GABAergic mechanisms of excitation and hypersynchrony in adult rat hippocampus

Marylka Uusisaari

Department of Biosciences, University of Helsinki
Finnish Graduate School of Neuroscience

Academic dissertation

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Supervised by:

Professor Kai Kaila, PhD
Department of Biosciences,
University of Helsinki, Finland

and

Professor Juha Voipio, PhD
Department of Biosciences,
University of Helsinki, Finland

Reviewed by:

Professor Rüdiger Köhling, MD,
Institut für Physiologie,
Westfälische Wilhelms-Universität Münster, Germany

and

Docent Jouni Sirviö, PhD
Orion Pharma

Opponent:

Prof.Dr.med. Andreas Draguhn
Institut für Physiologie und Pathophysiologie
Universität Heidelberg, Germany

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Abbreviations

2-AG	2-arachidonoylglycerol
ACF	autocorrelation function
AHP	afterhyperpolarisation
BDNF	brain derived neurotrophic factor
CA	carbonic anhydrase
CA1 / 3	cornu ammoni 1 / 3
CNS	central nervous system
DG	dentate gyrus
EPSP	excitatory postsynaptic potential
GABA	γ-aminobutyric acid
$GABA_AR$	$GABA_A$ receptor
$GABA_BR$	$GABA_B$ receptor
GDNSP	GABAergic depolarising non-synaptic potential
GDPSP	GABAergic depolarising post-synaptic potential
GIRK	G-protein coupled inward rectifying K^+ channel
iGluR	ionotropic glutamate receptor
GIE	GABAergic ictal-like event
ING	interneuron network gamma (oscillation)
IPSP	inhibitory postsynaptic potential
LTP	long-term potentiation
mGluR	metabotropic glutamate receptor
PDS	paroxysmal depolarising shift
pH_i, pH_o	intracellular pH, extracellular pH
PGO	population gamma oscillation
TLE	temporal lobe epilepsy

The drugs used in the present work are listed in Table 2.

Abstract

Tetanisation, i.e. high-frequency electrical stimulation of afferent fibers, is a common *in vitro* experimental paradigm in studies concerning network activity and plasticity, even though the relevance of such a strong input for physiological conditions is not always clear. Still, it has been a significant tool in elucidating the mechanisms of classical synaptic learning in the case of long-term potentiation (LTP) of glutamatergic transmission. Glutamate is the main excitatory transmitter in the central nervous system, and it has been traditionally seen to be countered by inhibitory action of GABA – mediated transmission. However, it has become clear during the last years that GABAergic synaptic transmission can also be excitatory in nature. For instance, in response to the aforementioned tetanisation a biphasic (inhibitory - excitatory) postsynaptic potentials are elicited and thus this view has had to be re-evaluated.

In the present work the high-frequency stimulation (HFS) –induced GABAergic non-synaptic depolarising potentials (GDNSPs) were dissected pharmacologically into hyperpolarising and depolarising parts. Activity-induced bicarbonate-dependent efflux of potassium and the direct electrical coupling between interneurons are shown to be the key elements of the depolarising response by its sensitivity to gap junction blockers and QX-314. In addition, the stimulus-induced $GABA_A$ receptor -mediated synchronous field oscillations are shown to require gap junction coupling between interneurons, while hyperexcitatory glutamatergic field response to external stimulation seen in the presence of $GABA_A$ receptor antagonists does not involve gap junctional communication.

It is further shown that the synchronisation of interneuronal network via gap junctions and synaptic communication can result in spontaneous GABAergic ictal-like activity in the absence of ionotropic glutamate receptor –mediated synaptic transmission under conditions where the autoinhibition of GABA release via the presynaptic $GABA_B$ receptors has been blocked pharmacologically. Furthermore, prolonged blockade of the $GABA_B$ receptors leads to long-term synaptic modifications that can be overcome by cannabinoid and opioid receptor activation.

Gamma-range oscillations are thought to be important in cognitive functions. Carbachol-evoked gamma oscillations are examined as another model of spontaneous hippocampal activity, and are shown to be sensitive to GABAergic modulation as well. Hypocapnia-induced increase of pH_o results in sharpening of the synaptic GABAergic response and enhanced temporal stability of gamma-band oscillations. In contrast, drugs that prolong the kinetics of $GABA_A$ receptor mediated response decrease the stability. Intracellular

alkalosis, on the other hand, increases oscillation frequency along with a decrease in oscillation amplitude. It is likely that this effect of the rise of pH_i is mediated via the enhancement of gap junctional coupling, which could allow faster spread of interneuronal voltage signals.

The results strongly support the view that in the adult CNS, interneurons modulate and pattern network behaviour via GABA – and bicarbonate – mediated processes instead of simply inhibiting the activity of pyramidal cells. Gap junctional coupling between interneurons enhances synchronicity and spatial extent of this patterning. Also, it is likely that depolarising GABA responses play a significant role in generation and maintenance of spontaneous activity as well as synaptic plasticity.

1. Introduction

The mammalian hippocampus is a structure in the central nervous system (CNS) that is involved in mnemonic functions. Since the ability to form and recall memories constitutes a significant part of what is usually described as 'consciousness', it is no wonder that the hippocampus has been target for intense physiological studies for decades. In addition, the ordered structure of the synaptic circuits of the hippocampus permits well-defined experimental manipulations. Another reason for the current interest in the hippocampus is that it seems to be involved in the processes leading to temporal lobe epilepsy. The ability of hippocampal neural networks to generate spontaneous, synchronous activity can in pathological conditions lead to uncontrolled, wide-spreading bursts of activity, or seizures. Despite significant efforts to elucidate the processes involved in epileptogenesis, medical treatment of epileptic patients is often ineffective. There is thus a need for further clarification of the mechanisms that produce abnormal spontaneous activity in order to develop more efficient therapies for this disorder.

In addition to synaptic transmission, neurons communicate via nonsynaptic means, including direct electrical communication via gap junctions and shifts of ions, especially potassium, chloride and bicarbonate. Furthermore, proton concentration (usually quantified as pH) modulates the function of various membrane channels, transporters and enzymes. Importantly for the studies to be presented in this work, the equilibrium condition for bicarbonate, a significant current carrier, is set by the pH gradient.

Signals in the CNS are processed by the two 'classical' interacting classes of neurons: the principal neurons and interneurons. These two groups are associated with different synaptic transmitters: the principal cells use glutamate and interneurons rely on GABA as the neurotransmitter molecule. In addition, there are numerous other differences that result in significant differences in the functional properties of these neuronal populations. In older literature, principal cells have been generally thought to be the information-processors in the hippocampus, while the interneurons have generally had to be content with a less-glorified role of cellular brakes that prevent the activity in principal cells running out of control. This view has been challenged by several observations, including the key role

of interneurons in generation of oscillatory network activity as well as excitatory effects of interneuronal input to principal cells.
This study focuses on the role of GABAergic transmission, in particular, on the generation and maintenance of spontaneous activity. By using electrophysiological and pharmacological methods the synaptic and nonsynaptic mechanisms are examined and shown to influence each other in addition to modulating the network activity on different time and space scales.

2. Literature Review

2.1 Principles of chemical synaptic transmission

2.1.1 Receptors

As reviewed in a tremendous amount of literature starting from Katz & Miledi's work in the 70's, depolarization of the presynaptic terminal results in exocytosis of transmitter molecules into the synaptic cleft in a calcium influx controlled manner (the most important processes of synaptic transmission are summarised in Figure 1). The transmitter molecule then diffuses, binds to and activates its receptor. All receptors relevant for the present work are membrane-spanning proteins that mediate their effect within the target cell either directly by gating an ion channel (ionotropic) or indirectly via second-messenger cascades (metabotropic).

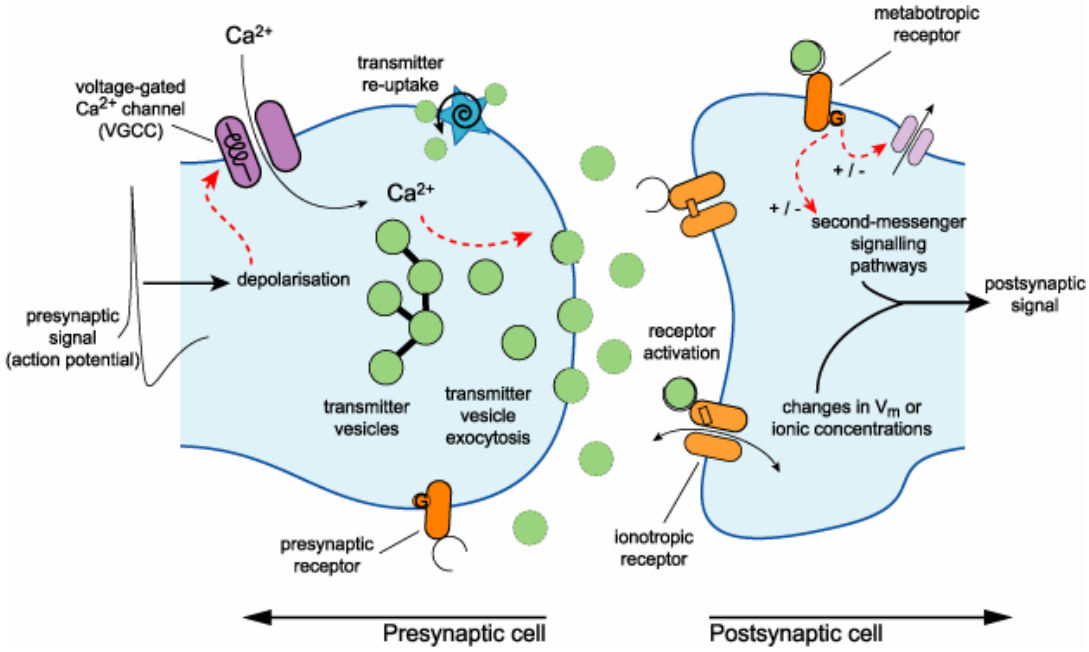

Figure 1. Synaptic transmission. Generally, a depolarising (action) potential arriving at a presynaptic terminal (left) results in an influx of Ca^{2+} via voltage-gated Ca^{2+}-channels. Vesicles containing the transmitter are exocytosed in response to the calcium signal, and the transmitter is then diffused across the synaptic cleft and binds to its receptors on the postsynaptic cell. Activation of the receptor results in a change in the cells internal state, thus transmitting the presynaptic signal into the postsynaptic cell (right).

The diffusion of the transmitter molecule is efficiently restricted by transmitter uptake mechanisms which ascertain that the presynaptic signal is transmitted precisely timed to the target synapse.

The main ionotropic receptors in the CNS are activated by synaptically released glutamate (NMDA, AMPA and kainate receptors) or GABA ($GABA_A$ receptor). Membrane response to ligand binding-induced channel opening is fast, and since the duration of exposure to the transmitter is efficiently controlled (by transmitter diffusion, binding and uptake; see Clements, 1996), ionotopic receptor-mediated signalling can be precisely timed and usually spatially well restricted. The result of ionotropic receptor activation depends on the properties of the associated ion channel (selectivity, rectification, conductance, and kinetics; see Hille, 2001) determined by channel subunit composition. Most ligand-gated channels have rather broad ionic selectivity but the main current is carried by only a few ionic species.

The second-messenger cascades associated with metabotropic receptors constitute diverse pathways of intracellular communication, with overlapping patterns of effects ranging from gating of ion-channels to synaptic plasticity and modulation of gene expression. The effect of metabotropic receptor activation is slower than those of ionotropic receptor activation and often modulatory rather than driving. For instance, several glutamate-activated metabotropic receptors (mGluRs; reviewed by Coutinho & Knöpfel, 2002) may induce a tonic depolarisation of a neuron, thus influencing its response to other inputs.

The term 'synapse' was coined by Sherrington (1906) but the membrane area responsible for the functional effect of the axon on the dendrites of the target cell was not defined before the era of electron microscopy and identification of membrane specialisations and machinery underlying the transmitter release (Peters & Palay 1996). Nevertheless, a number of studies have shown fully functional receptor groups residing predominately at the synapse border (Lujan & al., 1996) and on extrasynaptic membranes (particularly $GABA_A$ receptors, see Somogyi & al.,1989) suggesting that membrane areas outside the postsynaptic membrane specialisation should not be excluded from the definition of a synapse.

In addition to synaptic and extrasynaptic postsynaptic receptors, there are receptors on the presynaptic terminals, such as metabotropic $GABA_B$ autoreceptors and metabotropic glutamate receptors that may regulate the probability of transmitter release upon arrival of an action potential. Furthermore, self-innervating connections on the

cell itself are known to exist, forming autapses that modulate the cell firing patterns.

2.1.2. Membrane responses to synaptic transmission

Opening of an ion channel can have different electrical effects on the cell membrane depending on the activated current. Influx of cations or efflux of anions (inward current) can result in a depolarisation while anion influx or cation efflux (outward current) is able to hyperpolarise the membrane, provided that the membrane resistance is sufficient. It is important to understand that according to Ohm's law, stronger currents will be need to produce significant polarisation if the cell resistance is very low (i.e., membrane is short circuited). On the other hand, the polarisation induced by a channel-mediated current may last longer than the associated decrease in resistance if the membrane repolarises slowly (depends on the membrane time constant τ) (see Gulledge & Stuart, 2003).

Transmitter-induced changes of postsynaptic membrane potential can be classified according to their effect on action potential generation probability: those that increase it are called excitatory postsynaptic potentials (EPSP) and the decreasing ones inhibitory postsynaptic potentials (IPSP). Since the effect of membrane depolarisation is usually excitatory and hyperpolarisation drives the cell away from action potential threshold, the terms depolarisation and hyperpolarisation have been (incorrectly) used as synonyms for excitation and inhibition, respectively. Activation of a channel that depolarises the postsynaptic cell may well have net inhibitory result if the cell resistance drops enough to prevent further depolarisation and action potential generation (e.g. Kaila & al., 1993).

2.2. Modulation of neuronal signalling

2.2.1. Ionic modulation

The changes in the concentrations of ions, particularly H^+ (usually quantified as pH), bicarbonate (HCO_3^-), Ca^{2+} and K^+ brought about by neuronal activity can impose a feedback on the active cells themselves. In this section the ionic modulation of neuronal signalling will be briefly discussed, as it will be essential for understanding the network interactions in the hippocampus.

Even though membrane potential changes can be brought about with very small ionic fluxes, channel activation may result in significant changes in intracellular ion concentrations. All electrical signalling in neurons is based on

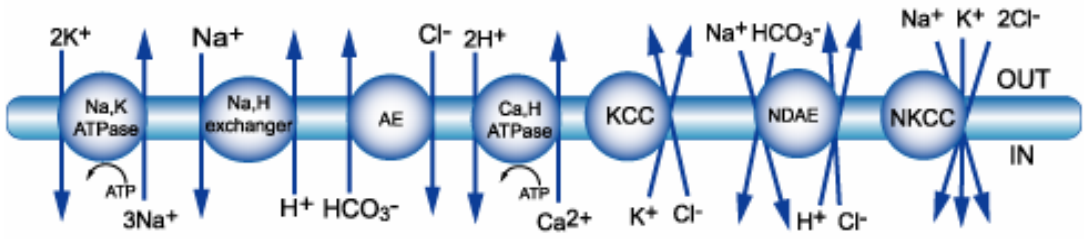

Figure 2. The main ion transporters present in neurons. The individual isoforms are not shown. The localisation on the cellular membrane as well as the activation/inactivation kinetics varies between transporters, so not all transporters are functioning at the same time and place. AE – anion exchanger; KCC – K^+ / Cl^- cotransporter; NDAE – Na^+-dependent anion exchanger; NKCC – $Na^+/K^+/Cl^-$ -cotransporter.

strictly controlled ion gradients established by an array of ion transporters and slightest shifts in ion concentrations can launch regulatory mechanisms transporting ions across the cell membrane (the key molecules together with the stoichometry of transport are schematised in Figure 2). Net movement of ions (chloride, potassium, sodium, hydrogen and bicarbonate) are followed by water (osmosis) and thus volume changes are often associated with neuronal activity in addition to ionic and pH shifts. The swelling and shrinkage of cells can be quantified as changes in the volume fraction, the ratio of intracellular to extracellular volume, which is around 17-22% under 'control' conditions (Jefferys, 1995).

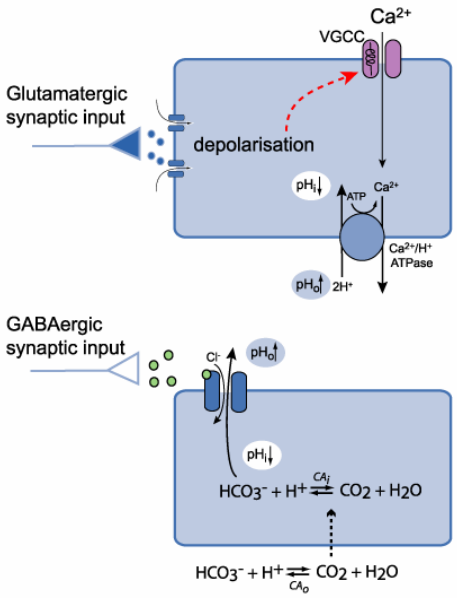

Figure 3. Mechanisms behind activity-induced pH shifts in response to glutamate – and GABA-mediated synaptic input. Inhibition of carbonic anhydrase increases the pH shifts brought about by glutamatergic synaptic transmission (by reducing the buffering power) and decreases those resuling from GABAergic activity (by inhibiting the reaction permitting significant bicarbonate efflux).

2.2.1.1. pH

Under resting conditions, the intracellular pH (pH_i) is maintained at a much more alkaline level than would be expected if the protons would be passively distributed across the membrane. This electrochemical gradient is maintained by transmembrane acid-extrusion mechanisms, such as the Na^+/H^+ exchanger and the Na^+

dependent Cl^-/HCO_3^- exchanger (Figure 2). During neuronal activity significant shifts (in the range of few tenths of a pH unit; see Ballanyi & Kaila (1998) and Kaila & Chesler (1998) for detailed reviews on the subject) in intra – and extracellular pH are seen; both glutamate– and GABA– mediated activity are associated with extracellular alkalosis and intracellular acidosis even though the mechanisms are different (see Figure 3). Glutamate receptor activation-induced pH shifts result probably of stimulation of Ca^{2+}/H^+ -ATPase in response to influx of calcium (Ballanyi & Kaila, 1998). GABA-induced pH transients result from bicarbonate (HCO_3^-) efflux through the $GABA_A$ receptor-gated channel. Bicarbonate is an anion of a weak acid (carbonic acid, H_2CO_3), and if P_{CO2} is constant, its equilibrium potential is equal to the H^+ equilibrium potential (around –10 to –20 mV). Thus, opening of $GABA_A$ channels leads to bicarbonate efflux. Carbonic anhydrase (CA) catalyses the reaction

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+. \quad (1)$$

Since the cell membrane is highly permeable to CO_2, efflux of bicarbonate is immediately compensated by the reaction above, leading to further intracellular acidification because of production of H^+.

Acid-base equivalents are neither generated de novo nor consumed in the extracellular space. Thus, intracellular acid shifts must naturally be reflected as extracellular alkalosis. The mechanistic differences of acid-base equivalent movements in GABA – and glutamatergic neuronal signalling result in differences in the sensitivity of extracellular alkalosis to inhibitors of CA. The glutamate-induced flux of protons into active cells produces an extracellular alkalosis which is enhanced when carbonic anhydrase is inhibited, as the buffering power of extracellular space decreases. In contrast, the GABA –induced extracellular alkalinization requires not only on the efflux of bicarbonate but fast anhydration of carbonic acid (reaction (1) to the right) and thus is decreased by inhibitors of CA (Chesler & Kaila, 1998). Interestingly, intracellular CA activity is not seen in the hippocampal pyramidal cells of very young animals (Ruusuvuori & al., 2003), suggesting a different role for bicarbonate in the modulation of synaptic transmission during the development of these cells.

Activity of a neuron can have feed-back influence on its own activity via activity-dependent pH changes, since intracellular enzymes, ion channels, gap junctions are sensitive to slight changes in $[H^+]_i$. Acid extrusion by exchangers and

transporters leads to direct changes in concentrations of other ions; a fall in pH_i inhibits several voltage-gated channels (Tombaugh & Somjen 1998) leading to changes in neuronal excitability. A rise in intracellular pH enhances gap junctional coupling and can lead to increased synchrony in activity. Interestingly, termination of non-synaptically synchronised seizure activity in the dentate gyrus has been shown to correlate exactly with acidification of the intracellular environment (Xiong & al., 2000),
Some antiepileptic drugs in clinical use (azetazolamide and topiramate; Wyllie, 1997) are known to have CA-inhibiting effects. Among the several mechanistic explanations proposed for their antiepileptic effects (Stringer, 2000; Sabers & Gram 2000), it has also been suggested that inhibition of the CA-dependent GABA-mediated alkaline pH shifts by these drugs could decrease local excitability and the degree of synchronisation between neurons, thus increasing seizure threshold (Aribi & Stringer, 2002).
Extracellular pH shifts are also known to affect neuronal excitability (Somjen & Tombaugh, 1998): acidification and alkalinization have depressing and even proconvulsant actions, respectively. Transient pH changes may be confined to localized regions of the extracellular space and thus may play a role in neuronal signalling by locally altering neuronal excitability. Several channels, for example the NMDA receptor-gated, are also inhibited by a fall in the pH_o leading to further depression of neuronal communication. Extracellular pH has multiple distinct effects on $GABA_AR$ mediated conductances; the differences are mostly due to differences in subunit compositions.

It is worth mentioning one more mechanism for pH modulation in the CNS: voluntary hyperventilation leads to a fall in pCO_2 in the blood and also raises the pH. This respiratory alkalosis can result in hyperexcitability and if high enough, precipitate seizures in epileptic patients (Foerster 1924), especially in children.

2.2.1.2. Other ions

Several of the shifts of ions found in the extracellular fluid can contribute to neuronal excitability (Jefferys 1995; Jensen & Yaari 1997; Heinemann & al., 1990). For instance, extracellular calcium and magnesium are involved in membrane stabilization by charge screening, where the divalent cations attracted to negative charges on the neuronal membrane increase the electric field sensed by the channels in the membrane. Lowering $[Ca^{2+}]_o$ results in large and synchronous field discharges that can last for seconds, even though in

the absence of calcium all chemical synaptic transmission is blocked. Magnesium has also a specific role on the NMDA receptor function as it exerts a voltage-dependent block of the associated channel.

Two ions, namely, potassium and chloride, require a more detailed discussion as they significantly influence many of the signalling mechanisms examined in the present work.

The resting level concentrations of potassium (approximately 3 mM in the extracellular space and 120 mM intracellularly) result in a negative equilibrium potential (around –90 mV) compared to the resting membrane potential (-60 - -70 mV). The concentration gradient is maintained by the primarily active Na^+/K^+-ATPase (Figure 2) that uses the energy stored in ATP to extrude sodium and accumulate potassium inside the cell. The resulting strong outward driving force for potassium is used as the energy source for transporting other ions across the membrane. One such transporter is the potassium – chloride cotransporter isoform 2 (KCC2; Payne 1997; Rivera & al., 1999; Gulyas & al., 2001; Payne & al., 2003), a secondarily active transporter that uses the K^+ concentration gradient as the driving force for chloride extrusion. Under physiological conditions, KCC2 is close to its thermodynamic equilibrium and thus slight changes in the ion concentrations on either side of the membrane can modulate its action. Extracellular potassium has a direct (although slightly sub-Nerstian) effect on membrane potential and therefore, strong extrusion of intracellular potassium can result in a significant depolarisation of the surrounding cells, as will be later shown.

Importantly, an increase in the extracellular potassium concentration decreases the driving force for KCC2-mediated extrusion of chloride. The transmembrane driving force for chloride depends under constant membrane voltage on the combined effects of passive chloride leak and KCC2. As will be discussed later (chapter 2.6.1, $GABA_A$ receptors), chloride gradient influences the $E_{GABA-A,}$ and thus partakes in determining the membrane response to $GABA_A$ receptor activation. High intracellular concentrations of chloride can also modify G-proteins (Nakajima & al., 1992; Lenz & al., 1997), thereby affecting metabotropic signalling and influence neuronal function on a longer time scale.

2.2.2. Activity-induced modulation

2.2.2.1. Ephaptic modulation

Volume changes resulting from neuronal activity can act as a

feedback or feed-forward signalling mechanism by itself. Changes in the extracellular volume fraction affect ephaptic coupling properties (Jefferys, 1995), that is, the sensitivity of a cell to extracellular field effects. Currents produced by one neuron can affect the membrane potential of another, given that the communicating cells are electrically close enough. Cell swelling increases extracellular resistance and can facilitate ephaptic communication, sometimes resulting in very tight synchronisation of activity. Dendritic swelling in response to intense activity has been reported the CA1 area (Andrew & MacVicar, 1994); this suggests that the signal processing properties of dendrites can be modulated at a local scale. In addition, it has been proposed that intrinsic firing properties and NMDA receptors can be influenced by swelling of the spines (Paoletti & Ascher 1994).

2.2.2.2. Synaptic plasticity

Glutamatergic synaptic transmission can be facilitated in response to repetitive activation, as a result of elevated presynaptic calcium levels remaining from the previous synaptic events (Kamiya & Zucker, 1994). This phenomenon is called paired-pulse facilitation. On the other hand, synapses that have a high release probability are subject to short-term synaptic depression, particularly when the density of release sites is high. This paired-pulse depression probably results from transmitter vesicle depletion, since reducing release probability decreases the depression seen in synaptic transmission during a train of presynaptic spikes (Brenowitz & al, 1998). Other possible mechanisms include presynaptic autoreceptor activation and postsynaptic receptor desensitisation.

Activity-dependent long-term changes in synaptic efficacy are thought to be the cellular mechanism underlying learning and memory formation. Since the hippocampus has a crucial role in memory consolidation in the mammalian brain, it is useful to review very briefly some of the processes by which synapses are modified in structure and function with emphasis on the plasticity at GABAergic synapses. Long-term modulation is also the key to understanding the development of both normal and pathological states of neuronal excitability (for instance, epilepsy).

The classical models of activity-dependent synaptic plasticity at glutamatergic synapses, long-term potentiation (LTP) and long-term depression (LTD) have for decades been under extensive studies inspired by Hebb's (1949) theory of the synaptic basis of learning (e.g.

Bliss & Lømo 1973; Bear & Malenka 1994; Kullmann & al., 1996; Lüscher & al., 2000). Long-term changes in GABAergic synaptic strength (reviewed by Gaïarsa & al, 2002), on the other hand, have until recently received much less attention. Similar to glutamatergic synapses, the plastic changes at GABAergic synapses might be triggered by rises in intracellular Ca^{2+} concentration (McLean & al., 1996). The actual mechanisms must differ, however, as one of the key molecules in the synaptic plasticity of glutamatergic synapses, the Ca^{2+}/calmodulin-dependent kinase II (CaMKII), is absent from interneurons (Sik & al., 1998, but see also Minichiello & al., 2002 for the role of another isoform, the CaMKIV). Both long-term potentiation and depression can be experimentally induced at GABAergic synapses, depending on the conditioning protocol and the relative amounts of Ca^{2+} entering the neurons (Aizenman & al., 1998). GABAergic synapses can be modulated postsynaptically by changing the amount of available postsynaptic receptors (Nusser & al, 1998), or presynaptically by modifications in the GABA release probability or number of functional release sites (Gubellini & al, 2001).

Clearly, LTD of a GABAergic synapse may facilitate LTP induction of a glutamatergic synapse and massive depression of GABAergic inhibition may lead to pathologic conditions (epilepsy), but the overall effect of plastic changes depends on the specific type of the GABAergic neuron and connection in question. Therefore, to correctly interpret the influence of plasticity at a GABAergic synapse on network activity, the subgroup of the interneuron must be identified.

2.2.2.3. Retrograde signalling

In addition to forward (anterograde) signals spreading from pre– to postsynaptic cells, it is known that the postsynaptic cells are able to signal back (in a retrograde manner) to the presynaptic cell.

Depolarisation of CA1 pyramidal cells leads to transient suppression of incoming $GABA_A$ receptor-mediated IPSPs. This phenomenon, the depolarisation-induced suppression of inhibition (DSI) was described by Pitler & Alger (1990) and requires a retrograde messenger diffusing back to the presynaptic GABAergic neuron. In search for the molecular components of this phenomenon, it was found that activation of the hippocampal cannabinoid receptor, CB_1, inhibits GABA release (Davies & al., 2002).

The endogenous agonists of the CB_1 receptors, endocannabinoids (arachidonoylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG)), are released in response to

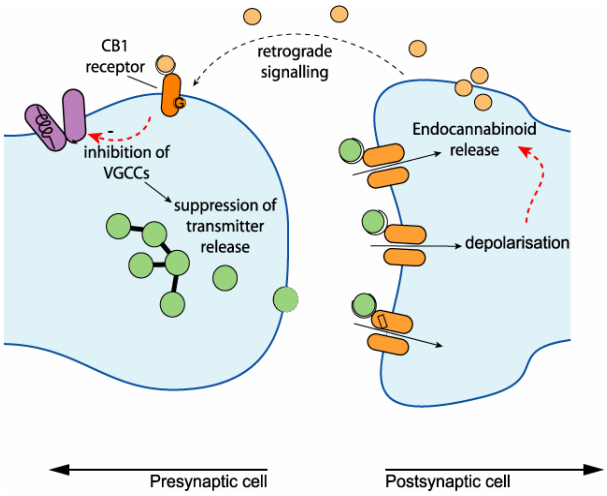

Figure 5. Depolarisation-induced suppression of GABA release. The postsynaptic depolarisation activates endocannabinoid (anandamide or 2AG) synthesis and release in a Ca^{2+} -dependent manner. The released compound traverses retrogradely the synaptic cleft and activates G-protein –coupled CB_1 receptors. The resulting inhibition of transmitter release can be seen as a suppression of inhibition after a strong postsynaptic depolarisation.

depolarisation (DiMarzo & al.,1998) and thus could implement DSI (see Figure 3). The cannabinoid receptor in the hippocampus, CB_1, is localised to the presynaptic terminals of a subset of GABAergic neurons, especially to the CCK-containing basket cells (Hájos & al., 2000; Irving & al., 2000; Katona & al., 1999, 2000; Freund 2003). CB_1 receptors are metabotropic receptors coupled to $G_{i/o}$ proteins which block voltage-gated calcium channels. Although this is a possible mechanism by which cannabinoids may inhibit transmitter release, metabotropic receptor activation triggers numerous intracellular signalling cascades as explained previously

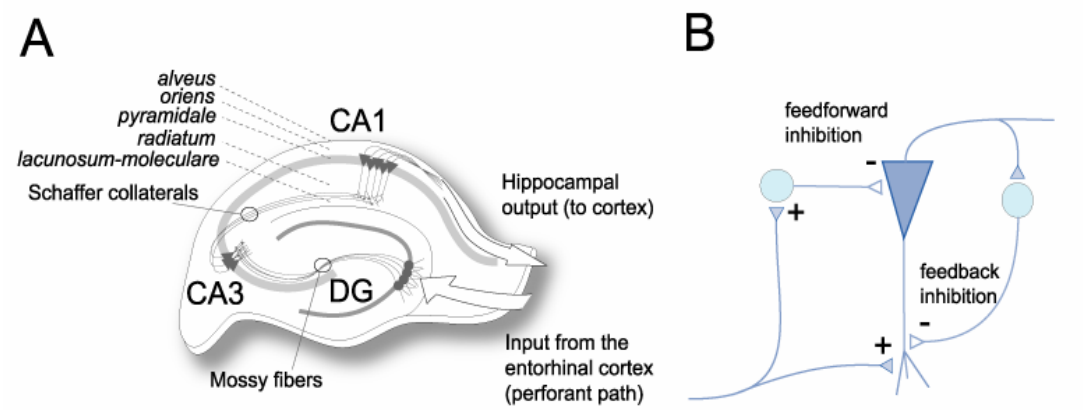

Figure 4. A. Synaptic connectivity of the hippocampus. The trisynaptic circuit brings afferent input from the entorhinal cortex to the granule cells of the dentate gyrus (DG). The main output of these cells, the mossy fibers, innervate the proximal dendrites of the pyramidal cells in the CA3. In addition to the recurrent excitatory connections within the CA3 area, the pyramidal cells send axon collaterals further into the hippocampus and terminate in the dendritic region of the CA1. The circuit is completed by axons of the CA1 pyramidal cells, which extend back to the cortex.

B. Local circuits of pyramidal cells and interneurons comprise of feedforward and feedback connectivity.

(chapter 2.1.1, Receptors) so the response to CB_1 receptor activation is not likely to be simple. Indeed, a number of potassium channels are modulated by CB_1 activation thereby affecting net excitation.
Metabotropic cascades are overlapping and signalling from one source can be modified by another signal; GABA itself acts through a G-protein coupled receptor (the $GABA_B$ receptor, see chapter 2.6.2) and therefore may interfere with or modulate its own release. Curiously, release of endocannabinoids in response to depolarisation may be enhanced by activation of postsynaptic mGluRs (Wilson & Nicoll, 2001) suggesting a weave of second messenger communication underlying the fast, ionotropic neuronal communication.

2.3. Structure of the hippocampus

The hippocampus has been particularly useful in revealing basic principles of synaptic organisation in the cortex, due to arrangement of the cell bodies of principal cells into a single layer and segregation of different functional parts of the circuitry into seperate areas.
Lorente de No (1934) established the division of hippocampus into CA (cornu ammonis) 1 through 3 and the dentate gyrus (DG), according to cell body and projection characteristics. Perpendicularly to the pyramidal cell layer the CA1 region is divided to several lamina, with stratum oriens on the 'external' side of stratum pyramidale, and stratum radiatum and stratum lacunosum-moleculare extending to the upper blade of the DG (Figure 5a). The dentate gyrus receives sensory information from the entorhinal cortex via the perforant pathway. Principal cells of the DG, granule cells, send their main axons (mossy fibers) to the CA3 subfield where they form terminals on the proximal dendrites of pyramidal cells. In the CA3 area, pyramidal cells are interconnected with heavily recurrent arborisation of pyramidal cell axons. The major output of CA3 area extends in the form of Schaffer collaterals to CA1 pyramidal cells and interneurons. This so-called trisynaptic circuit, even though overly simplistic, is still thought of as the principal route of signal flow through hippocampus. In addition, all subfields of the hippocampus receive modulatory subcortical projections from various sources (e.g. serotonergic and noradrenergic projections). On a local scale, the neurons form intricate networks with synaptic connections restricted to only a certain portion of the dendritic tree. The connections can be roughly divided to feedforward and feedback connections (Figure 5b) where a signal is driven in feedforward manner and modulated

by feedback-connections (for a detailed review on the basic circuit properties of the hippocampus, see Somogyi & al., 1998).

2.4. Gap junctions

In addition to chemical synapses, neurons can be coupled via electrical synapses that are formed of gap junctions (GJs). Recently the significance of GJ communication in neuronal synchronisation has attracted a lot of attention together with the diversity and specificity of GJ connections.

2.4.1. Structure and expression

GJs connect the cytoplasm of a cell directly to another via gap junction channels. Each cell of the coupled pair contributes a half-channel (connexon) formed of six homologous subunits called connexins. Functional gap junction channels are normally formed only of homologous connexons. Connexins belong to a large family of genes; there are many connexins expressed in the brain, including Cx32, Cx36, Cx43 and Cx47, but only one, Cx36 is unequivocally known to be expressed in adult hippocampal CA1 interneurons (Condorelli & al., 2000; Rash & al., 2000). The expression of gap junctions is not much altered in the neocortex during development (Meyer & al., 2002), but the strong GJ coupling between neonatal hippocampal interneurons, glia and principal cells decreases during maturation. In the neocortex there are at least two different networks of electrically coupled interneurons: fast-spiking (FS) and low-threshold spiking (LTS) GABAergic cells are coupled among themselves via gap junctions (Galarreta & Hestrin 1999; Gibson & al., 1999). In the hippocampus, the parvalbumin (PV) –positive interneurons (mainly basket cells targeting pyramidal neuron somata and initial segments of axons) form a dense electrically coupled network covering large areas (Fukuda & Kosaka, 2000) and thus can exert control over the generation of interneuronally paced oscillations (see section 2.7.3.1., Interneuron network oscillations; see also Freund, 2003).

2.4.2. Conductance

Gap junction channels allow the movement of electrical signals as well as ions and small molecules (up to 1 kDa) from cell to cell; for instance, intercellular Ca^{2+} signalling can take place via gap junctions. The range given for electrical conductance of a single GJ is usually 30-300 pS (Carlen & al., 2000; but see Traub & al., 2002 for discussion of the difficulties in direct measurements of GJ conductances), and an array of GJs at an electrical synapse can result in nS -scale conductance. Most gap junctions are symmetrical, i.e., the

coupling strength is roughly equal in both directions, if the capacitative load on the two sides of the junction is equal. Signal transmission is however frequency-dependent (Galarreta & Hestrin 1999), with low-frequency signals showing greater coupling ratios (see 2.7.2, Resonance). It should be noted that the electrical signal attenuates while propagating through a GJ-coupled chain of neurons according to the electrotonic length and upon crossing over a gap junction. Therefore a network of electrically coupled neurons does not necessarily 'see' all the activity if the dendrites act like passive cables. Interneuronal dendrites, however, are known to possess the capability to actively propagate action potentials (Martina & al., 2000).

2.4.3. Gating

Under normal circumstances GJ channels are mostly in an open state (Peracchia & al., 2000), but like other channels, GJ channels gate in response to physiological conditions, changing the extent of coupling between cells and modifying the topography of electrically coupled neural networks. For instance, intracellular acidification decreases and alkalinization increases gap junctional coupling (Spray & Scemes 1998) in the physiological pH range; also, elevated cytoplasmic concentrations of Ca^{2+} can reduce coupling. The gap junction channel is well insulated from the extracellular space and thus extracellular pH and other modulators do not directly affect GJ coupling. Even though chemical gating of gap junctions via neurotransmitters is in some cases known to occur (e.g. via kinases (Lampe & Lau 2000) and calmodulin (Peracchia & al., 2000)), lack of specific GJ blockers has been a major obstacle in elucidating the gap junctional communication in neural network. The conventionally used GJ blockers, heptanol, octanol, halothane (an anaestethic agent) and carbenoxelone have side effects not related to their GJ blocking action but similar action of these drugs has been interpreted as an indication of GJ involvement. Quite luckily, the antimalarial drug quinine (Srinivas & al., 2001) was found to specifically block gap junctions formed of the connexin 36 (Cx36), providing means for studying the role interneuronal gap junctions in synchronization of the network.

2.4.4. Physiological role

Several studies have succeeded in correlating gap junctions directly to the synchronous activity of central neurons, starting from Llinas and coworkers in 1974. The spontaneous firing of hippocampal interneurons in the absence of

glutamatergic transmission is initiated and synchronized by gap junctions (Yang & Michelson 2001), and they can promote action potential generation in the connected neurons and synchronize their firing within 1 ms (Gibson & al., 1999, Galarreta & Hestrin 1999). The GJ-coupled LTS network of cortical interneurons is also known to be able to produce synchronized inhibition and coordinate the firing patterns of cortical neurons over a large distance when activated by metabotropic receptors (Beierlein & al., 2000). Accuracy of timing is accentuated by the spatial proximity of GABAergic synapses and GJs (Támas & al., 2000), and indeed it has been shown by modelling studies (Traub & al., 2001) that GJs between interneurons can enhance gamma synchrony.

As electrical synapses can transmit signals on a time scale much faster than chemical synapses, they can synchronize neural activity at higher frequencies than chemical synapses. Mathematical models (Traub & al., 1999) suggest that in the absence of chemical synaptic signalling it is enough for a CA1 cell to be electrically coupled to only two other neurons to produce coordinated ripple oscillations (see chapter 2.7.3., Oscillations) in the CA1 region of the hippocampus. The GJs would be most efficient in promoting high-frequency oscillations if they were located on the axons of CA1 cells (Draguhn & al., 1998), where low capacitative load, high input resistance and the density of voltage-gated Na^+ channels would promote the generation of active depolarizations and coordinated activity of pyramidal neurons despite the inhibition of CA1 somata by the active network of interneurons. Inspired by these simulations and the finding that gap junction blockers inhibit high-frequency ripple oscillations (Draguhn & al., 1998), Schmitz & al., (2001) sought and found evidence for axo-axonal electrical synapses between the CA1 pyramidal neurons. Moreover, in the recently constructed Cx36-deficient mice (Deans & al., 2001; Hormuzdi & al., 2001; Maier & al., 2002) the strength and spatial extension of synchronous neuronal activity was significantly altered. Even though there seems to be some controversy in the effect of Cx36 deletion on gamma – and high-frequency oscillations, the role of gap junctions in generation and maintenance of synchronous activity seems increasingly important.

2.5. Interneurons

The classical picture of interneurons being an inhibitory counterpart of excitatory principal cells has been based on the term 'GABAergic' used as a synonym for 'inhibitory', and the fact that all interneurons are taken to be GABAergic. This view has finally giving way to understanding the importance of interneurons as modulators of cortical and hippocampal activity. Interneurons are considerably less abundant (~10%) in the hippocampus and dentate gyrus than pyramidal neurons. However, the massive connectivity of interneurons (each interneuron may contact thousands of postsynaptic target cells) permits the generation and control of the rhythmic output of the hippocampal network. In contrast to the rather uniform population of principal cells in any of the hippocampal subfields, the diversity of interneurons in terms of morphology, chemical and physiological characteristics is so vast that classification into clear subpopulations is very demanding if not impossible. In a heroic study by Parra and co-workers (1998), over 50 different subclasses were found when these properties were taken into account! Owing to the diversity and fine-tuning of properties, each interneuron can perform multiple computational functions depending on localisation, timing, input parameters and target identity.

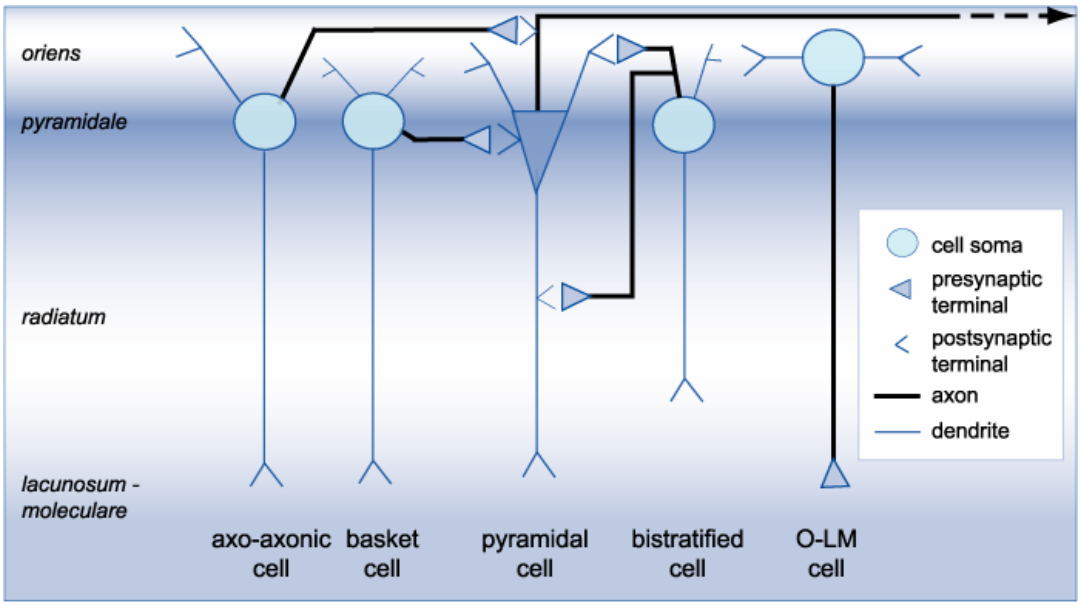

Figure 6. Schematic examples of the afferent and efferent connections of the interneurons in the CA1 region. Schaffer collaterals from the CA3 pyramidal neurons terminate in the stratum radiatum.

2.5.1. Physiological properties of the interneurons

Eccles (1969) already suggested that interneurons fire in a different way than pyramidal neurons. Their high spontaneous firing rates, short spikes, large AHPs, weak spike frequency accommodation and high input resistance together with the locally projecting anatomy render the interneurons clearly a group with functions differing from the pyramidal cells. A review by Freund and Buzsáki (1996) provides an excellent introduction in to the properties and functions of interneurons, some of which are briefly discussed below.

Unlike in the principal cells, the interneuronal AMPA channels are significantly permeable to Ca^{2+} and fast excitatory transmission through interneuronal AMPA receptors is more rapid and of greater amplitude (McBain & Fisahn, 2001). Also, interneuronal voltage-gated channels have important differences in subunit composition as well as spatial distribution if compared to the principal-neuron counterparts, allowing fast spiking (due to different potassium channel kinetics) and dendritic action potentials (density of Na^+ and K^+ currents in dendrites), two features associated with interneurons and sometimes used as a fingerprint of an interneuron. While recurrent pyramidal-to-interneuron connections are mediated by one synaptic junction, interneurons usually form multiple synapses with a particular target neuron, making synaptic transmission more reliable (Buhl & al., 1994). Unlike pyramidal neurons, interneurons are heavily interconnected to neurons of their own type by gap junctions, creating complex electrically coupled networks (Gibson & al., 1999; Galarreta & Hestrin, 1999).The diverse properties of interneurons can be used to classify them into subgroups (reviewed by McBain & Fisahn, 2001).

One of the most useful characterisations of interneurons has been based on neurochemical content; Presence or absence of calcium-binding proteins (parvalbumin (PV), calbindin, calretinin) and neuropeptides (somatostatin, neuropeptide Y (NPY), cholecystokinine (CCK)) that likely modulate synaptic responses of target neurons can be verified by labeling methods and this labelling can be combined with other characterisations. For instance, parvalbumin-positive interneurons of the hippocampus have been electrophysiologically characterized as a group of fast-firing, non-plastic 'clockwork' cells that has very likely a specific role in hippocampal information processing (Freund, 2003).

The afferent and efferent connections of interneurons show

great variation (Figure 6; McBain & al., 1999) even though they all use GABA as their fast-acting neurotransmitter. Some interneuron subtypes are innervated exclusively by extrahippocampal afferents (partaking only in feedforward signalling) but most are innervated both in feedforward and feedback manner. Effect of interneuronal input on a principal cell depends on the location of the GABAergic synapse. For instance, the somatic connections of basket cells on pyramidal cells regulate the local generation of Na^+ -dependent action potentials (Miles & al., 1996) and may remove inactivation of subthreshold inward currents (Cobb & al., 1995). Dendritic inhibition by bistratified (O-LM) cells modulates synaptic plasticity and provides source-specific inhibition by shunting dendritic currents and suppressing dendritically generated Ca^{2+} -dependent action potentials. The different origins of somatic and dendritic inhibition may become important in development of pathological conditions, as dendritic inhibition is specifically decreased in experimental epilepsy (Cossart & al., 2001). In addition, axon initial segment-projecting interneurons can influence the ability of a neuron to initiate an action potential. Interestingly, there is a correlation between interneuronal connectivity and the neurochemical and morphological phenotype; for instance, axo-axonic interneurons are also often PV-positive, and calretinin-containing interneurons specifically innervate other interneurons (Gulyás & al., 1996).

Interneurons can be divided into subgroups based on functional properties. In the neocortex, the interneurons have been classified by Gibson and co-workers (1999) to fast-spiking (FS; with narrow action potentials, deep and brief AHP, high firing rate, no frequency adaptation) and low-threshold spiking cells (LTS; broader action potentials and some frequency adaptation). These groups contribute to different circuits, as FS but not LTS interneurons make inhibitory synapses with cells of the same type. Also, FS neurons selectively mediate feedforward inhibition, whereas LTS cells seem to be involved in local (recurrent) inhibition. In the hippocampus different interneuronal subgroups have been proposed to participate in feedback and feedforward inhibition (Nurse & Lacaille, 1997) and according to the patterns of activity, interneurons can be grouped as regular-firing, irregular-firing, clustered-firing (Parra & al., 1998).

2.5.2. Physiological roles of interneurons

In the absence of glutamatergic transmission, interneurons can fire in synchrony using $GABA_A$

receptor-mediated synaptic excitation (Michelson & Wong, 1991) and electrical coupling (Yang & Michelson, 2001) for synchronization. Interneuron network-mediated oscillatory activity has been proposed to provide a rhythm which allows precise temporal coding for the hippocampus, and "super networks" of interneurons have been hypothesized to link different regions of the brain by providing a pattern against which other activity takes place. Thus, networks of interneurons may impose a co-ordinated oscillatory 'context' for the 'content' carried by principal cells (Freund & Buzsaki, 1996). For instance, by regulation of NMDA receptors and synaptic plasticity the interneurons can take part in 'deciding' whether the strength of a synapse is to be modified upon arrival of synaptic input or the incoming signal just passed on, thus switching between processing modes.

Interneurons become postmitotic earlier than pyramidal cells. At birth only 5% of the interneurons are silent in contrast to the 80% of silent principal neurons, and GABAergic synapses are established first during development (Tyzio & al., 1999). Interneurons that innervate the apical dendrites of pyramidal neurons mature before those innervating cell body. When considering together with the fact that the functional properties of GABAergic transmission in the immature brain are different than in the adult brain (Rivera & al., 1999; Ben-Ari, 2002), the physiological role played by interneurons is necessarily different at early developmental stages.

2.6. Ionic mechanisms of GABA-mediated responses

The two major transmitters in the brain, glutamate (reviewed by Ozawa & al., 1998) and GABA (reviewed by Kaila, 1994) are usually presented in text books of neurobiology as excitatory and inhibitory transmitters, respectively. This concept is based on the equilibrium potential of glutamate-activated receptor channels being positive enough to shift the membrane potential towards action potential threshold, while the equilibrium potential of chloride – the first recognised current carrier for ionotropic GABA-activated channels – is often more negative than the resting membrane potential due to active extrusion of intracellular chloride by KCC2. However, the $GABA_A$ channel is significantly permeable to HCO_3^- and GABA-mediated synaptic activity may result in membrane depolarisation, as will be discussed shortly. While the entire output of the cerebellar cortex is GABAergic

(Ito, 1984), only a fraction of hippocampal cells – interneurons - use GABA as neurotransmitter. Nevertheless, the importance of GABAergic communication is all but nonsignificant, as slight alterations in the GABA–mediated inter-neuronal control of pyramidal cells leads to severe disturbances of hippocampal function.

GABA exerts its function mainly via two receptors, the $GABA_A$ and $GABA_B$ receptors (see Figure 7) mediating $IPSP_A$ and $IPSP_B$, respectively. The two receptors have nothing in common in evolutionary terms as well as in kinetics or currents carried. As they are activated by the same neurotransmitter and often reside on the same neuronal membrane, the postsynaptic GABA response in intact synaptic network *in vitro* can be a combination of these two mechanisms. Nevertheless, the distribution of these two receptor types is somewhat different on the pyramidal cells with $GABA_B$ receptors residing predominantly in the dendritic region unlike the more uniformly expressed $GABA_A$ receptors (Lopez-Bendito & al., 2002). Also, there is some evidence that $GABA_A$ and $GABA_B$ receptors might be associated with distinct inhibitory circuits, so that feedback inhibition would be mediated via $GABA_A$ receptors only (Nurse & Lacaille, 1997) and it has been shown that in the neocortex $GABA_B$ receptor-mediated IPSPs originate from unitary sources (Tamás & al., 2003). In any case, both receptor types should be considered in parallel when examining the activity of GABAergic interneurons.

As with other fast synaptic transmission, transmitter uptake mechanisms restrict the spatial range of GABAergic transmission in addition to enzymatic degradation of GABA when synchronous release occurs from a population of synapses. The ability of GABA molecules to reach the abundant extrasynaptic receptors depends on the synapse morphology and efficacy of uptake mechanisms, which, intriguingly, can differ between somatic and dendritic areas (Isaacson & al., 1993).

2.6.1. $GABA_A$ receptors

The $GABA_A$ receptor is a pentameric membrane protein composed of several distinct subunits (Figure 7) with several sites for allosteric modulation of activity. In addition to endogenous and exogenous ligands, the $GABA_A$ receptor function is modulated by calcium and zinc ions as well as the extracellular pH, with alkalinization enhancing and acidification suppressing GABA-activated currents (Huang & Dillon, 1999). The normal activation of the receptor is thought to require the

cooperative binding of two molecules of GABA; of the inhibitors, picrotoxin (PiTX), a noncompetitive antagonist, and bicuculline (Bic), a competitive agonist, are worth mentioning.

The $GABA_A$ receptor-associated ion channel is permeable to chloride and importantly, bicarbonate. As discussed earlier (section 2.2.1.1, pH), the concentration of intracellular bicarbonate is set by the pH_i and is higher than what would be if dependent only on passive distribution. Thus, opening of a $GABA_AR$ associated channel results in an efflux of bicarbonate together with the chloride influx. Even though the bicarbonate permeability of the $GABA_A$ channel is much lower than chloride permeability (Kaila, 1994), at the resting membrane potential the driving force for bicarbonate is significantly stronger than for chloride. For instance, during activation the $GABA_A$ current in resting neocortical pyramidal neurons is carried by HCO_3^- to a greater extent than by chloride (Kaila & al., 1993; Gulledge & Stuart, 2003).

Movement of these negatively charged anions through the $GABA_A$ channel results in a shift in membrane potential towards the channel reversal potential (E_{GABA-A}), which is approximately 10 – 20 mV more positive than the equilibrium potential of chloride (E_{Cl}) due to the inward current carried by bicarbonate (Voipio & Kaila, 2000). In addition, the intracellular anion content increases as the bicarbonate leaving the cell is compensated by the reaction catalyzed by carbonic anhydrase (Roos & Boron, 1981) while chloride ions flow in.

It has been known for decades that GABA can have a biphasic (hyperpolarising - depolarising) effect on membrane potential (e.g. Alger & Nicoll, 1979). This has been attributed to pharmacologically different $GABA_A$ receptors (Alger & Nicoll, 1982; Perkins & Wong, 1996), as well as to an intracellular chloride gradient (Misgeld & al., 1986) or to the dissipation of chloride gradient during intensive activity (Staley & al., 1995) due to influx of Cl^-. During the last few years evidence has been presented for a nonsynaptic mechanism of GABAergic depolarisation, which will be discussed later.

In a recent study, Gulledge & Stuart (2003) shed further light on the long-discussed difference between somatic and dendritic $GABA_A$ receptor activation (Alger & Nicoll, 1982; Kaila, 1994; and others). It turned out that the excitatory action of GABAergic synapses depends on the spatial or temporal isolation of the conductance increase during activation from other depolarising inputs, while no qualitative differences in dendritic and somatic

GABA responses were found. In fact, responses to brief GABA applications or stimulation-evoked IPSPs were always depolarising irrespective of the location of activated site. In addition, $GABA_A$ receptor-mediated activity can result in post-inhibitory rebound activation of the target pyramidal neuron (Cobb & al., 1995) that simultaneously synchronises a population of pyramidal cells.

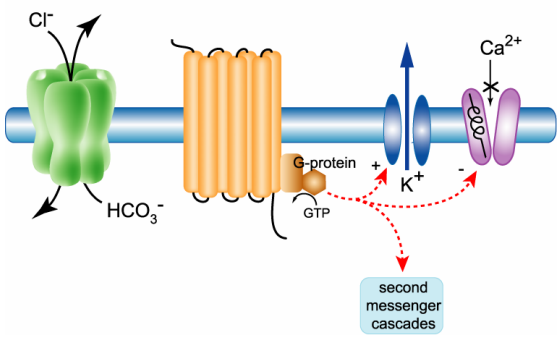

Figure 8. Schematic presentation of the GABA receptors and their immediate effects. Note that the ion channels modulated by the $GABA_B$ receptor-mediated response are in reality residing on different sides of the synapse: potassium conductance is increased postsynaptically, while the inhibition of calcium influx is a presynaptic process.

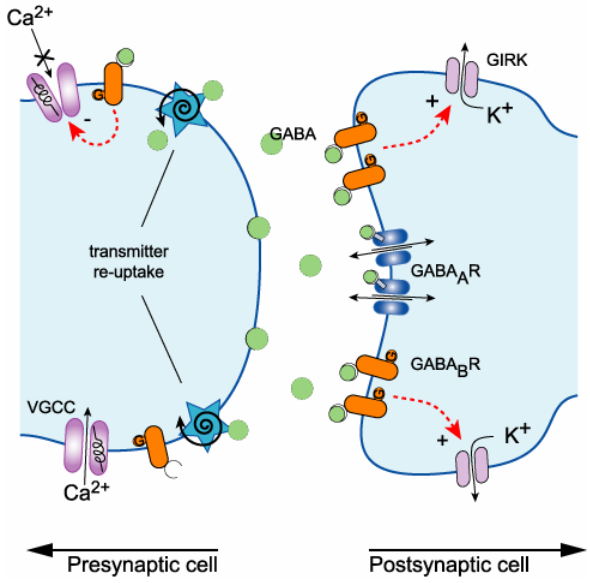

Figure 8. $GABA_B$ receptor-mediated signalling. Intense activity releases enough GABA to activate the more distant presynaptic receptors.

Due to the developmental regulation of expression of the KCC2 and the low level of this extruder in rats younger than postnatal day (P)6 (Rivera & al., 1999), intracellular chloride concentration is much higher than in adult animals. This results in a less negative E_{GABA-A}, and subsequently, more strongly depolarising $GABA_AR$ responses. Another key component in generation of the adult $GABA_A$ receptor mediated response, the bicarbonate efflux, is also dependent on a developmentally regulated factor: intracellular carbonic anhydrase (Pasternack & al., 1993). Very recent studies (Ruusuvuori & al., 2003) show that in neonatal animals this intracellular enzyme is not expressed. Interestingly, the stimulus – induced oscillations (discussed later in chapter 2.7.3) develop at a strikingly similar time course.

2.6.2. $GABA_B$ receptors

The $GABA_B$ receptors (reviewed by Misgeld & al., 1995, and Mott & Lewis, 1994) differ fundamentally from $GABA_A$ receptors in that they are metabotropic receptors, that is, the effect of receptor activation is

mediated by a G-protein (Figure 7). In addition to the endogenous GABA, this receptor is activated by exogenously applied baclofen and selectively blocked by antagonists such as CGP55835A and phaclofen. During low-frequency activity, GABA released synaptically by a single presynaptic interneuron activates postsynaptic mainly $GABA_A$ receptors, since $GABA_B$ receptor activation often requires synchronous activity of several presynaptic cells (see for example Scanziani, 2000 but also Támas & al., 2003 for a recent update) which may occur during hippocampal rhythmic activity. Activation of the postsynaptic $GABA_B$ receptors generate a slow hyperpolarisation, mediated by G-protein –dependent activation of outward potassium currents of the GIRK type. GABA is efficiently cleared from the synaptic cleft by transmitter re-uptake and thus the more distant presynaptic $GABA_B$ receptors are not activated unless the presynaptic signal lasts longer. During intensive activity, sufficient amount of GABA is released to diffuse retrogradely (see chapter 2.2.2.3., Retrograde signalling) to the presynaptic $GABA_B$ receptors and results in G-protein mediated inhibition of GABA and glutamate release by a reduction in voltage-gated calcium influx (Cobb & al., 1999). In the case of inhibiting GABA release transmitter exocytosis can be directly suppressed by $GABA_BR$ activation (Lei & McBain, 2003).

The $GABA_B$ receptor-mediated responses, particularly the presynaptic ones, are not uniform. Throughout the mammalian nervous system, the downstream mechanisms of presynaptic $GABA_B$ receptor modulation appear to be highly heterogenous and differ from synapse type to synapse type. In addition to inhibition of calcium influx, presynaptic $GABA_B$ receptor mediated effects may be induced via activation of presynaptic K^+ channels producing hyper-polarisation, direct interaction with transmitter exocytosis and phosphorylation of various targets. The affinity of presynaptic $GABA_B$ receptors for GABA can be higher than that of the postsynaptic ones (Isaacson & al., 1993) suggesting different subunit composition for the pre– and postsynaptic receptors, which may also underlie the observation that presynaptic $GABA_B$ receptor mediated inhibition acts with a somewhat slower time course than the postsynaptic counterpart. In addition, at least some evidence suggests that the postsynaptic $GABA_B$ receptor functionality matures later than presynaptic (Nurse & Lacaille, 1999; Lei & McBain, 2003).

According to Lei & McBain (2003), composition of Ca^{2+} currents contributing to presynaptic

inhibition of GABAergic and glutamatergic terminals differ; more P/Q-type current is involved in inhibition of GABA release while both N - and P/Q-type currents are modulated glutamate transmission inhibition. Also, sensitivity of presynaptic $GABA_B$ receptors on inhibitory terminals for GABA is higher than on excitatory ones, thus rendering autoinhibition of synaptic GABA release stronger than heterosynaptic inhibition of glutamate release. This may also result from the obvious reason that GABA can reach excitatory synapses only by diffusing from inhibitory synapses, and the diffusion is restricted by transmitter uptake mechanisms. In accordance with this, GABA uptake blockers enhance the $GABA_B$ receptor mediated responses; it has also been proposed (Isaacson & al., 1993) that GABA uptake is less efficient in the dendritic region thus allowing wider inhibition of transmitter release. Interestingly, the $GABA_B$ receptors seem to reside mainly in dendritic rather than somatic regions in the rat somatosensory cortex (Tamás & al., 2003).

This presynaptic inhibition is usually strongest when the presynaptic terminal is stimulated at around 5 Hz. It has been shown that $GABA_B$-receptor mediated frequency-dependent depression of $GABA_A$ IPSCs is necessary for induction of LTP in pyramidal dendrites (Davies & al., 1991). In addition, $GABA_B$ receptor activation is involved in numerous other neuronal processes, including modulation of rhythmic activity in the hippocampus (Scanziani, 2000). The interneurons producing $GABA_B$ responses in the CA3 are electrically coupled (Yang & Michelson, 2001), and thus may be capable of long-range synchronous $GABA_BR$ mediated transmission. Presynaptic $GABA_B$ receptor activation reduces the likelihood of transmitter depletion during high-frequency activity, resulting in an enhancement of synaptic strength by preventing frequency-dependent synaptic depression (Brenowitz & al., 1998; Lei & McBain, 2003).

Even though the result of presynaptic $GABA_B$ receptor activation seems to be predominantly a disinhibition of the postsynaptic cell, there are numerous examples of anticonvulsant action of $GABA_B$ receptor activation (Higashima & al., 2000; Morrisett & al., 1993; Scanziani & al., 1994). Several studies of epilepsy models (e.g. Mangan & Lothman, 1996; Billinton & al., 2001) have also reported that the function or expression of $GABA_B$ receptors is decreased. The report by Cossart and co-workers (2001) of a selective impairment of $GABA_A$-mediated inhibition in the dendritic region of hippocampal CA1 in experimental temporal lobe

epilepsy (TLE) and increased somatic inhibition is intriguing. Together with the possible segregation of $GABA_B$ receptors into the dendritic areas it suggests a specific role for $GABA_B$ receptor mediated inhibition in epileptogenesis and thus a possible target for therapeutic manipulations. On the other hand, the absence epilepsy-type seizures are often alleviated with blockers of $GABA_B$ receptors (e.g. Liu & al., 1992).

2.7. Network mechanisms of emergent activity patterns

2.7.1. Synchrony

Synchronization of neuronal activity is fundamental in the operation on neural networks. EPSPs synchronized within a few milliseconds are more effective in triggering postsynaptic spikes than EPSPs dispersed over longer intervals (Stevens & Zador, 1998) due to kinetics of EPSPs and the back-propagating dendritic spikes as well as sensitivity of firing threshold to the rising slope of depolarization (Singer, 1999). Large-scale synchrony, with thousands of neurons firing together, can be detected in the EEG-signal. All classifications of brain states, from sleep and attentive levels to epileptic seizures are based on the activity patterns of enormous groups of neurons taking part in synchronous discharges. Synchronicity serves as a tag of relatedness (Singer, 1999) since it increases the saliency of synchronized responses, which in turn favours their joint evaluation (binding) at following processing stages. This applies to external (sensory) stimuli as well as to internally generated synchrony, grouping internal processes into functional (task-oriented) units.

Fast, synchronous firing of putative interneurons can be recorded *in vivo*, and multielectrode recordings have shown that synchronization occurs even between distant neurons, several millimetres apart. Synchronous activity in a neuronal network can result from common (sensory) input to the network or of an emergent population oscillation depending on the properties of neurons in the network. This internally generated synchronization can be as precise as externally generated, and network mechanisms for emergent synchronous activity will be discussed in the following sections.

2.7.2. Resonance

The frequency of synchronous activity may, of course, be modulated by external inputs, but more important for the emergent frequencies are the resonant properties of neurons participating in oscillation. Resonance (Hutcheon

& Yarom, 2000) characterizes the frequency at which neurons respond best to inputs of injected currents. In order for neuron or population of neurons to have resonance, two distinct frequency–dependent properties are needed: one that attenuates incoming signals below a threshold value (high-pass) and another that reduces responses to high-frequency input (low-pass). These result in a notch filter, and if in addition combined with a signal amplifier with preferred frequency range an efficient resonator unit emerges. The low-pass properties of neurons result from the well-documented passive membrane properties that attenuate responses to fast inputs, and slowly (in respect to the membrane time constant) activating voltage-gated currents that oppose changes in membrane voltage (e.g. 'delayed rectifier'-type potassium channel) act as high-pass filters. The resonance emerging is in a frequency range set by the time constants of these two processes and amplified by quickly activating voltage-gated channels enhancing membrane potential changes (or, for instance, the NMDA receptor–mediated current). The dominating resonant properties may depend on membrane potential. As an example, neocortical pyramidal cells are resonant at 1-2 Hz near the resting membrane potential but when depolarised more than to –55 the main resonant range is between 5-20 Hz (Hutcheon & al., 1996; Gutfreund & al., 1995). The resonant properties of hippocampal pyramidal and interneuronal cells differ as well within the interneuronal subpopulations (Pike & al., 2000). Pyramidal neurons fire preferentially in response to high delta – theta frequency (2-7 Hz) input, while a subgroup of interneurons, the fast-spiking interneurons, responds best to inputs in gamma range.

2.7.3. Oscillations

Synchronized cortical discharges are often associated with an oscillatory patterning with the frequency of these oscillations covering a broad range. This periodic patterning reduces the probability of spurious correlations and enhances substantially the precision with which the discharges of different neurons are synchronized. Synchronization can be established very rapidly, even in time less than an oscillation cycle, as neurons are able to delay their output relative to the incoming EPSPs and the ongoing oscillatory network activity. The population of neurons taking part in oscillation may vary from one cycle to another (cycle skipping) even if the power of an oscillation stays constant. For instance, in some oscillatory modes an individual pyramidal cell may fire only in 5% of cycles.

Oscillatory activity can narrow the time window for LTP *in vitro*: the polarity of synaptic gain change depends on the phase relations between pre – and postsynaptic discharges (see Aizenman & al., 1998). It is possible, that the interneuronal IPSP arriving synchronously with pyramidal cell action potential enhances spike backpropagation by accelerating the recovery of activity-dependent depression, and thus promotes plastic changes.

2.7.3.1. Interneuron network oscillations

It has been shown that when ionotropic glutamate receptor mediated transmission is blocked pharmacologically, oscillatory trains of IPSPs are generated in pyramidal neurons and interneurons (Whittington & al., 1995; Jefferys & al., 1996) if sufficient excitatory drive to the interneurons is present, for example in the form of metabotropic glutamate receptor activation. These spontaneous synchronous IPSPs tend to occur perisomatically (Soltesz & al., 1995) and thus are most likely originating from basket cells intercoupled with gap junctions (see below). The frequency of the emerging oscillation is modulated by properties of the GABAergic synapses and generally falls into the γ (gamma) range (30–80 Hz). This network phenomenon, generated by mutually inhibitory interneurons, is called Interneuron Network Gamma, or ING (Traub & al., 1998). Heavy interconnections have been shown experimentally in the sensory neocortex (Tamas & al., 1998) and hippocampus (Cobb & al, 1997), especially between cholecystokinin (CCK) – and parvalbumin (PV) –positive basket cells (Sik & al., 1995).

The frequency of a single interneuron firing depends on the properties of the cell, especially the afterhyperpolarisation (AHP) delaying the next spike, but the frequency of interneuron network oscillation depends on the properties of $GABA_A$ responses, which suppress the next population spike until the inhibition has faded out. Thus, prolongation of the decay time constant (τ) of $GABA_A$ responses (eg. with barbiturates) slows down the oscillation frequency.

2.7.3.2. Population gamma oscillations

γ-frequency oscillations can be induced by tetanic stimulation (usually 8 – 40 pulses at 100 Hz) of the CA1 region of hippocampal slices. The oscillations are superimposed on post-tetanic depolarisation and slow from the initial γ-range to beta band (around 10-30 Hz). During this activity, pyramidal cells as well as

interneurons depolarize tonically and fire regularly at gamma frequencies. As a distinction from ING, this mode of oscillation is called Population Gamma oscillations (PGO). PGO can be induced under blockade of ionotropic glutamatergic transmission and the tonic depolarisation is then the result of massive GABA release (see discussion for the mechanism). It has been suggested (Bracci & al., 1999) that this depolarisation induces rhythmic activity in the pyramidal cells at a frequency dependent on the intrinsic resonant properties of cells, and that neurons would synchronise between themselves via ephaptic field effects. The oscillations are abolished if the GABAergic output of interneurons is blocked which also suggests a role for the synaptic activity of interneurons in network synchronisation.

Although axon conduction velocity is relatively slow in the cortical structures (in the range of 0.5 meters sec^{-1}), the population gamma oscillations can synchronise within 1 ms over distances of several millimetres. In order to solve the mechanism of such a precise synchronization, Traub and co-workers (1996) devised a model which predicted long-range synchrony in a network of neurons if the synchronized output of excitatory cells would be strong enough to induce a second spike in the inhibitory cells. It was then confirmed *in vitro*, that during long-range synchronized oscillations, most interneurons indeed fired in doublets (Traub & al., 1999). The EPSPs also stabilize the oscillation, so that modification of IPSP parameters or heterogenity in driving forces has less of an effect on oscillation frequency in the presence of fast excitatory transmission than in pure ING.

2.7.3.3. Carbachol-induced gamma oscillation

Bath – applied cholinergic agonist carbachol induces population oscillations with a prominent γ frequency component (Fisahn & al., 1998) with phase-locked EPSPs and IPSPs seen with whole-cell patch-clamp recordings. This 'carbachol-gamma' differs from tetanically induced PGO in that pyramidal cells skip many cycles of the oscillation, firing often in no more than 5% of the cycles (over 80 % of cycles in PGO). Under these conditions, the degree of tonic excitation of the interneurons is not sufficient to maintain ING and phasic excitation originating in the recurrent feedback loops assumes a greater role in maintaining the oscillation. On the other hand, the oscillation frequency is set by the kinetics of IPSPs and oscillation is abolished if $GABA_AR$ mediated

synaptic events are blocked pharmacologically. This model of gamma oscillations relies, therefore, on the interplay of the interneuronal and pyramidal cell networks and is probably closest to the normal *in vivo* situation.

2.7.3.4. System-level oscillations

γ oscillations may play an important role in the function of hippocampus, which receives a major cholinergic input from other brain regions. As cholinergic activation can induce gamma–range oscillations and it has been shown (Whittington & al., 1997) that gamma oscillations enhance recurrent excitatory connections between CA1 pyramidal neurons, the functions of hippocampus in memory formation may well depend on gamma-frequency activity.

Oscillatory activity is not restricted to these rather small networks of *in vitro* preparations, but is seen as EEG oscillations in awake, behaving animals. One of the great mysteries of last decades, the 'binding problem' related to distributed processing of sensory information (for review, see Singer 1999) is thought to be solved by relating in-phase signals with each other. It has also been realized that different behavioral and perceptual states are associated with different brain rhythms.

Taking into account the importance of bicarbonate availability on GABAergic synaptic transmission (see chapter 1.6.) and the putative roles of the interneuronal network in synchronizing and pacing the described models, it should be of no surprise that bicarbonate is also necessary for the gamma oscillations. As described earlier (chapter 1.2.1.1.), bicarbonate levels are regulated together with pH and can be modified with respiration thus allowing for system-level regulation of gamma oscillations.

Apart from the γ-band oscillations, high-frequency (100 - 200 Hz) rhythmic oscillations have been recorded from hippocampi of freely moving rats, mainly during sleep or rest. It has been hypothesized that these 'ripple' oscillations are involved in memory consolidation as they result in widespread activation of hippocampal targets. Ripples arise from the high-frequency phase-locked firing of inhibitory interneurons; they are so fast that their coordination across many cells in the hippocampus would be difficult to achieve with chemical synapses. Indeed, they are reported to persist in the absence of chemical synaptic transmission (Draguhn & al., 1998). Even though the interneurons fire at ripple frequencies, most CA1 cells fire only one spike in a ripple; so, even this model of synchronous

oscillation seems to depend on interneuronal activity. As was discussed in an earlier chapter, interneurons communicate with each other by means of direct electrical synapses, further – in addition to frequent mutual synaptic connections – increasing their possibilities for coordinated and synchronous activity, especially in the high-frequency range.

2.8. Epilepsy

"Epilepsy" is a term for remarkably diverse collection of disorders, affecting around 0.8% of the population worldwide. Sadly, a significant part of the cases are young people and children, and as severe seizures may result in developmental retardation, aggressive therapeutical approaches are taken as far as to surgical

Epilepsy	Partial - focal initiation Behavioral manifestations determined by the cortical region affected by seizure	Simple	Consciousness preserved
		Complex	Consciousness impaired
	Generalized - widespread cortical activation	Absence	Cessation of ongoing activity for <30 s
		Myoclonic	Muscle contractions
		Tonic-clonic	Sustained muscle contractions alternating with relaxation periods
Seizure: unusual sensations, behaviours or movements resulting from abnormal electrical discharges in the brain Convulsion: the violent uncontrollable contractions of muscles, often caused by a seizure			
Seizures (*in vivo*)	Epileptic	Intrinsically triggered in the brain	
	non-epileptic	Evoked by external triggers, e.g. electric shocks or convulsant drugs (in animal models)	
(Abnormal) synchronous activity	Ictal (discharges)	Associated with a seizure *in vivo*; sustained (up to minutes) depolarisation *in vitro*, with tonic or tonic-clonic bursts	
	Inter-ictal (spikes)	Between seizures *in vivo*; Fast, short (tens of ms) depolarisations	

Table 1. Classification and terminology related to epilepsy. McNamara 1994 and McCormick & Contreras 2001

removing of the brain tissue triggering seizures. Still, the rates of recovery are depressingly low, as the therapy is symptomatic (based on symptoms rather than known causes) and is sometimes chosen by a 'trial-and-error' method. The lack of efficient treatments stems form the diversity of 'epilepsies', which could in fact be treated as different conditions of aberrant activity in the central nervous system. Over 40 recognised types of clinical epileptic syndromes are known; however, Table 1 summarizes the basic terminology and classification used in the epilepsy literature.

2.8.1. Phenomenology

Clinical epilepsy, in general, is a pathological state characterized by the periodic and rather unpredictable occurrence of seizures, which are defined as transient behavioral or sensory changes attributable to the synchronous and rhythmic firing of populations of neurons in the CNS. During an epileptic seizure, thousands of cellular elements are spontaneously and synchronously active; a seizure can be precipitated by external stimuli in susceptible individuals or initiate without any apparent external trigger.

Two patterns of neuronal discharge are typically generated by epileptic cortex. Interictal bursts are brief (up to 100 ms) synchronous neural discharges that originate and remain in a relatively defined locus. Although interictal bursts and the associated spikes in the EEG occur frequently and regularly, they are usually not associated with significant disturbances of behavior. In contrast, ictal paroxysms tend to spread over wide cortical areas, last up to several minutes and result in severe clinical seizures. While the interictal bursts in the hippocampus depend on non-NMDA –type glutamate receptors (de Curtis & al., 1999) and the recurrent excitatory connections among pyramidal cells of CA3, ictal events probably require the interplay of both glutamatergic and GABAergic transmission and are generated in the CA1 area only. It has been shown that activity of a single CA3 neuron can be sufficient to initiate an interictal event (Miles & Wong, 1983), which can then propagate to the CA1 (Avoli & al., 2002).

The presence of interictal spikes in patients' EEG is taken as a symptom of clinical epilepsy and they may aid in localising the epileptogenic focus, but the actual relationship between interictal spikes and ictal seizures is not clear. In some cases, interictal and ictal discharges may be closely related and an interical event can actually initiate an ictal episode (interictal-to-ictal transition, Ayala & al., 1973). However, the interictal spikes do not always correlate at all with ictal activity. It has been proposed (de

Curtis & Avanzini, 2001; Jensen & Yaari 1988) that the interictal spikes in fact protect against the occurrence of ictal discharges by strong after-inhibition, and in some models simulation of interictal activity by electrical stimulation prevents the generation of ictal discharges (Avoli & al, 2002).

2.8.2. Role of GABAergic inhibition

The most common histological abnormality in TLE is Ammon's horn sclerosis, where a significant amount of principal cells of the hippocampus is lost. A leading hypothesis in the 1980s was that the GABAergic interneurons in the hippocampus die and the resulting loss of inhibition leads to hyperexcitable neural network and epileptic seizures. Also, pharmacological compounds that increase GABAergic inhibition are used to attenuate epileptic convulsions (Prince, 1978). Intriguingly BDNF, a brain-derived neurotrophic factor known to be up-regulated in *in vivo* kindling model (Binder & al., 2001) weakens maintenance of the intracellular chloride homeostasis by KCC2 (Rivera & al., 2002) and could thereby further suppress $GABA_AR$ mediated inhibition. Still, it is now known that not only are the GABAergic interneurons more resistant to seizure-induced death (Sloviter, 1987) but also somatic inhibition is increased in experimental TLE (Cossart & al., 2001) even though tonic inhibition in the pyramidal cell dendrites is compromised. Clearly, simple potentiation of GABAergic inhibition can not alone antagonize all abnormalities in TLE, but a more precicely targeted approach must be invented to find an effective cure.

Many of the *in vitro* and animal models of seizures developed during the last decades rely on either increasing neuronal excitability (for example, by elevating the extracellular potassium (Traynelis & Dingledine, 1988) or applying the K^+ channel blocker 4-AP (Avoli & al., 2002)), or on suppressing the fast GABAergic inhibition by pharmacological blockade (for instance by bicuculline or picrotoxin). However, epileptic-like activity can develop in the brain with intact GABAergic communication, as it is now becoming increasingly clear with several seizure models.

In the rat kindling model seizures are induced by initially subthreshold electrical stimuli delivered daily to limbic areas over several days or weeks. This model is characterized by an enhanced functional inhibition by GABA in the dentate gyrus, probably partly due to significant reduction of $GABA_BR$-mediated autoinhibition of GABA (Buhl & al., 1996) and to an increase in the number of $GABA_A$ receptors

on the somatic and axon initial segments of principal cells (Nusser & al., 1998). The ictal-like afterdischarges induced in hippocampal slices by repetitive tetanic stimulation (Higashima & al., 2000), are abolished and potentiated by $GABA_A$ receptor antagonists and enhancers, respectively. On the other hand, $GABA_B$ receptor activation seems to play a suppressive role in the generation of these discharges, perhaps by inhibiting excess GABA release. Therefore, it seems that increased GABAergic transmission, rather than decreased, is at least in part responsible for the hyperexcitability in these models.

In the rat model of the complex febrile seizures, prolonged hyperthermia-induced seizures in young animals lead to a persistent increase in perisomatic inhibition of CA1 pyramidal cells together with a decreased threshold for seizures. This is a result of a depolarising shift in the activation of a hyperpolarisation-activated cation current I_h (see Hille, 2001), which then depolarises neurons after the enhanced bursts of IPSPs (Chen & al., 2001).

Further contrasting the 'Yin-Yang' dogma of epileptogenesis, mutant mice lacking functional $GABA_B$ receptors constructed in two separate laboratories (Schuler & al., 2001; Prosser & al., 2001) show clear and severe epileptic symptoms even though lack of $GABA_B$ receptor mediated autoinhibition of GABA release might be expected to result in overly depressed excitation.

2.8.3. Gap junctions in epilepsy

Hippocampal neurons can generate highly synchronous spontaneous discharges in the absence of chemical synaptic transmission, suggesting that other interactions can have a significant role in epileptogenesis. As gap junctions are essential for fast network synchronization (see chapter 2.4.4.), it is not surprising that many experimentally induced states of epileptic-like activity depend on gap junctions: the 0-Ca^{2+} -induced spontaneous field bursts (Perez Velazquez & al., 1994), 0-Mg^{2+} -induced secondary bursts (Köhling & al., 2001) and the 4AP-induced synchronous GABAergic potentials (Yang & Michelson, 2001; Szente & al., 2002) are inhibited by GJ blockers. Jahromi and co-workers (2002) showed, on the other hand, that the maintenance of epileptiform discharges after a tetanic stimulus requires gap-junctional coupling. As discussed earlier (see 2.2.1.1, pH modulation), alkalinization increases neuronal excitability, and together with the increased GJ-coupled neuronal synchronization can further promote precipitation of epileptic seizures (see for example Köhling &

al., 2001). Furthermore, intense activity results in intracellular acidification which can decouple gap junctionally connected cells. Indeed, it has been shown (by Xiong & al., 2000) that seizures induced by 0-Ca^{2+} are directly terminated by fall of pH_i.

Despite of the present, rather convincing evidence for gap junctional coupling contributing to pathological hypersynchrony in the brain – for instance, levels of connexin mRNA are elevated in surgically removed brain tissue from epileptic patients (Naus & al., 1991) and an up-regulation of Cx32 is seen in epileptic mouse hippocampus (Li & al., 2001) – no clinical anticonvulsants are in use that would target GJ communication, as no connexin-specific GJ modulators have been found – with the one and only exception to date being quinine (Srinivas & al., 2001). Before rushing into devising complex anti-convulsant strategies based on decreasing gap junctional hypersynchrony, it must be kept in mind that hypersynchrony is only one side of epileptic-like activity: the classical hyperexcitability still produces the drive for seizures. The question of GJ communication in epilepsy is complicated by the finding of Margineanu and Klitgaard (2001) that some gap junction blockers inhibit not only hypersynchrony but also hyperexcitability, while others selectively depress hypersynchrony. Also, even though the immature brain exhibits more extensive GJ coupling than the mature brain (Peinado & al., 1993) and is much more susceptible for epileptiform activity (e.g. Sperber & al., 1992), different mechanisms account for epileptogenesis in neonatal tissue (for instance, based on the higher intracellular chloride concentration) that need not be related to GJ coupling efficacy.

3. Aims of the study

The first goal of the present study was to seek out how to dissect the hyperpolarising and depolarising parts of the biphasic GABA responses in order to be able further examine their mechanisms in isolation. Next, based on the findings and using the pharmacological dissection, the roles played by GABAergic transmission in oscillatory activity (using the carbachol oscillation model) and spontaneous ictal-like activity were to be examined. Specifically, it was to be found out whether $GABA_A$ receptor-mediated transmission alone is capable of initiating and maintaining spontaneous ictal-like activity in the absence of external stimulation or pharmacologically enhanced excitability.

Synchronisation of interneuronal activity is known to partly depend on the coupling via gap junctions. We sought to elucidate the role of GJs in both stimulus-evoked $GABA_A$-mediated depolarising responses as well as in spontaneous activity. Using the new tool for dissecting the depolarising and hyperpolarising phases in the first part of the study, namely, quinine, which was meanwhile shown to specifically block interneuronal gap junctions formed of connexin (Cx) 36, we compared the influence of interneuronal gap junctions in analogous GABA – and glutamatergic situations of both evoked and spontaneous activity.

4. Materials and methods

The questions above have been addressed using transverse (I, III, IV) or horizontal (II) hippocampal slices from adult (postnatal day (P) 24 – 60) Wistar rats of either sex. Electrophysiological methods used comprised of field potential, membrane potential and ion-selective measurements and they all were performed in an interface-type recording chamber, while the optical imaging of intracellular pH (in II) was done in a submerged-type chamber. The perfusing solution was continuously gassed with 5% CO_2 / 95% O_2 (naturally, in the experiments where hypocapnia was induced the pressure of CO_2 was decreased) and pH set to 7.4. Details for the construction of different type electrodes and dye loading as well as the apparatus used are given in the original articles. Table 2 lists drugs and the concentrations they were used at.

AP5	Competitive NMDA-R antagonist	40 – 60 μM	Tocris Cookson
4-aminopyridine (4-AP)	K+-channel blocker	50 μM	Sigma
Baclofen	$GABA_BR$ agonist	10 – 50 μM	Sigma
Benzolamide (BA)	CA_o inhibitor	10 μM	E. Swenson, Univ. Washington Medical Center, Seattle, WA, USA
Bicuculline	$GABA_AR$ antagonist	10 μM	Tocris
Carbachol	Cholinergic agonist	5-10 μM	RBI
CGP35348	$GABA_BR$ antagonist	100-200 μM	Ciba-Geigy, Switzerland
CGP55845A	$GABA_BR$ antagonist	0.5 – 5 μM	Ciba-Geigy, Switzerland
DAMGO	μ-opioid agonist	1 μM	Tocris
Ethoxyzolamide (EZA)	CA_i inhibitor	50 – 100 μM	Sigma
Ketamine	Non-competitive NMDA-R antagonist	50 μM	Sigma
Midazolam (MDZ)	Benzodiazepine	20 μM	Sigma
Muscimol	$GABA_AR$ agonist	ionto phoretic	Tocris
NBQX	AMPA / kainate –R antagonist	10 μM	Tocris / NOVO Nordisk
Octanol	Gap junction blocker	0.5 mM	Sigma
Picrotoxin (PiTX)	$GABA_AR$ antagonist	100 μM	Sigma
Pentobarbital (PB)	Upmodulator of GABAAR-gated channel	10 – 100 μM	Sigma
Quinidine	Connexin 36 –specific gap junction blocker	100 μM	Sigma
Quinine	- " -	200 – 500 μM	Sigma
QX-314	Reduces cell sensitivity to $[K^+]_o$; I_q blocker	50 μM	Astra (Södertälje, Sweden) / Sigma
Tetraethylammonium (TEA+)	K^+ channel antagonist	10 mM	Sigma
UL-FS 49	I_q blocker	50 μM	H.-C. Pape, Univ. Madgeburg
WIN 55,212-2	CB_1R antagonist	1 μM	Sigma

Table 2. Drugs, used concentrations and manufacturers.

5. Results

5.1. Dissociation of synaptic and nonsynaptic components of the GABAergic depolarising non-synaptic potential (Original publication I)

The initial goal of the study was to find a way to dissect the two components of a HFS-evoked GABAergic response of a CA1 pyramidal neuron so that further analysis of the components would be feasible. The direct $GABA_A$ receptor-mediated component was selectively inhibited by intracellular perfusion with fluoride as the major anion and no ATP, while internal perfusion with QX-314 abolished the late GABAergic depolarising post-synaptic potential (GDPSP; Figures 1 and 2 in the original publication). Also, bath application of the anti-malarial drug quinine was found to block the GDPSP. With these tools we confirmed the previous conclusions (Kaila & al., 1997) that the GDPSP depends on non-synaptic communication, namely, activity-induced increase in extracellular potassium and that the bicarbonate-dependent accumulation of intracellular chloride (anionic redistribution, Kaila & al., 1989; see also Staley & al., 1995) is of lesser importance. Therefore, the late phase of this GABAergic response was re-named as GABAergic depolarising non-synaptic potential (GDNSP). In the key experiments it was shown that the $E_{GABA\text{-}A}$ is negative in respect to the membrane potential at the time of GDNSP peak (Figure 4d in original publication) if the GDNSP response of the cell was blocked. Also, the initial increase in membrane conductance during the fast hyperpolarising component faded away during the GDNSP, thus showing that the initial and late components of the response are mechanistically different.

The finding of a 'specific GDNSP-blocker', quinine, let us perform further experiments elucidating the actual mechanisms behind the phenomenon. It inhibited both the GDNSP and the activity-induced potassium rise that was already in the earlier studies proposed as one key element in the GDNSP generation, thus strengthening the causal link between the two events. In the concentration and duration range of quinine applications used, its effects were not due to a decrease in the cell's sensitivity to external potassium (i.e. by blockade of potassium channels), as pressure injections of potassium evoked similar depolarising responses in the absence and presence of quinine. Also, as the reversal potential of $GABA_A$ responses were not shifting in quinine the activity of the KCC2

was unaffected by this drug. Since the GDNSP is a bicarbonate-dependent response, it was asked whether quinine exerts its inhibitory action by depressing the depolarising bicarbonate efflux. The alkaline transient during a GDNSP was not affected, thus showing that the inward current carried by HCO_3^- is still present under quinine-induced blockade of the GDNSP but it can not – alone, at least – be responsible for the significant depolarisation seen in the absence of quinine.

At the time of publication, the gap junction –blocking effect of quinine was not known and thus the actual mechanism behind quinine's inhibitory action on the GDNSP was not revealed.

5.2. Synaptic and nonsynaptic modulation of oscillatory activity (Original publication II)

It was shown that changes in PCO2 can have a significant role in modulating hippocampal function. Temporal stability of the carbachol-induced gamma-oscillations was significantly increased (Figure 1 in original publication) during hypocapnia induced by switching the gassing of physiological solution from 5% to 1% CO_2, as was evident from the prolongation of the autocorrelation function (ACF) decay time. At the same time, a modest increase in frequency and decrease in amplitude of the oscillations was observed.

To elucidate the mechanisms by which hypocapnia influences the properties of gamma oscillations, the effects of hypocapnia on physical and physiological parameters of the hippocampus were assessed. First of all, hypocapnia resulted in a monophasic alkalinisation of both intra- and extracellular space. The pH_i was found to increase slower and to lesser extent than the pH_o. Second, an increase in the single-pulse –evoked IPSCs in the presence of blockers of ionotropic glutamate and $GABA_B$ receptors was observed (Figure 4 in original publication). There was no change on input resistance, and thus hypocapnia increased the $GABA_A$ receptor-mediated hyperpolarisation seen by the postsynaptic cell. Importantly, the decay kinetics of IPSC were unaltered during hypocapnia, producing a sharper postsynaptic response. Next, we sought to find out whether the hypocapnia-induced increase in IPSC amplitude is required for the strengthening in oscillation stability. Indeed, when the increase in $GABA_A$ receptor mediated IPSC under hypocapnic conditions was reversed with a low concentration of bicuculline, the decay of the ACF returned close to the control conditions.

The dependence of oscillatory properties on the properties of $GABA_A$ receptor-mediated responses were examined with the two up-modulators of $GABA_A$ receptors, midazolam (MDZ) and pentobarbital (PB), both of which increase amplitude and duration of IPSCs (Segal & Barker, 1984; Poncer & al., 1996). Consistent with the significance of $GABA_A$ receptor kinetics on setting gamma-oscillation frequency (Jefferys & al., 1996; Traub & al., 1998), prolongation of the IPSC decay lead to decrease of the oscillation frequency, and, perhaps due to frequency decrease, to an increase of gamma band amplitude. Pentobarbital also significantly decreased the temporal stability of oscillations.

Further, it was found that pH changes in the intracellular and extracellular space had differential effects on the oscillations. When pH_i was increased selectively by applying TriMA, gamma oscillation amplitude and frequency were affected as when PCO2 was reduced, although somewhat more significantly (Figure 3 in original publication). Strikingly though, the stability of oscillation was affected in an opposite direction and the intrinsic phase-correlation was decreased. Thus, intracellular alkalinisation could not explain the hypocapnia-induced effects on temporal stability.

5.3. Mechanisms of $GABA_A$ receptor-mediated spontaneous epileptic-like activity (Original publication III)

Another novel model of spontaneous activity examined during this work consisted of synchronous GABAergic Ictal-like Events (GIEs) and provided an opportunity to elucidate the mechanisms for interneuron network synchronisation and plasticity.

As the ability of GABAergic neural transmission to produce depolarising responses leading to synchronous spiking of the pyramidal cells is known, we proceed to establish whether this excitatory action can result in epileptic-like activity. Avoli and co-workers severalfold demonstrated (for review, see Avoli & al., 2002) that if the excitability of neurons is pharmacologically (4-AP) enhanced, GABAergic synaptic activity is sufficient for developing synchronous spontaneous activity in the mature hippocampus. However, we found that this drug-induced excitability increase is not necessary as the intrinsic tendency of intereurons for spontaneous activity is often enough for a GABAergic epileptic-like condition to develop, provided the down-regulation of transmitter release via presynaptic $GABA_B$ receptors is blocked

pharmacologically (Figure 1 in original publication).

The spontaneous GABAergic ictal-like events (GIEs) resemble in many ways the GDNSP (see Kaila & al., 1997 and original publication I), including their dependence on the availability of bicarbonate, and other models of ictal activity. For instance, the GIEs are sensitive to interictal events (Figure 6 in original publication; compare for example with Jensen & Yaari, 1988) and depend on gap junctional coupling for synchronisation. The spontaneous GIEs were seen synchronously over large areas of the hippocampal CA1 region and appeared to spread by the interplay of synaptic and nonsynaptic mechanisms.

During the period of GIE induction, plastic changes of the hippocampal circuitry are possibly taking place as removal of the inducing conditions ($GABA_B$ receptor antagonists, elevated pH) did not reverse the GIE development. Based on the inhibitory action of μ-opioid and cannabinoid receptor antagonists, these long-term modifications were most likely localised on the presynaptic terminals where a persistent increase of GABA release could act as the pivotal point for the positive feedback loop. Also, after a GIE had passed, the spontaneous activity was suppressed for several tens of seconds, probably via a depolarisation– induced suppression of inhibition (DSI; Pitler & Alger, 1994; Davies & al., 2002) that further points towards presynaptic processes in the generation of spontaneous GIEs.

The enhancement of GIE activity by a transient alkalosis led us to examine the role of GJ coupling. Combined with the previous finding that quinine blocks the stimulus-induced GDNSP (original publication I), the inhibitory activity of quinine and octanol on GIE activity confirmed that the spontaneous GABAergic activity is dependent on gap junction coupling and the depolarising effects are mediated by nonsynaptic ('GDNSP') means. Furthermore, it was found that quinine (or octanol) had no effect on epileptic-like activity induced by blockade of synaptic inhibition (by PiTX), thus demonstrating that the GABA- and glutamatergic epileptic-like conditions depend on different cellular mechanisms for synchronisation and excitation.

5.4. Dependence of GDNSPs on inter-interneuronal gap junction communication (Original publication IV)

In addition to the spontaneous GIEs examined in Original publication I, quinine was found to

block HFS-induced GABA-mediated field responses ('field GDNSPs') as well as the associated $[K^+]_o$ transient transient, thus further providing evidence for the mechanistic similarity of evoked GDNSPs and spontaneous GIEs. The action of quinine on the network response of interneurons was not a result of interference with the $GABA_A$ receptor agonist binding to its receptor, since quinine did not influence the strength of V_m (or $[K^+]_o$; unpublished data) responses induced by GABA or muscimol application in the absence of glutamatergic transmission. The fact that the post-stimulus synchronous oscillations were abolished upon quinine application shows that even though the tetanization effectively synchronises the interneurons (at 100 Hz), they can not maintain synchronisation to produce the needed long-term depolarising drive for the pyramidal cells without direct electrical communication.

In the absence of GABAergic communication (when it was blocked by PiTX), the post-tetanic field response was much shorter than what was seen in the absence of ionotropic glutamatergic transmission. No post-stimulus oscillations were seen suggesting that interneuronal pacing activity is needed for gamma oscillations to develop or, neuronal spiking was actually absent despite the significant rise in extracellular potassium. Quinine had no inhibitory effects on any stimulus-evoked glutamate-only responses, again stressing the mechanistical differences between these two systems in generating activity.

6. Discussion and conclusions

6.1. GABAergic non-synaptic depolarisation unveiled (I, IV)

Bicarbonate-dependent accumulation of intracellular chloride (anion redistribution; Voipio & al., 1991; Kaila & al., 1993; Staley & al., 1995) can not alone explain the HFS-induced long depolarising GABAergic response, as it does not result in shifting of $E_{GABA\text{-}A}$ positive enough (Kaila & al., 1997). In reality, activation of $GABA_A$ receptors in the target cell is not even necessary for GDNSP in a tightly packed neuronal tissue, since GDNSP can be evoked in an individual cell where the $GABA_A$ receptor mediated responses are blocked pharmacologically, and the surrounding neurons generate the K^+ transient by the $GABA_AR$-dependent mechanism.

The fact that the two components of GABAergic response to a HFS can be dissected shows that the phenomenon depends on two separate mechanisms which operate at different temporal (from 100's of milliseconds to seconds) and organisational (from synaptic to network) levels. The relative contributions of synaptic and network mechanisms depend on the conditions of the experiment, and the smooth transition from one dominating mechanism to another contains an intermediate phase where the synaptic and network-level effects are overlapping. During the transition from synaptic to volume transmission, the increase in postsynaptic membrane conductance fades away and has returned to resting levels at the time the GDNSP peak has been achieved. It should be noted that the experiments were performed on cells more than 200 μm away from stimulation site; the drop in input resistance in cells closer than that has been shown to last longer (Bracci & al., 1999).

It has been recently shown by Srinivas and co-workers (2001) that, in addition to quinine's known inhibitory effects on various potassium conductances it also specifically blocks gap junctions formed of the connexin 36 (Cx36). Interneuronal gap junctions are precisely of this type (Deans & al., 2001), and as show in original publication IV, GABA- but not glutamate-driven population activity is inhibited by quinine. The gap junction mediated effect was confirmed by the experiments where similar results were obtained with the broad-spectrum GJ blocker octanol. Also, the possibility that quinine would interfere with agonist-receptor interactions at the level of GABAergic synaptic transmission was rejected as the

drug did not modify the cellular response to exogenous GABA application. The fact that post-stimulus synchronous oscillations were abolished upon quinine application shows that even though the tetanisation provides an efficient synchronizing drive to the interneurons, they desynchronize quickly without direct electrical intercommunication.

Thus the mechanism of GDNSP generation proposed in the original publication could be further elaborated with the participation of inter-interneuronal gap junctions and can be schematised as in Figure 9 (see also Ruusuvuori & al., 2003).

The initial pyramidal cell response to HFS is a neuronal hyperpolarisation formed of fused IPSPs generated by the firing of GABAergic interneurons and activation of $GABA_A$ receptor-gated channels (see chapter 2.6.1). The increase in anion content of the intracellular space would result in cell swelling as water inevitably follows, if no volume-regulation would be present. One of such regulatory mechanisms, the potassium – chloride cotransporter isoform KCC2, is activated by the increase of $[Cl^-]_i$ and extrudes the excess chloride together with potassium as discussed previously with ionic modulation of synaptic transmission (section 2.2.1.2). In the

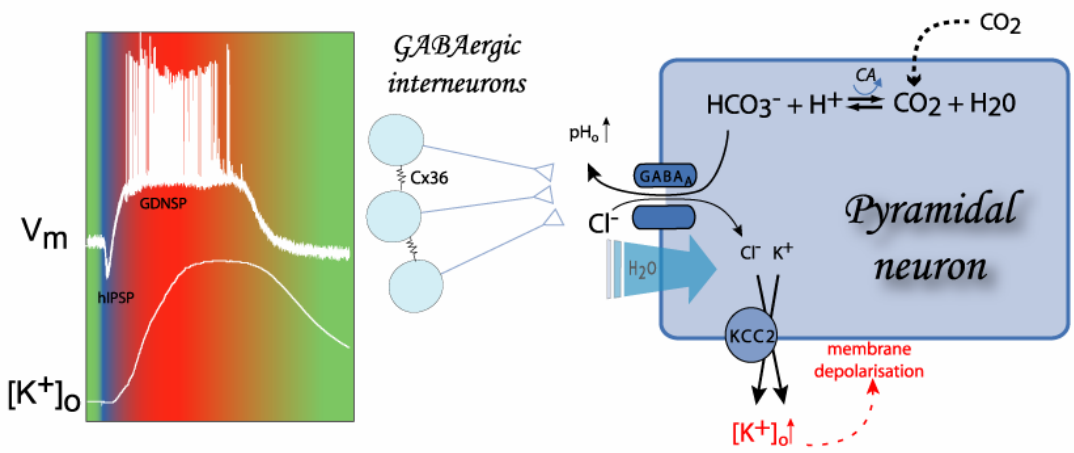

Figure 9. Schematisation of the mechanism of GDNSP in the presence of iGluR and $GABA_BR$ blockers. The GDNSP (V_m, left) begins with a hyperpolarising IPSP (hIPSP) (on blue background) during which not much potassium is extruded. Continuous $GABA_AR$ activation results in an initial increase in $[K^+]_o$ and a depolarisation of the membrane (transition between blue and red). When sufficiently strong, membrane depolarises over the action potential threshold and vigorous activity of cells further increases extracellular potassium concentration. The high $[K^+]_o$ impairs the ability of the KCC2 to extrude chloride, which leads to a positive shift in E_{Cl} and a reduction in the hyperpolarisation that the activation of $GABA_A$ receptors can induce.

presence of gap junction coupling between interneurons, GABAergic activity can be sufficiently synchronous across large areas of the hippocampal slice to result in a significant increase in the extracellular potassium, which is fast enough to influence the ongoing neuronal signalling. This volume transmission affects not only the cells postsynaptic to the population of active interneurons but all nearby neurons as well as glial cells.

We were not surprised to find that quinine had no effect on the glutamatergic depolarising field response to stimulation, since, unlike interneurons, hippocampal CA1 pyramidal cells are not as extensively interconnected via gap junctions and use different connexin subtypes. The results are in agreement with the recent study by Bikson and co-workers (2001), who reported that quinine in the concentrations used has no effect on cell input resistance, resting membrane potential or action potential threshold. On the other hand, prolonged (over 60 minutes) quinine application is known to impose non-specific effects on cell excitability and therefore we did not extend our quinine exposure times over half an hour.

Consistently with previous reports (e.g. Bracci & al., 1999), the glutamatergic post-tetanic depolarisation (seen in the field recording as a negative deflection) was much smaller and shorter than a regular GDNSP (less than a second vs. up to several seconds) even though the stimulation resulted in a prominent rise in the extracellular potassium. It is possible that this results from the activation of postsynaptic $GABA_B$ receptors and the subsequent increase in potassium conductance, which 'shunts' the depolarising effect of elevated $[K^+]_o$ and drives the membrane towards the E_K.

6.2. GABAergic mechanisms of spontaneous and rhythmic activity (II, III)

To assess the functional significance of network and synaptic properties of GABAergic communication, we studied two models of pharmacologically induced spontaneous synchronous activity, both of which depend on the availability of bicarbonate.

While the tonic carbachol-induced spontaneous field activity is initiated in the CA3 region and dependent on non-NMDA receptor activation, the GABAergic ictal events (GIEs) were mainly triggered in the CA1 area and relied only on $GABA_A$ receptor-mediated chemical transmission. This is in agreement with the known anatomical differences between the CA3 and CA1 areas: the main characteristic

of the CA3 subfield is its strong recurrent excitatory connections among pyramidal cells, while the CA1 area stands out with the divergent interconnections between pyramidal cells and interneuronal subgroups. Despite these differences, properties of spontaneous activity in both models are modulated by the activity of interneurons and manipulations that affect the degree of interneuronal synchronisation and transmission efficacy modify the emergent network behaviour.

In the carbachol model of spontaneous oscillatory activity, amplitude and frequency seem to be set by network-level properties while stability of the oscillation depends on the kinetics of synaptic transmission. Lowering PCO2 results in an alkalosis both intra – and extracellularly, but the pH changes on the opposite sides of cell membrane modulate different oscillatory properties. The increase in IPSP amplitude induced by extracellular alkalinisation provides stronger termination of postsynaptic depolarisations and thus permits sharper resetting of oscillation phase. It is possible that IPSP decay has to stay within certain limits for stable oscillation in a given frequency, and prolongation of IPSP kinetics by pentobarbital can shift it out of that range. The observed increase in oscillation frequency during hypocapnia-induced intracellular alkalosis can result from enhanced gap junctional communication by allowing faster spread of neuronal excitation through the network. It has been proposed that higher frequency oscillations are dependent on fast gap junctional coupling. Furthermore, an increase in oscillation frequency may itself decrease oscillation amplitude.

Enhanced activity of the GABAergic, gap junction coupled interneuronal network can be sufficient to trigger epileptiform activity in adult hippocampus in the absence of ionotropic glutamatergic transmission. The GABAergic 'ictogenesis' begins from a state where virtually all the pyramidal cells in a given area receive synchronising input from the interneuronal network, demonstrated by the fact that the spontaneous field IPSPs (sfIPSPs; see Figure 3a in original publication III) were always accompanied by an intracellular hyperpolarising. The development of regular GIE activity comprises of synaptic and network components that can be separately targeted in future studies. Blockade of $GABA_BR$ mediated autoinhibition of GABA release (synaptic effect) results in an increase of transmitter release, which gradually reaches levels needed for generating spontaneous GDNSPs (that depend, as discussed previously, on network activity).

The synaptic component of the ongoing spontaneous activity could be strengthened pharmacologically (e.g. with pentobarbital) and the amount of synchronously active interneurons could be increased by manipulations that enhance gap junctional coupling by alkalosis.

6.3. Implications for epilepsy research

The role of GABAergic transmission in generating epileptic-like activity has been thoroughly revised by recent work, and in addition to participating in pacing of epileptic-like activity, GABAergic transmission can initiate and maintain ictal events. The emerging picture offers several new paths for epilepsy research. For instance, specific GJ blockers should be taken as a target for future research for anticonvulsant drugs.

Together with the glutamatergic transmission's singular leading role of in epileptogenesis, hyper-excitability is also losing significance to hypersynchrony as the main pathological change in functionality of neurons leading to epileptic-like activity. Since synchronized discharge of a group of neurons can be seen as a PDS while the same amount of discharge asynchronously is lost in the noise, abnormally strong interneuronal activity – in the extreme case, forcing most of the pyramidal cells to be active at the same time - can indeed lead to epileptic-like states. It is now known that excitability and synchronicity are two distinguishable properties of neuronal network. Indeed, synchronisation can be achieved through several non-synaptic means, such as electrotonic coupling via gap junctions or field effects and ionic changes in the extracellular space, and it can be decreased without effecting intrinsic excitability of the connected neurons. Despite the recent interest in the role of GJ coupling-mediated synchronisation, GJs are not an answer to everything regarding synchronising neuronal activity as long-term stability seems to require synaptic activity. Direct electrical coupling provides only short-term spread of activation, while the maintenance of synchronisation seems to require regenerating input to the network.

Even though the data presented in this and other papers suggests that epileptic-like activity can be the result of high levels of interneuronal activity, enhanced GABAergic transmission per se is not sufficient for epileptogenesis. For instance, output of cerebellar networks is purely GABAergic, but without the countering pyramidal cell network, epileptic-like activity is seldom seen in this structure. Thus, epilepsy seems to be the outcome of aberrant interplay between the two

principal neuronal groups: the pyramidal cells providing the fast excitatory drive and interneurons timing the activity, normally allowing processing of interrelating data but in pathological situations results in hypersynchrony and aberrant network behaviour.

The recent flood of genetically modified animals in the field follows the biotech-era notion of genetics as the key to eradication of all of the intractable medical conditions. The goal of generating mice with point mutations (e.g. mice deficient in the connexin Cx36 (Deans & al., 2001 and independently, Hormuzdi & al., 2001), $GABA_{B1}$ (Prosser & al., 2001 and, also independently, Schuler & al., 2001), KCC2 (Hübner & al., 2001) and ClC-2 (Bösl & al., 2001)) is to elucidate the functional significance of the altered gene. Many of such studies have resulted in intriguing observations as in the case of the mice deficient in functional $GABA_B$ receptors; even though there are differences in the phenotype of the mice in the two laboratories, both studies report epileptic-like symptoms in the mice and most certainly inspire further work. On the other hand, with the present knowledge about the crucial role of chloride homeostasis for GABAergic signalling and thereby for neuronal maturation, the animals with ClC-2 knocked out are surprisingly normal and not the least epileptic. On the other hand, the importance ClC-2 has recently been implicated in etiology of human idiopathic generalised epilepsy (Haug & al., 2003). A lesson to be learned from these studies together with the present work is that in a system as complicated as the brain, concentration on a single molecule or process will not likely give the correct picture of the actual processes involved and even less often lead to successful clinical tools – if not by accident. As an examples, interictal activity has been proposed to act *in vitro* both as in an anti – and proconvusant manner, while in clinical practise, the disappearance of interictal activity is often taken as a sign to discontinue anticonvulsive drug treatment.

6.4. Conclusions: the paradox of GABA-driven excitation is passé

As demonstrated by Cobb and co-workers in 1995, IPSPs originating from interneurons can induce post-inhibitory 'rebound' action potentials in the postsynaptic cells after a constant interval, effectively synchronising the output of a large number of pyramidal cells. Thus, even though the action potentials are suppressed in postsynaptic cells during the IPSP and the following afterhyperpolarisation, the net outcome at the network level can be

a massively synchronous population action potential after the suppression has faded away.

Following evidence presented since late 80's that undermine the classical role of GABAergic synaptic transmission as a solely inhibitory signal in the CNS, recent works have provided mechanistic explanations for GABA-mediated depolarisation. Thus, it is time to drop the word 'paradoxical' when describing GABAergic depolarising or even excitatory activity. The paradox was related to the traditional 'Yin-Yang' view (see Uusisaari & al., 2002), where interneurons – the suppressing Yang – are countering the activity of the excitatory, information-processing Yin-like pyramidal neurons. Even though the effect of $GABA_AR$ activation in a pyramidal cell in 'textbook conditions' can be indeed reducing the efficacy of simultaneous EPSPs, the relevant outcome of interneuronal activity is always a combination of synaptic and network effects and depends on the precise activity patterns on both sides of the synapse.

7. References

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