2001; 29(3):703-715.
86. Gutierrez R, Romo-Parra H, Maqueda J et al. Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 2003; 23(13):5594-5598.
87. Bergersen L, Ruiz A, Bjaalie JG et al. GABA and GABAA receptors at hippocampal mossy fibre synapses. Eur J Neurosci 2003; 18(4):931-941.
88. Gómez-Lira G, Lamas M, Romo-Parra H et al. Programmed and induced phenotype of the hippocampal granule cells. J Neurosci 2005; 25(30):6939-6946.
89. Zander JF, Münster-Wandowski A, Brunk I et al. Synaptic and vesicular coexistence of VGLUT and VGAT in selected excitatory and inhibitory synapses. J Neurosci 2010; 30(22):7634-7645.
90. Safiulina VF, Fattorini G, Conti F et al. GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. J Neurosci 2006; 26(2):597-608.
91. Sivakumaran S, Mohajerani MH, Cherubini E. At immature mossy-fiber–CA3 synapses, correlated presynaptic and postsynaptic activity persistently enhances GABA release and network excitability via BDNF and cAMP-dependent PKA. J Neurosci 2009; 29(8):2637-2647.
92. Dzhala VI, Talos DM, Sdrulla DA et al. NKCC1 transporter facilitates seizures in the developing brain. Nat Med 2005; 11(11):1205-1213.
93. Sipilä ST, Schuchmann S, Voipio J et al. The cation-chloride cotransporter NKCC1 promotes sharp waves in the neonatal rat hippocampus. J Physiol 2006; 573(Pt 3):765-773.
94. Ben-Ari Y, Gaiarsa JL, Tyzio R et al. GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. Physiol Rev 2007; 87(4):1215-1284.
95. Voronin LL, Cherubini E. 'Deaf, mute and whispering' silent synapses: their role in synaptic plasticity. J Physiol 2004; 557(Pt 1):3-12.
96. Durand GM, Kovalchuk Y, Konnerth A. Long-term potentiation and functional synapse induction in developing hippocampus. Nature 1996; 381(6577):71-75.
97. Danbolt NC. Glutamate uptake. Prog Neurobiol 2001; 65(1):1-105.
98. Rozas JL, Paternain AV, Lerma J. Noncanonical signaling by ionotropic kainate receptors. Neuron 2003; 39(3):543-553.
99. Rodríguez-Moreno A, Sihra TS. Metabotropic actions of kainate receptors in the CNS. J Neurochem 2007; 103(6):2121-2135.
100. Fletcher EJ, Lodge D. New developments in the molecular pharmacology of alphaamino-3-hydroxy-5-methyl-4-isoxazole propionate and kainate receptors. Pharmacol Ther 1996; 70(1):65-89.
101. Semyanov A, Kullmann DM. Kainate receptor-dependent axonal depolarization and action potential initiation in interneurons. Nat Neurosci 2001; 4(7):718-723.
102. Amaral DG, Dent JA. Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J Comp Neurol 1981; 195(1):51-86.
103. Dan Y, Poo MM. Spike timing-dependent plasticity: from synapse to perception. Physiol Rev 2006; 86(3):1033-1048.
104. Caporale N, Dan Y. Spike timing dependent plasticity: a Hebbian learning rule. Annu Rev Neurosci 2008; 31:25-46.

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The co-existence and co-release of different neurotransmitters from the same fiber has been well documented in several brain structures including the retina, the spinal cord and the auditory system (Hnasko and Edwards, 2012). Although mossy fibers (MFs), the axons of dentate gyrus granule cells, release glutamate and GABA early in postnatal development, (Walker et al., 2001; Gutiérrez et al., 2003; Safiulina et al., 2006), they normally provide monosynaptic glutamatergic excitation and disynaptic GABAergic inhibition to the CA3 hippocampal field in adults. But in some conditions, such as kindling or activity-dependent processes, MFs can transiently resume a GABAergic phenotype in adulthood. This suggests that MFs can switch in a developmentally and activity-dependent regulated way the type of neurotransmitter released (Gutiérrez, 2005).

Studies using immunohistochemistry and electron microscopy have revealed that MFs possess the full machinery to synthesize, store, and release GABA. Glutamic acid decarboxylase (GAD67/65), the enzyme that catalyzes GABA synthesis, as well as GABA itself, and the vesicular GABA transporter (VGAT) have all been detected within MF terminals. Importantly, immunogold experiments have demonstrated that AMPA and $GABA_A$ receptors are sometimes co-localized on postsynaptic sites in close apposition to MF terminals, strongly suggesting that MFs may convey a GABAergic signal to their targets (Gutiérrez, 2005). Consistent with this hypothesis, monosynaptic GABAergic currents have been recorded in CA3 principal cells upon granule cells stimulation in the dentate gyrus in acute hippocampal slices from newborn (Safiulina et al., 2006) or juvenile animals (Gutiérrez *et* al., 2003; Walker et al., 2001). The evoked responses fulfill the criteria for identification of MF inputs: strong paired pulse facilitation, short term frequency-dependent facilitation, and sensitivity to group II and III mGluR agonists (Safiulina et al., 2006).

The data discussed above have been challenged, however. Specifically, Uchigashima et al. (2007) questioned the GABAergic nature of MF-CA3 responses on the basis that, at least in young animals, the stimulation protocol generally used to activate MFs might co-activate adjacent GABAergic terminals, thus causing misinterpretation of the results. Given the complex nature of the neuronal network of the dentate gyrus and CA3 area, the possibility that interneurons with axonal or dendritic projections to the dentate gyrus could be activated with minimal stimulation protocol is plausible. This controversial issue may be solved by performing paired recordings from interconnected granule cells-CA3 principal neurons.

In a study recently published in *The Journal of Neuroscience*, Cabezas et al. (2012) used paired recordings of interconnected neurons in organotypic hippocampal slice cultures from GAD67-EGFP transgenic mice to explore the possibility that MFs can co-release glutamate and GABA onto CA3 principal cells. First, by performing immunocytochemical experiments from the hippocampus of postnatal day 15 (P15) old mice, the authors found that GAD67 is expressed only in a subset of MF terminals immunopositive for ZnT3, a selective MFs marker. Interestingly, GAD67 positive granule cells showed signs of immaturity, including expression of

doublecortin but not calbindin, low membrane capacitance, moderate input resistance, and small amplitude action potentials.

Next, in organotypic hippocampal slices prepared from P7-P8 old mice and kept in cultures for 10–12 days, Cabezas et al. (2012) used local photolysis of caged glutamate to examine postsynaptic responses evoked by photo-stimulation of individual, visually identified granule cells. In spite of the expression of GAD67 in a sub-population of immature granule cells, no unitary $GABA_A$-mediated synaptic currents were detected in any of the 39 pairs of interconnected granule cells-CA3 principal cells examined, casting doubts on previous findings obtained from acute hippocampal slices (Walker et al., 2001; Gutiérrez et al., 2003; Safiulina et al., 2006). Based on their reversal potential and pharmacology, unitary currents were identified as mediated by AMPA/kainate receptors.

Notwithstanding the lack of GABA-mediated postsynaptic currents, however, Cabezas et al. (2012) showed that GAD67-positive granule cells release GABA. They demonstrated that GABA released from MF terminals acts upon presynaptic $GABA_B$ receptors, reducing MF excitability. Thus, repetitive activation of EGFP-positive neurons in the dentate gyrus (a train of 30 orthodromic action currents at 25 Hz elicited by depolarizing voltage pulses through the patch pipette) transiently reduced the probability of evoking antidromic action currents by extracellular stimulation of MFs in stratum lucidum, and this effect was blocked by selective $GABA_B$ receptor antagonists. Therefore, it appears that, in juvenile animals, a transient MF GABAergic phenotype is important for instructing presynaptic rather than postsynaptic elements of synapses.

Although the data presented by Cabezas et al. (2012) favor the hypothesis that monosynaptic GABAergic currents recorded from CA3 pyramidal cells in acute slices (upon stimulation of granule cells) originate from direct activation of hilar interneurons and not dentate gyrus granule cells, it is worth noting that Cabezas et al. (2012) used a different preparation than previous studies. Specifically, whereas earlier studies (Walker et al., 2001; Gutiérrez et al., 2003; Safiulina et al., 2006) used acute hippocampal slices, Cabezas et al. (2012) used organotypic hippocampal slices kept in cultures for 10-12 days before recording. Because it is impossible to determine the exact developmental stage of MFs in culture, making direct comparison between the two preparations is extremely difficult. Furthermore, although organotypic hippocampal slices maintain some local circuitry intact, they develop in isolation and hence they lack experience-dependent plasticity characteristic of behaving animals. Because MF-dependent GABAergic transmission is strongly activity-dependent (Gutierrez et al., 2003), the lack of extrinsic afferents in slice cultures may interfere with the acquisition of a functional GABAergic phenotype from granule cells. In addition, compared to acute slices, organotypic cultures exhibit enhanced glutamatergic connectivity as demonstrated by the four- to five-fold increase in the frequency of glutamatergic, but not GABAergic miniature postsynaptic currents (De Simoni et al., 2003). Moreover, the fact that astrocytes do not reach a full maturation in organotypic cultures (Derouiche, 1993) might alter the normal glutamate and GABA metabolism, possibly

lowering the level of GABA released from MF terminals. Finally, it is unclear whether, $GABA_A$ receptors facing MF terminals remain present and functional in organotypic cultures.

In addition to the caveats listed above, the observations of Cabezas et al. (2012) are difficult to reconcile with a recent report by Beltran and Gutiérrez (2012), which examined synaptic responses evoked by stimulation of single identified MF boutons attached to the apical dendrites of mechanically isolated pyramidal cells. Such stimulation produced synaptic currents that, like typical MF-evoked responses, showed a high degree of facilitation upon repetitive stimulation and were blocked by group II mGluR agonists. Interestingly, whereas stimulation of MF boutons in neurons dissociated from adult animals generated exclusively glutamate receptor-mediated responses, stimulation of MF boutons from younger animals produced either GABAergic or mixed GABAergic and glutamatergic responses.

In summary, the question of whether activation of immature MF terminals elicits monosynaptic GABAergic responses in CA3 pyramidal cells remains unsolved. Recently developed tools may help to clarify this issue. For example, expressing Channelrhodopsin-2 (ChR2) *via* a retroviral vector in hippocampal granule cell progenitors would allow one to selectively activate ChR2-positive granule cells by photostimulation and to identify the nature of the neurotransmitter released by MFs onto patched CA3 principal cells. Classical criteria for MF identification could then be used to ascertain that ChR2-driven synaptic responses were truly MFs.
Alternatively, by sequentially uncaging glutamate with the beam-multiplexed two-photon laser, a novel optical method developed by Nikolenko et al. (2007), it would be possible to selectively activate (in acute hippocampal slices from GAD67-EGFP transgenic mice) up to a thousand GAD67-positive granule cells, causing them to fire. This would allow investigation of the nature of monosynaptic evoked responses in CA3 targeted pyramidal cells.
Either of these approaches would permit one to selectively activate granule cells, as well as to overcome the difficulty of finding connected pairs of neurons. Such experiments might finally elucidate the role of GABA in MF terminals.

References

Beltrán JQ, Gutiérrez R (2012) Co-release of glutamate and GABA from single, identified mossy fibre giant boutons. J Physiol 590(Pt 19):4789-800. doi: 10.1113/jphysiol.2012.236372. Epub 2012 Jun 18.

Cabezas C, Irinopoulou T, Gauvain G, Poncer JC (2012) Presynaptic but not postsynaptic GABA signaling at unitary mossy fiber synapses. J Neurosci 32(34):11835-40. doi: 10.1523/JNEUROSCI.5543-11.2012.

Derouiche A, Heimrich B, Frotscher M (1993) Loss of layer-specific astrocytic glutamine synthetase immunoreactivity in slice cultures of hippocampus. Eur J Neurosci 5(2):122-7.

De Simoni A, Griesinger CB, Edwards FA (2003) Development of rat CA1 neurons in acute versus organotypic slices: role of experience in synaptic morphology and activity. J Physiol 550:135-147.

Gutiérrez R (2005) The dual glutamatergic-GABAergic phenotype of hippocampal granule cells. Trends Neurosci 28(6):297-303.

Gutiérrez R, Romo-Parra H, Maqueda J, Vivar C, Ramìrez M, Morales MA, Lamas M (2003) Plasticity of the GABAergic phenotype of the "glutamatergic" granule cells of the rat dentate gyrus. J Neurosci 23(13):5594-8.

Hnasko TS and Edwards RH (2012) Neurotransmitter corelease: mechanism and physiological role. Annu Rev Physiol 74:225-43. Epub 2011 Oct 31.

Nikolenko V, Poskanzer KE, Yuste R (2007) Two-photon photostimulation and imaging of neural circuits. Nat Methods 4(11):943-50. Epub 2007 Oct 28.

Safiulina VF, Fattorini G, Conti F, Cherubini E (2006) GABAergic signaling at mossy fiber synapses in neonatal rat hippocampus. J Neurosci 26(2):597-608.

Uchigashima M, Fukaya M, Watanabe M, Kamiya H (2007) Evidence against GABA release from glutamatergic mossy fiber terminals in the developing hippocampus. J Neurosci 27(30):8088-100.

Walker MC, Ruiz A, Kullmann DM (2001) Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. Neuron 29(3):703-15.

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