

Perisomatic-targeting interneurons control the initiation of hippocampal population bursts

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Abstract

Replay of spike sequences can be seen during sharp wave – ripple population burst activity in the hippocampus. It is thought that this activity, which occurs during rest and sleep, is involved in memory consolidation. The cellular mechanisms underlying the initiation of these replay events are not well understood. To investigate this, a hippocampal slice model, showing spontaneous sharp wave – ripple activity, and a combination of planar multi-electrode array recordings and whole-cell patch-clamp recordings of anatomically identified hippocampal neurons were used.

Firstly, the spatial and temporal profile of sharp waves *in vitro* was analysed in detail. Sharp waves were generated by changing subpopulations of pyramidal neurons in the CA3 region and had characteristics similar to those found *in vivo*. Secondly, four major receptor types present in hippocampal CA3, namely NMDA, AMPA, GABA_A and GABA_B receptors, were investigated for their involvement in sharp wave generation. Surprisingly, not only AMPA receptor-mediated events, but also phasic GABA_A receptor-mediated inhibition, were necessary for sharp wave generation. Thirdly, single perisomatic-targeting interneurons were activated. This experiment showed that induced spiking activity of an individual perisomatic-targeting interneuron can both suppress and subsequently enhance local sharp wave generation. Spiking activity of other neuron types (i.e. pyramidal neurons, dendritic-targeting interneurons and interneuron-selective interneurons) had no significant effect on sharp wave incidence. Finally, it is suggested that this post-inhibitory enhancement of sharp wave generation can be mediated by a transient increase in the ratio of excitation to inhibition in the local network.

In conclusion, these results suggest a new role for perisomatic-targeting interneurons in controlling the local initiation of sharp waves by selectively suppressing and subsequently enhancing recruitment of a subpopulation of pyramidal neurons. These results further imply that interneurons may play an integral part in the local information processing that takes place in the hippocampal network.

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Abbreviations

ACSF	Artificial cerebrospinal fluid
AMPA	L-alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
CA	Cornu Ammonis
CB	Calbindin
Cbx	Carbenoxolone
Cx	Connexin
CCK	Cholecystokinin
CR	Calretinin
DAB	3,3'-diaminobenzidine
DG	Dentate gyrus
DTI	Dendritic-targeting interneuron
EC	Entorhinal cortex
EEG	Electroencephalogram
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
fEPSP	Field excitatory postsynaptic potential
GABA	Gamma-aminobutyric acid
HFS	High-frequency (or tetanic) stimulation
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
IR-DIC	Infrared differential interference contrast
ISI	Interneuron-selective interneuron
LIA	Large irregular amplitude activity
LTD	Long-term depression
LTP	Long-term potentiation
mGluR	Metabotropic glutamate receptor
NMDA	N-methyl-D-aspartate
NREM	Non rapid eye movement sleep
O-LM	Oriens-lacunosum moleculare
PBS	Phosphate buffered saline
PC	Pyramidal cell
PSC	Postsynaptic current
PTI	Perisomatic-targeting interneuron
PV	Parvalbumin
REM	Rapid eye movement sleep
RMS	Root mean square
SD	Standard deviation
SEM	Standard error of the mean
STDP	Spike timing-dependent plasticity
TBS	Theta burst stimulation

Main Introduction

What is the cellular correlate of a ‘memory’? This must surely be one of the big questions in modern neuroscience. A present working hypothesis is that a memory is stored within a population (or assembly) of neurons as changes in the synaptic weights between those neurons (Hebb, 1949). It has been shown that such assemblies get reactivated during rest and sleep and it has been suggested that this might help consolidate the memories they contain (Buzsaki, 1998). It is presently not well known what governs the reactivation of such assemblies. In this thesis I have therefore used an *in vitro* model to investigate the cellular mechanisms governing the initiation of such reactivation.

In this chapter I will, firstly, introduce the hippocampal formation; a structure essential for many memory processes (section 1.1). Secondly, I will discuss hippocampal network activity seen during memory related behaviours and subsequent rest and sleep (section 1.2). Thirdly, I will discuss how network patterns could subserve hippocampal mnemonic function (section 1.3). Lastly, I will discuss several *in vitro* models of physiological and pathological population burst activity which have helped us to study the cellular mechanisms of burst initiation in more detail (section 1.4).

1.1 The hippocampus

This sea horse (*Greek: hippocampus* (Aranzi, 1564)) shaped structure (at least in humans), situated deep within the brain, has been instrumental in shaping our current thinking about memory processing in the brain. The crucial role for this structure in forming new memories was first revealed after surgical removal of the hippocampus in patient H.M. (Scoville & Milner, 1957) resulted in permanent anterograde amnesia (Corkin, 2002). This inability to form new memories, following removal of the hippocampus, was later confirmed by a combination of lesion and behavioural studies in rodents (Morris *et al.*, 1982).

The discovery of both long term potentiation (Bliss & Lomo, 1970; Bliss & Gardner-Medwin, 1973) and later long term depression (Dudek & Bear, 1992) within this structure, suggested a biochemical process by which connections between neurons could be strengthened and weakened. This selective strengthening and weakening of synapses could enable a network of neurons to store information, as was suggested much earlier by Donald Hebb (Hebb, 1949).

It is now generally agreed that the hippocampus is essential for at least spatial memory in rodents (Morris, 2006) and possibly other forms of memory as well (Eichenbaum *et al.*, 1999).

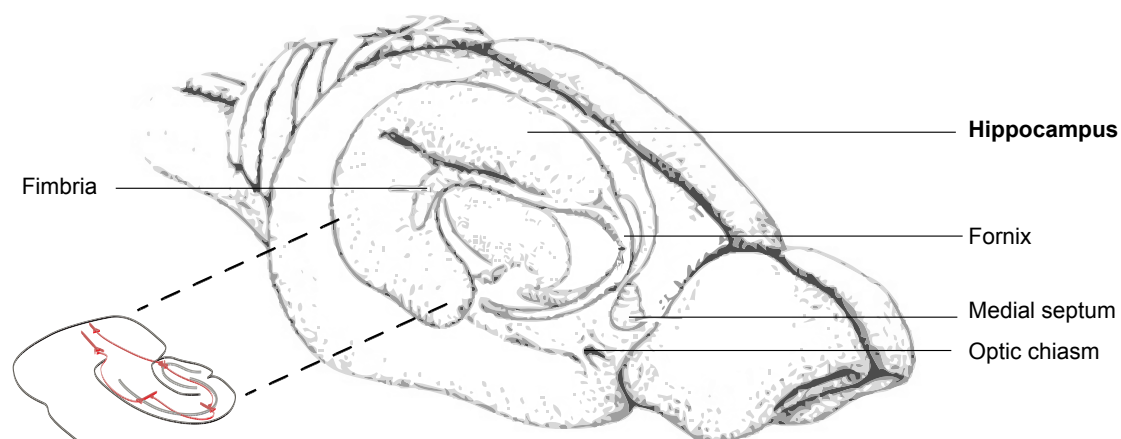


Figure 1.1 Hippocampal formation of the rat brain

Diagram of a rat brain looking at the right hemisphere (rostral is to the right) and showing the hippocampus as a sausage shaped structure situated within the brain. Notice a similar sausage shaped structure in the left hemisphere also. Indicated is an example of a brain slice taken from the hippocampus. Modified from Amaral & Witter (1995).

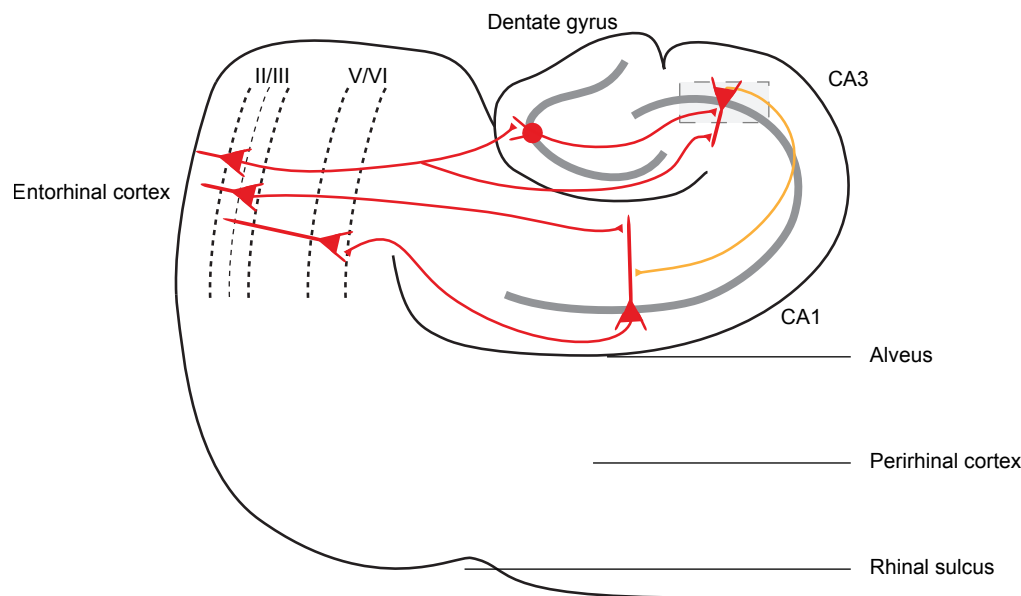


Figure 1.2 The tri-synaptic loop

Diagram of a hippocampal slice containing the tri-synaptic loop. In red are indicated the main excitatory neurons of the entorhinal cortex and hippocampus and their projections. The Schaffer collateral projection of a CA3 pyramidal neuron to a CA1 pyramidal neuron is indicated in orange.

Layer II and III of the entorhinal cortex predominantly projects towards the hippocampus, whereas layers V and VI of the entorhinal cortex predominantly receive output from the hippocampus.

1.1.1 The tri-synaptic loop

The hippocampal formation in rodents is a sausage shaped structure (Figure 1.1) consisting of the dentate gyrus, hippocampus proper (consisting of the Cornu Ammonis (CA) 3, CA2 and CA1 region) and subiculum. This structure receives its main input from layer II and III of the entorhinal cortex (Witter & Moser, 2006). Via the entorhinal cortex the hippocampus is connected to all the other cortical regions (Burwell & Amaral, 1998b, a; Witter *et al.*, 2000)

A predominant direction of excitatory transmission through the hippocampus is via the so called tri-synaptic loop (Figure 1.2). Layer II cells of the entorhinal cortex, and to a smaller degree layer V and VI cells (Steward and Scoville, 1976), make their synapses (first of the tri-synaptic loop) predominantly on the granule cells of the dentate gyrus (besides making some synapses as well on the distal ends of the apical dendrites of the CA3 pyramidal neurons). The granule cells project their axons (mossy fibres) to the CA3 region of the hippocampus and make their synapses (second of the tri-synaptic loop) on the proximal part of the dendrites of the CA3 pyramidal neurons. The CA3 pyramidal neurons project their axons (Schaffer

collaterals) to the CA1 region of the hippocampus where they make their synapses (third of the tri-synaptic loop) on the CA1 pyramidal neurons. The CA1 neurons project either directly or via the subiculum back to neurons in layer V and VI of the entorhinal cortex (Figure 1.2).

Although the tri-synaptic loop is a dominant pathway through the hippocampus, there are other projections also. It has been shown that layer III of the entorhinal cortex projects directly to CA1 and subiculum (so called temporoammonic pathway) (Amaral & Witter, 1989). Furthermore, CA3 pyramidal neurons in the temporal region of the hippocampus project back to the dentate gyrus granule cells, although these connections are sparse (Amaral & Lavenex, 2007).

1.1.2 Hippocampal layers

The hippocampus proper (consisting of the CA3, CA2 and CA1 region) can be divided in several well defined layers (Figure 1.3). Starting from the outside of the temporal region of a rodent brain, after removing the 6 layers of the perirhinal / postrhinal cortex, it is possible to see the alveus; a thin layer of myelinated fibres, covering the hippocampus. The alveus contains both intrinsic connections (e.g. the CA1 – entorhinal pathway) as well as extrinsic connections to and from subcortical sites (e.g. connections joining the fimbria-fornix pathway to and from the medial septum). The alveus covers the stratum oriens of the CA1 region of the hippocampus. This region contains the basal dendrites of the CA1 pyramidal neurons whose cell bodies are in the subsequent stratum pyramidale. The apical dendrites of the CA1 pyramidal neurons are located in the stratum radiatum, and it is in this layer that most of the synapses from the Schaffer collateral pathway (the axons from the CA3 pyramidal neurons) are made. The CA1 pyramidal cells do not tend to form recurrent connections (CA1 pyramidal cell interconnections) as can be seen in CA3. Finally, the stratum lacunosum moleculare contains the distal ends of the CA1 pyramidal neuron apical dendrites, which receive input from layer III of the entorhinal cortex via the perforant (or temporoammonic) pathway.

The CA3 region of the hippocampus contains the same layers. The CA3 stratum lacunosum moleculare contains the distal ends of the apical dendrites of the CA3 pyramidal neurons on which the perforant path (i.e. the axons from layer II entorhinal cortex cells)

synapses. The stratum radiatum is composed of the apical dendrites of the CA3 pyramidal neurons and this is where most of the synapses from both the associational pathway (CA3 pyramidal neuron interconnections in the same hemisphere) and commissural pathway (CA3 pyramidal neuron interconnections from the other hemisphere) are found and which form the recurrent network of CA3. This network is highly recurrent with a single CA3 pyramidal neuron making synapses with 30 000 – 60 000 neighbouring CA3 pyramidal neurons (Li *et al.*, 1994). Both pathways project beyond CA3 and form the Schaffer collateral pathway which makes its synapses in CA1 such that CA3a pyramidal neurons make connections with proximal CA1, CA3b with medial CA1 and CA3c with distal CA1 (Ishizuka *et al.*, 1990). Between the CA3 stratum radiatum and the CA3 stratum pyramidale is a layer not found in CA1, namely the stratum lucidum. In this layer the mossy fibres, the axons from the granule cells in the dentate gyrus, make their synapses on thorny excrescences (specialized large spines) which are located on the basal portion of the apical dendrites of CA3 pyramidal neurons (Cajal,

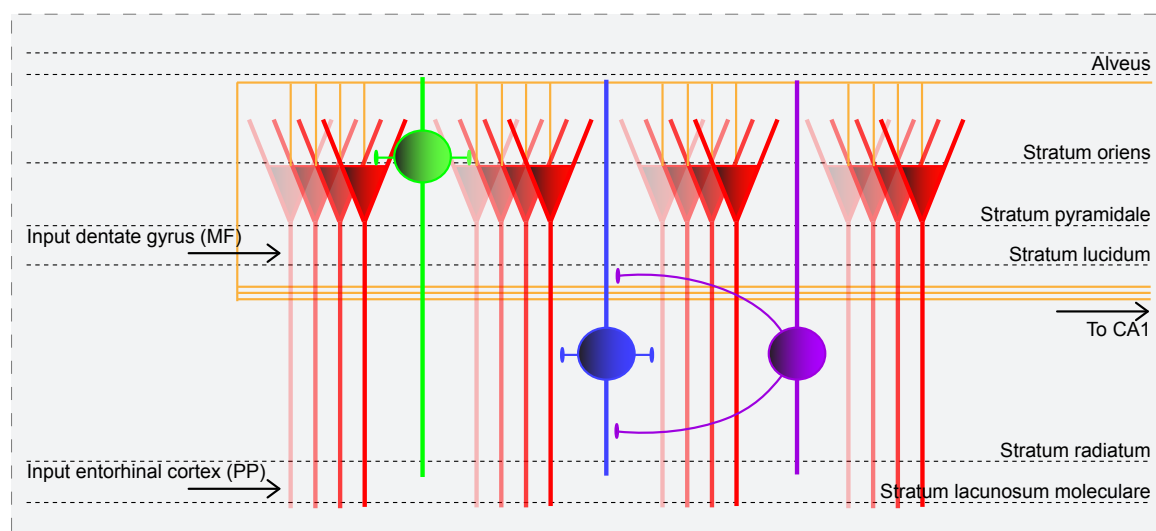


Figure 1.3 Hippocampal CA3 circuitry

Diagram of the circuitry of CA3. Indicated on the left are the inputs CA3 receives from the dentate gyrus (mossy fibres (MF)) and the entorhinal cortex (perforant path (PP)). Indicated on the right are the different layers of CA3. Coloured red are the CA3 pyramidal neurons which are interconnected by the associational fibres (orange), which also form the Schaffer collateral pathway projecting to CA1. CA3 pyramidal neurons receive input via the commissural fibres from the contralateral hemisphere as well, but this is not indicated in the diagram. In green, blue and purple I have indicated GABAergic interneurons. Green coloured are the perisomatic-targeting interneurons. Blue coloured are the dendritic-targeting interneurons. Purple coloured are the interneuron-selective interneurons.

1893; No, 1934; Blackstad & Kjaerheim, 1961). This layer has a relatively clear appearance in fresh tissue, from which it derives its name. This clear appearance has been suggested to result from the fact that the mossy fibres are non-myelinated, but could be the result of the specialized synapses between the mossy fibre boutons and thorny excrescences as well. The border between CA3 and CA2 is demarcated by cessation of the stratum lucidum; the mossy fibres do not extend into CA2 and CA1. The CA2 region is a relatively little studied region of the hippocampus proper containing pyramidal neurons with similar morphology to CA3 pyramidal neurons. They do not, however, receive mossy fibre input and express different neurochemical markers (Baimbridge & Miller, 1982; Leranth & Ribak, 1991; Sloviter *et al.*, 1991). The CA3 stratum pyramidale contains the cell bodies of the CA3 pyramidal neurons. The CA3 pyramidal neurons extend their basal dendrites into stratum oriens which also contains some of the Schaffer collateral fibres going towards CA1.

1.1.3 Hippocampal neurons

CA3 and CA1 pyramidal neurons of the hippocampus proper form the main excitatory cell types of this structure. They have their somata in the stratum pyramidale or principal cell layer and extend their apical dendrite into the stratum radiatum and a variety of basal dendrites into the stratum oriens (Spruston, 2008). Whereas CA3 pyramidal neurons are varied in size and shape, the CA1 pyramidal neurons are very homogenous with total dendritic length and dendritic configuration similar between different CA1 pyramidal neurons (Pyapali *et al.*, 1998). The CA3 pyramidal neurons are larger than CA1 pyramidal neurons and have one or two apical spiny dendrites extending radially from the superficial pole of the pyramidal neurons through the stratum lucidum, up through the stratum radiatum towards the stratum lacunosum moleculare (Ishizuka *et al.*, 1990; Freund & Buzsaki, 1996; Spruston, 2008). Numerous oblique spiny basal dendrites extend from the deep end of the pyramidal cells into the stratum oriens and towards the alveus (Ishizuka *et al.*, 1990; Turner *et al.*, 1995)(also see Figure 2.2B, page 29). The axons originate from the deep pole of pyramidal neurons either from the soma or a primary basal dendrite.

Besides these glutamatergic neurons the hippocampus proper contains a family of

GABAergic neurons (Ribak *et al.*, 1978; Freund & Buzsaki, 1996), so called interneurons, which are mainly inhibitory (Figure 1.3). The ratio of pyramidal cells to interneurons is approximately 10:1 (Vizi & Kiss, 1998), but despite the lower number they are able to influence the entire excitatory network. This has proven to be a very diverse family of neurons, difficult to classify as their morphology (e.g. arborisation of the dendrites), electrophysiological features (e.g. action potential properties) and biochemical markers (e.g. Ca^{2+} binding proteins such as parvalbumin) vary extensively. There are ongoing attempts to classify them (Maccaferri & Lacaille, 2003; Ascoli *et al.*, 2008).

One useful way to classify the interneurons is according to which cell type and where on the cell they make their synapses. Using this classification they can then be divided in perisomatic-targeting interneurons, dendritic-targeting interneurons and interneuron-selective interneurons (Freund & Buzsaki, 1996) for GABAergic neurons making their synapses on respectively the perisomatic (near or on the soma) region of the pyramidal neurons, the dendritic (apical and/or basal) region of the pyramidal neurons or on other interneurons.

The family of perisomatic-targeting interneurons is comprised of parvalbumin-positive basket cells (Kosaka *et al.*, 1987), cholecystokinin-positive basket cells (Somogyi *et al.*, 1984) and parvalbumin-positive axo-axonic cells (Somogyi, 1977). All three often have their soma close to the pyramidal cell layer but can be found in the stratum oriens and the stratum radiatum also. The basket cells make their axons on the soma and perisomatic region of the pyramidal neurons, excluding the axon initial segment (Sik *et al.*, 1995), whereas the axo-axonic cells make their axons solely on the axon initial segment of the pyramidal neurons (Somogyi, 1977; Buhl *et al.*, 1994b). Both have their dendrites extending through all hippocampal layers and can receive input from all major glutamatergic sources that excite pyramidal cells. As their dendritic tree spans all layers, this enables them to provide both feedforward and feed-back inhibition (Wierenga & Wadman, 2003a, b). It has been estimated that a single basket cell contacts 1500 – 2000 pyramidal neurons in rat CA1 (Sik *et al.*, 1995) with a single CA1 pyramidal neuron receiving approximately 100 synapses on the perisomatic region (Megias *et al.*, 2001). The locations where perisomatic-targeting interneurons make their synapses makes them especially suited to influence the timing of action potential firing

in large populations of pyramidal neurons (Cobb *et al.*, 1995).

The family of dendritic-targeting interneurons is very diverse and consists of, among others, bistratified cells, trilaminar cells, oriens-lacunosum moleculare cells and ivy cells. Bistratified cells extend their axonal arbour in the stratum oriens and the stratum radiatum and have their dendritic arbour spanning all hippocampal layers, except the stratum lacunosum moleculare (Buhl *et al.*, 1994a). Trilaminar cells extend their axonal arbour in the stratum oriens and the stratum radiatum as well as the pyramidal cell layer and have their dendritic arbour spanning all hippocampal layers (Sik *et al.*, 1995). Oriens-lacusosum moleculare cells have their soma and dendrites in the stratum oriens and their axonal arbour in the stratum lacunosum moleculare (Sik *et al.*, 1995). They tend to receive local excitatory input from pyramidal neurons as well as project locally, suggesting a role in feedback inhibition (Blasco-Ibanez & Freund, 1995; Sik *et al.*, 1995). Ivy interneurons have been described recently and are suggested to be the most prominent interneuron of the hippocampus. Ivy interneurons have a fine and dense axonal arbour innervating mostly basal and oblique pyramidal cell dendrites and have their dendritic arbour spanning all hippocampal layers except the stratum lacunosum moleculare (Fuatealba *et al.*, 2008a). The list of dendritic-targeting interneurons is still expanding and includes more than 15 well described members (Klausberger & Somogyi, 2008). Overall, the location where dendritic-targeting interneurons make their synapses makes them suited to modify plasticity and dendritic integration at pyramidal cell dendrites. It must be noted that most, if not all, of the data on this family of interneurons derive from anatomical and immunocytochemical analysis of CA1 interneurons. Detailed knowledge about CA3 interneurons is sparser.

The third family of interneurons are the interneuron-selective (IS) interneurons (Acsady *et al.*, 1996; Gulyas *et al.*, 1996) which can be divided into three classes (IS-I, IS-II and IS-III) depending on their neurochemical expression and connectivity (Freund & Buzsaki, 1996). This family of interneurons makes their synapses preferentially on other interneurons and when active can disinhibit. One example is the recently described enkephalin-expressing interneuron (Fuatealba *et al.*, 2008b). A distinguishing anatomical feature of this family of interneurons is their axon collaterals. These collaterals carry both en passant and drumstick-

like axon terminals (because they become varicose upon encountering an interneuron, soma or dendrite), which are distributed unevenly along the axon (Freund & Buzsaki, 1996).

This is by no means a complete list of the various types of interneuron within each family, but tries to emphasize the diversity of interneurons in the hippocampus compared to the relatively homogeneous population of pyramidal neurons. The differences in morphological, neurochemical and electrophysiological properties enable these neurons to control various aspects of the activity of the excitatory pyramidal neurons (Klausberger & Somogyi, 2008). It must be noted again that most of this work has been done in the CA1 region of the hippocampus and these descriptions refer to interneurons in CA1. It is at present not known how different or similar the three families of interneurons are in CA3.

1.2 State dependent network patterns

Having given a brief overview of the basic structure and principal components of the hippocampus I would now like to describe the synchronous activity, generated by populations of pyramidal neurons and interneurons, which can be observed during different behavioural states.

When one places an electrode in the hippocampus of a freely moving rodent it is possible to observe the activity of large populations of neurons simultaneously similar to making an electroencephalogram (EEG) from the scalp of humans (Buzsaki, 2004). Experiments like these have shown that during different behavioural states the hippocampus produces different types of network activity. In 1969 Vanderwolf described three distinct states: the rhythmical theta state (Green & Arduini, 1954), the large irregular amplitude activity (LIA) and the small irregular amplitude activity (SIA) (Vanderwolf, 1969). The SIA state often occurs during behavioural transitions (e.g. when an animal awakens) and little is known about its possible physiological function.

During memory related behaviour (i.e. active exploration) both the theta activity and the more recently described gamma activity (Bragin *et al.*, 1995) can be observed in the hippocampus. LIA activity, also referred to as sharp wave – ripple activity, has classically been described to occur during rest (e.g. awake immobility or eating) and slow-wave sleep (O'Keefe & Nadel, 1978; Buzsaki *et al.*, 1983), but has more recently been seen during exploratory behaviour also (O'Neill *et al.*, 2006).

1.2.1 Hippocampal theta and gamma oscillations

Theta activity in rodents describes the 6 – 10 Hz oscillatory pattern which can be recorded from the hippocampus of freely moving animals when they are actively exploring their surroundings (making 'translational' movements, i.e. movements which change the location of the head of the animal with respect to the environment) (Vanderwolf, 1969), but also during rapid eye movement (REM) sleep (Winson, 1972). The term theta activity describes a variety of oscillatory patterns at this frequency band and can be further divided, for example

in slow atropine-sensitive theta and fast atropine-insensitive theta (Buzsaki, 2002).

Theta activity in CA1 is thought to be the result of synchronous membrane potential fluctuations of large numbers of pyramidal neurons. It is important to distinguish between the current-generator and the rhythm-generator when investigating network oscillations. Whereas the current-generator produces the transmembrane current flow underlying the observed field oscillation, it is the rhythm-generator that controls the oscillatory pattern and frequency (Buzsaki, 2002). It has been shown that an isolated pyramidal neuron can exhibit a membrane potential oscillation at theta frequency (Alonso & Llinas, 1989; Strata, 1998; Buzsaki, 2002), but it is thought that all these individual theta oscillations are stabilised and synchronised by a set of inhibitory feedback circuits (Buzsaki *et al.*, 1983; Leung & Yim, 1986; Soltesz & Deschenes, 1993; Cobb *et al.*, 1995; Klausberger *et al.*, 2003). Furthermore, input arriving via the fornix – fimbria pathway from the medial septum and brainstem would enable the hippocampal neurons to enter the oscillatory state and could also act as rhythm-generator by driving the theta rhythm (Petsche & Stumpf, 1962; Stewart & Fox, 1990). Theta generators have been described in other regions as well, e.g. in the entorhinal cortex (Fox *et al.*, 1986) and the CA3 region of the hippocampus (Buzsaki *et al.*, 1986).

Often it is possible to see a higher frequency oscillation (30 – 100 Hz), referred to as gamma frequency, nested within the theta oscillation, but it is also possible for gamma frequency oscillation to occur independently during non-theta epochs (e.g. during urethane-anesthesia) (Bragin *et al.*, 1995; Penttonen *et al.*, 1998; Csicsvari *et al.*, 2003). Recently, it has been shown that local neocortical gamma oscillations can be phase modulated by hippocampal theta activity (Sirota *et al.*, 2008). It is thought that gamma oscillations are generated by a recurrent feedback mechanism between pyramidal neurons and perisomatic-targeting interneurons (Mann *et al.*, 2005; Oren *et al.*, 2006; Bartos *et al.*, 2007).

Both activities can not only be seen in rodents but also occur in humans and especially during memory related behaviour. For example, theta activity is increased during working memory tasks in humans (Kahana *et al.*, 1999; Kahana *et al.*, 2001; Raghavachari *et al.*, 2001; Jensen & Tesche, 2002; Kahana, 2006) and gamma activity in humans is increased during a variety of verbal and non-verbal tasks (Kahana, 2006; Womelsdorf *et al.*, 2007).

In rodents it was found that interfering with theta activity (Rawlins *et al.*, 1979) resulted in deficits in learning and recall of certain hippocampus dependent tasks (Winson, 1978; Givens & Olton, 1990; Numan & Quaranta, 1990). Both the rodent and human data on theta and gamma activity suggest that they could serve an important function in mnemonic processes.

1.2.2 Sharp wave – ripple activity

During rest (e.g. awake immobility or eating) and slow-wave sleep the hippocampus produces intermittent population bursts at a frequency between 0.1 – 2 Hz. Initially described as LIA (Vanderwolf, 1969; O'Keefe & Nadel, 1978) they were later dubbed sharp wave – ripple complexes (Buzsaki *et al.*, 1983). They have been seen in recordings from the hippocampus of the rat (Vanderwolf, 1969; O'Keefe & Nadel, 1978; Buzsaki *et al.*, 1983), the macaque monkey (Skaggs *et al.*, 2007) as well as humans (Clemens *et al.*, 2007; Axmacher *et al.*, 2008; Le Van Quyen *et al.*, 2008).

Sharp wave – ripple complexes are composed of two parts. The sharp wave part is seen as a negative voltage deflection of around 1 mV and a duration of 50 to 100 ms in the stratum radiatum of CA1. The polarity reverses in the stratum pyramidale and the stratum oriens and the sharp wave is seen as a low amplitude positive potential in these layers (Buzsaki, 1986). Current source density analysis in CA1 during sharp wave – ripple activity revealed large sinks and sources similar to that seen when stimulating the Schaffer collateral pathway (Ylinen *et al.*, 1995), suggesting that sharp wave activity is the result of input from the Schaffer collateral pathway corresponding to synchronous firing of a population of CA3 pyramidal neurons (Buzsaki, 1986). This hypothesis is supported by the observation that an increased spiking activity in CA3 precedes sharp wave – ripple activity recorded in CA1 (Csicsvari *et al.* 2000). Furthermore, it was shown that the location of small amplitude ripples in CA1 could be correlated with spiking activity of individual CA3 pyramidal neurons, which was not dependent on the exact location of, or the distance between, the CA3 pyramidal neuron and the particular CA1 region (Csicsvari *et al.*, 2000). Most of the work on sharp wave – ripples is done in the CA1 region of the hippocampus; it is easier to record from this region with tetrodes and it also has clearer sink – source distributions. Although it is suggested

that a synchronous burst in CA3 initiates the sharp wave – ripple as seen in CA1, not much is known about this synchronous burst in CA3 or the mechanisms underlying its initiation.

The second part of the sharp wave – ripple is composed of a fast oscillation with a frequency ranging from 120 to 200 Hz with the largest amplitude in the stratum pyramidale of CA1. Ripple activity seems to be synchronous over large areas (up to 4 mm) of the CA1 region within the same hemisphere (Buzsaki *et al.*, 1992; Ylinen *et al.*, 1995; Chrobak & Buzsaki, 1996). The cellular basis of the ripple oscillation is still very much a matter of debate. Several possible explanations have been proposed. Firstly, it has been suggested that the synchronous depolarisation of CA1 neurons, by CA3 pyramidal neuron activity, sets in motion a dynamic interaction between CA1 interneurons and CA1 pyramidal cells, resulting in the oscillatory field potential between 120 and 200 Hz as seen in the stratum pyramidale (Buzsaki *et al.*, 1992). Ripples were also seen in area CA3 concurrent with CA1 sharp wave – ripples, but they were observed less regularly and their frequency was lower (80 – 140 Hz) (Ylinen *et al.*, 1995). Furthermore, unit-activity in CA3 and ripple oscillations in CA1 were not correlated. These combined results suggested that CA1 ripple oscillations emerge from the CA1 cell population rather than being a passive response to high-frequency input from CA3 (Ylinen *et al.*, 1995). The specific synaptic currents mediating the ripple oscillation were suggested to be synchronised somatic IPSCs of CA1 pyramidal neurons (Ylinen *et al.*, 1995). Potentially, the interneurons could be synchronised by gap junctions (Katsumaru *et al.*, 1988) as halothane anaesthesia abolished ripple activity (Ylinen *et al.*, 1995).

A second proposed mechanism for the CA1 ripple oscillations involves a network of pyramidal neurons connected via axo-axonic gap junctions (Draguhn *et al.*, 1998). It was shown that the CA1 region of hippocampal slices could produce ripple oscillations which were not affected by block of both excitatory and inhibitory synaptic currents, but disappeared when proposed gap junction blockers (octanol, halothane and carbenoxolone) were used. It has been, shown, however that CA1 pyramidal neurons tend to burst at higher (~4 ms) frequencies than ripple oscillations, which would not be consistent with this hypothesis (Csicsvari *et al.*, 1999).

Lastly, it has been suggested that ripples observed in CA1 could be the result of the

synchronous firing of small groups of pyramidal neurons. These population spikes could be the result of intrinsically generated pyramidal neuron spiking, synchronized by recurrent connections (Dzhala & Staley, 2004). Of course a combination of the above would be possible as well.

1.3 Proposed functions of network patterns

1.3.1 Place cells

One particular type of hippocampal neuron has been shown to fire only when the animal is in a particular location in space and is virtually silent when the animal is elsewhere. These cells have appropriately been named place cells, first described by O'Keefe & Dostrovsky (1971), and correspond to the CA3 and CA1 pyramidal cells of the hippocampus. Place cells, like many neurons of the hippocampus, are modulated by and fire at different phases of theta and gamma oscillations. It was found that a place cell will fire earlier and earlier relative to theta phase as the animal traverses that cell's place field (O'Keefe & Recce, 1993) and this phenomenon has been named theta phase precession. This was the first experimental evidence that showed that the phase at which a cell fires could carry information. Huxter *et al.* (2003, 2008) demonstrated that, whereas the firing rate of place cells correlates well with the speed of the animal while traversing the place field, it is the phase at which the cell fires that correlates better with the location of the animal within the place field.

So when an animal explores an environment and moves from location to location, global theta activity can be observed in the hippocampus and concurrently several place cells will be sequentially active.

1.3.2 Sequence encoding during theta related behaviour

Theta and gamma oscillations as recorded in the hippocampus and other cortical regions have been suggested to act as a synchronizing mechanism locking the different parts of the hippocampal formation in one processing mode (Ward, 2003; Fries *et al.*, 2007). Furthermore, it has been suggested that they could assist in the induction of synaptic plasticity (Sejnowski & Paulsen, 2006). As discussed previously, concurrent with these network patterns it is possible to observe activity of single place cells (O'Keefe & Dostrovsky, 1971). It has been suggested that gamma and theta oscillations can time the spiking activity of place cells so that spike timing-dependent plasticity (STDP) can occur (Sejnowski & Paulsen, 2006). The precise timing of pre-synaptic spikes relative to the post-synaptic spikes has been shown to result in

potentiation or depression of that synapse during classic STDP (Markram *et al.*, 1997; Bi & Poo, 1998; Debanne *et al.*, 1998) as well as more elaborate forms of plasticity (Fusi *et al.*, 2005; Fusi & Abbott, 2007).

Such synaptic plasticity would facilitate the formation of transient groups of CA3 pyramidal neurons encoding information about the outside world (Buzsaki & Draguhn, 2004; Lin *et al.*, 2006; Morris, 2006). In the case of place cell activity; if two place cells have overlapping place fields representing a path in space, traversing the path will activate the sequence of firing within a single theta cycle (Skaggs *et al.*, 1996). Repeated sequential firing could then lead to plastic changes within the hippocampal network such that if an animal traversed the path and the sequence would be cell A – cell B – cell C – cell D, then later reactivation of cell A would lead to the reactivation of the remaining sequence (B, C, D) as a result of the increased synaptic strength at these synapses. Recently, it has been shown that place cells which co-fire frequently during exploration, because their place fields are close together and/or are visited often, tend to co-fire more frequently during subsequent sleep. It has been suggested that this is indicative of a Hebbian learning rule in which changes in firing associations between cells are determined by the number of waking coincident firing events (O'Neill *et al.*, 2008).

It has been suggested that the phase relative to theta provides the reference for the sequence timing, whereas the activity of different cell populations is separated by consecutive gamma cycles (Lisman, 2005). These cell populations can be referred to as cell assemblies, which have been suggested to form units of information, encoded by the simultaneous discharge of a population of pyramidal neurons within a 10 to 30 ms time window (Harris *et al.*, 2003; Harris, 2005). The precise unit of information or neural code which the brain uses is unknown; memory traces may be encoded through the firing patterns of individual cells as well as the synchronous firing of groups of cells or assemblies.

However, it has been shown that the sequence of activity get reactivated and replayed during subsequent REM sleep (Louie & Wilson, 2001), during sharp wave – ripple activity in NREM sleep (Lee & Wilson, 2002) and in-between bouts of theta activity (O'Neill *et al.*, 2006).

1.3.3 Sequence reactivation during sharp wave – ripples

The first evidence that waking firing patterns are reactivated in sleep was provided by the observation that if a particular place cell had been active during exploration, by confining a rat to the field of a particular place cell but not to a field of another place cell, it was only the place cell that had been active that increased its firing rate during subsequent slow-wave sleep (Pavlides & Winson, 1989). This was extended by the finding that if during exploration the place field of one place cell overlaps with the place field of another place cell, they tended to have an increased correlation in firing during subsequent sleep, and this increased correlation was not seen during sleep before exploration or between cells with non-overlapping fields (Wilson & McNaughton, 1994). This work was confirmed and extended by Kudrimoti *et al.* (1999) showing that joint firing patterns during exploration predicted joint firing during subsequent sleep. Skaggs & McNaughton (1996) showed that a sequence of place cells get reactivated during sleep. More recently it was found that entire spike sequences of a population of neurons get repeated during subsequent sleep stages (Lee & Wilson, 2002).

This replay of spike sequences can occur in both reverse and forward order relative to how they occurred during the exploration phase. It has been shown that sequence replay during sharp wave – ripples which occur during exploration, can be both forward replay (Diba & Buzsaki, 2007) and reverse replay (Foster & Wilson, 2006; Csicsvari *et al.*, 2007; Diba & Buzsaki, 2007). Sharp wave – ripples which occur during prolonged immobility or slow-wave sleep tend to be in forward order (Lee & Wilson, 2002). Reverse replay has been suggested to be initiated by cells of high excitability (close to last traversed place field) (Csicsvari *et al.*, 2007) and could facilitate backward associations (Diba & Buzsaki, 2007). Forward replay has been suggested to have a role in planning of upcoming trajectories (Diba & Buzsaki, 2007). One remarkable aspect of this reactivation is that the reactivated sequences can be temporally compressed approximately 20 times (Lee & Wilson, 2002), but this does not hold for all replay episodes (Diba & Buzsaki, 2007) or during REM sleep (Louie & Wilson, 2001).

Sequence replay during sharp wave – ripples should not be confused with ‘synfire’ reactivation. Synfire reactivation has been suggested to occur in the cortex and take the form

of ‘synfire’ chains consisting of very precise (ms precision) temporal sequential order of spikes which can last for 1 – 2 seconds (Abeles, 1991; Ikegaya *et al.*, 2004; Roxin *et al.*, 2008; Schrader *et al.*, 2008). From the published *in vivo* traces of sequence replay during sharp wave – ripples it is clear to see that this replay does not occur with millisecond time precision but has a substantial amount of jitter (Lee & Wilson, 2002). This suggests that the sequence information is retained, whereas the actual spike times are not, during sharp wave – ripples.

What could be the function of this replay of sequences? It has been proposed that cell assemblies, established during exploration, get reactivated during the sharp wave – ripple state (Buzsaki, 1989). This reactivation could strengthen intra-hippocampal associations (O'Neill *et al.*, 2006) as well as transfer information from the hippocampus to the cortex (Buzsaki, 1986; McClelland *et al.*, 1995). It has been shown that layer II and III of the entorhinal cortex are relatively silent during sharp wave – ripple activity whereas layer V of the entorhinal cortex (the hippocampal output layer) showed increased activity (Chrobak & Buzsaki, 1994). During the theta state the inverse is true and the output layers are relatively silent with layer II and III being more active (Chrobak & Buzsaki, 1994). More recently, correlated reactivation of spike sequences have been observed between the hippocampus and the visual cortex (Ji & Wilson, 2007). Reactivation of spike sequences have been also observed in the striatum (Pennartz *et al.*, 2004; Lansink *et al.*, 2008) and prefrontal cortex (Euston *et al.*, 2007).

Further evidence that some form of memory consolidation from the hippocampus to downstream targets might be occurring comes from behavioural studies in rodents and from human studies. In rodents, it has been shown that after exposure to a conditional associative learning task, gradual improvement in the associations between stimuli developed over time in the medial prefrontal cortex, without the need for further training, and became independent of the hippocampus (Takehara *et al.*, 2003; Takehara-Nishiuchi & McNaughton, 2008). Furthermore, human studies have suggested an important role for slow-wave sleep in memory consolidation in both the hippocampal – cortical network (Stickgold, 2005) and corticothalamic network (Steriade & Timofeev, 2003). For example, transcranial magnetic stimulation (TMS) of the brain at slow-wave frequency at the beginning of the NREM stage of sleep, in effect enhancing slow-wave sleep, slightly but significantly enhanced previously

learned wordpair recall the next day (Marshall *et al.*, 2006; Stickgold, 2006). Also, giving odour cues at the slow-wave stage of sleep, improved the retention of hippocampal-dependent declarative memories if they had been paired previously with the odour cue. The odour cue did not have an effect when given during REM sleep or affect hippocampus-independent procedural learning retention (Rasch *et al.*, 2007).

There is a lot of evidence supporting the hypothesis that sequence replay occurs during sleep and that this could have a role in consolidation of memory. However, it has also been suggested that sharp wave – ripple bursts might actually inhibit potentiation (Leonard *et al.*, 1987; Colgin *et al.*, 2004) or that sleep de-potentiates synapses (Vyazovskiy *et al.*, 2008).

In summary, pyramidal cells in the hippocampus are active during exploration and have a spatial preference. The sequence of activation of these place cells during exploration appears to be replayed during subsequent sleep stages and during sharp wave – ripple activity. This reactivation might facilitate the consolidation of memories within the hippocampus or in downstream cortical targets. It is likely that sharp wave – ripples in CA1 are initiated through synchronous activity from the CA3 region of the hippocampus during slow-wave sleep. However, the mechanisms underlying the initiation of these bursts of activity in CA3 are not well understood. It has been suggested that they can be generated intrinsically in the CA3 region and that triggering of sharp wave activity could be the result of a reduction in subcortical (Buzsaki, 1989) or cortical (Chrobak & Buzsaki, 1994) inputs during slow-wave sleep and immobility. It has been proposed that synaptic connectivity in hippocampal CA3 changes during exploration (Buzsaki, 1989; Buzsaki & Chrobak, 1995) and that these synaptic weights control burst initiation and recruitment of CA3 pyramidal neurons in subsequent rest and sleep stages (Behrens *et al.*, 2005), when they are released from subcortical and cortical input. Recent place cell activity could bias a population discharge through increased excitability (Csicsvari *et al.*, 2007). However, hippocampal CA3 contains both feedforward and feedback inhibitory circuits and it is at present not known how these circuits influence sharp wave – ripple initiation.

1.4 Hippocampal bursts *in vitro*

A useful tool for investigating the cellular mechanism underlying network activity in more detail is *in vitro* slice models, which can exhibit similar network activity but enable easier access to the network. Gamma frequency oscillations have been induced in the CA3 region of the hippocampus using pharmacological agonists (Fisahn *et al.*, 1998), whereas sharp wave – ripples (Kubota *et al.*, 2003) and epileptiform bursts (Staley *et al.*, 1998) have been induced by altered ionic conditions of the ACSF.

1.4.1 Epileptic bursts *in vitro*

Studies of epileptiform discharges in area CA3 of hippocampal slices *in vitro*, under conditions of reduced or absent inhibition (Miles & Wong, 1983; Menendez de la Prida *et al.*, 2006) or high K^+ concentration (Staley *et al.*, 1998), have revealed detailed mechanisms responsible for the initiation of pathological population bursts.

It has been shown that a single CA3 pyramidal neuron can initiate an epileptiform burst, under conditions of reduced or absent inhibition (Miles & Wong, 1983). It was suggested that activity of a single pyramidal neuron could spread to other neurons through a multi-synaptic excitatory pathway which would eventually lead to the participation of the whole population in a synchronous burst. Computer modelling of hippocampal CA3 showed this behaviour also (Traub & Wong, 1982). However, it was suggested that an excitatory network containing fast or slow inhibition would need a high level of connectivity and/or strong synaptic connections for full synchronization and burst generation to occur (Traub *et al.*, 1987). More recently it was shown, in a disinhibited hippocampal slice, that the excitatory activity and remaining slow GABA_B receptor-mediated inhibition in the CA3 network can have opposing effects on synchronized network activity. It was shown that after a population burst there is an increase (build-up) of excitatory activity in the network which reaches a plateau level. This plateau level has been suggested to be the result of opposing forces of excitation and slow inhibition. Only when excitation reached a threshold was a population burst initiated (Menendez de la Prida *et al.*, 2006).

Studies of epileptiform bursting induced by washing in artificial cerebrospinal fluid (ACSF) containing high concentrations of K^+ (8.5 mM) have suggested that pre-synaptic factors (probability of release and replenishment of releasable glutamate vesicles) play an important role as well in setting the probability and duration of synchronous discharges of the CA3 network (Staley *et al.*, 1998).

Combined, these studies have suggested that recruitment of a population of pyramidal neurons is the result of mutual synaptic excitation which is facilitated in a disinhibited or excited recurrent network (Traub & Wong, 1982; Korn *et al.*, 1987; McBain, 1995). It is at present not known if similar cellular mechanisms are involved in the initiation of sharp wave – ripple population bursts in hippocampal CA3 under conditions with intact inhibition.

1.4.2 Sharp wave – ripples *in vitro*

Several hippocampal models of sharp wave – ripples have been proposed which exhibit sharp wave – ripple-like activity in CA3 and CA1. From now on, I will refer to these *in vitro* bursts as sharp wave – ripples. Draguhn *et al.* (1998) showed that it was possible to obtain high frequency ripples in the CA1 region of hippocampal slices in conditions where both excitatory and inhibitory currents were blocked. It was suggested, by using a combination of gap junction blockers and computer modelling, that these oscillations could be produced by a small network of pyramidal neurons connected through axo-axonic gap junctions. How the network would start this activity was not shown however. Recent work has shown that it is possible to obtain sharp wave – ripples in mouse slices in CA1 spontaneously and it was suggested that they were dependent on a combination of excitation, inhibition and gap junctions (Maier *et al.*, 2003). Later, Nimrich *et al.* (2005) confirmed this and showed that inhibition was necessary for the sharp wave but not the ripple component as blocking both excitatory and inhibitory synaptic transmission did not block the induction of ripple oscillations by local application of 1 M KCl (Nimrich *et al.*, 2005). Wu *et al.* (2005) reported that sharp waves could be generated in the CA3 region of thick mouse hippocampal slices (1 mm) without the need for pharmacological or ionic manipulation (Wu *et al.*, 2005). These bursts were blocked by antagonists at AMPA receptors, while enhancing GABA_A receptor-

mediated events with the barbiturate methohexital could significantly reduce the incidence. It was suggested that sharp wave generation *in vitro* required a balanced interaction between glutamatergic excitation and GABAergic inhibition. However, blocking all inhibition did not significantly alter the waveform of the sharp wave, whereas normally, block of all inhibition leads to the generation of epileptiform bursting with very different characteristics (Miles & Wong, 1983; Menendez de la Prida *et al.*, 2006), suggesting that they might have been looking at epileptiform bursting instead of sharp wave – ripples.

Sharp wave – ripples have not only been studied in mouse slices but they have also been generated in rat slices. It was found that slices from rats could generate sharp wave – ripple population bursts spontaneously when using an altered ACSF (Kubota *et al.*, 2003). Recent work by Behrens *et al.* (2007) showed that it is possible to induce sharp wave – ripples in the CA3 region of the rat hippocampus by giving trains of high frequency electrical pulses to this region, a protocol commonly used to induce LTP (Bliss & Lomo, 1970; Bliss & Gardner-Medwin, 1973). They found that after such an induction protocol they could obtain stable sharp wave – ripple generation in CA3, where before there was none. This suggested that sharp wave – ripple burst initiation could be the result of an increase in synaptic weights or excitability in a population of CA3 pyramidal neurons, as had been suggested (Buzsaki, 1989; Buzsaki & Chrobak, 1995; Csicsvari *et al.*, 2007).

In summary, it has been suggested that a combination of synaptic excitation, synaptic inhibition and electrical coupling is necessary for the generation of sharp wave – ripples *in vitro*. Their relative importance, the source of inhibition and the detailed mechanisms underlying the initiation of sharp wave – ripples in the CA3 region of the hippocampus are not known, however. I therefore investigated this in detail using a rat hippocampal slice model which shows sharp wave – ripple activity spontaneously in CA3 in altered ACSF (Kubota *et al.*, 2003).

1.5 Aims of thesis work

The overall aim of this thesis was to investigate the cellular mechanisms underlying the generation of sharp wave – ripple population bursts in CA3 of the hippocampus.

The first objective was to investigate the characteristics of sharp wave – ripples *in vitro* in detail. Chapter 3 will discuss the conditions with which to obtain sharp wave – ripples in hippocampal slices in both interface and submerged style recording chambers. I will then compare both the temporal and spatial characteristics of sharp wave – ripples *in vitro* to those described for sharp wave - ripples *in vivo*.

The second aim of my thesis is to study the potential involvement of synaptic receptors and gap junctions in the generation of sharp wave – ripples. Chapter 4 will discuss the potential role of AMPA, NMDA, GABA_B and GABA_A receptors, as well as gap junctions, in the generation of sharp wave – ripples *in vitro*.

The third aim of this thesis is to study both the input of CA3 pyramidal neurons and CA3 interneurons during sharp wave – ripples, as well as the participation of CA3 pyramidal neurons during this activity (Chapter 5). This was studied by recording both the spiking activity and intracellular response of CA3 pyramidal neurons in cell-attached and whole-cell current-clamp mode during sharp wave – ripple activity. More detailed investigation of the excitatory and inhibitory currents was done by analysing these currents from whole-cell voltage-clamp recordings of both CA3 pyramidal neurons and CA3 interneurons during sharp wave – ripple activity.

The final aim of this thesis is to study the cellular mechanisms underlying the initiation of sharp wave – ripples in CA3. Chapter 6 will, firstly, discuss the observation that a single type of interneuron is capable of initiating a sharp wave. Secondly, it will propose a mechanism by which this might occur.

Experimental Procedures

2.1 Slice preparation

Transverse hippocampal slices (400 μm) were prepared from the ventral part of the hippocampus of postnatal day 14 – 24 Wistar rats. Rats were anaesthetized with isoflurane and, following both slowing down of respiration and cessation of pinch reflexes, decapitated using procedures licensed under the Animals (Scientific Procedures) Act (1986). The entire brain was quickly removed and placed in ice-cold cutting solution (0 °C), containing (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4 , 5 MgSO_4 , 3.4 CaCl_2 , 26 NaHCO_3 , and 10 glucose, pH 7.2-7.4, which had been bubbled with 95% O_2 / 5 % CO_2 (carbogen gas) for at least 30 minutes before use.

The cerebellum and rostral part of the cortex were removed with a scalpel (Figure 2.1B). The two hemispheres were separated (Figure 2.1C) and each hemisphere was flipped forward so that the caudal part of the brain (which now did not contain the cerebellum) was pointing upwards. A part of the basal cortex was removed for each hemisphere by making a cut at a 45 ° angle starting from the lower midline and going up towards the lateral side of the brain (Figure 2.1D). Both hemispheres were stuck to a microtome plate, on the surface of the last made cut, using cyanoacrylate adhesive. Transverse slices of 400 μm thickness were cut using a microtome (Leica VT 1000 S). The slices were transferred to a petridish containing room temperature cutting solution and trimmed of most extrahippocampal areas, before being transferred using a paintbrush to an interface-style recording chamber, or an interface holding chamber for multi-electrode array recording, containing ACSF (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4 , 1 MgSO_4 , 3 CaCl_2 , 26 NaHCO_3 , and 10 glucose, pH 7.2-7.4.

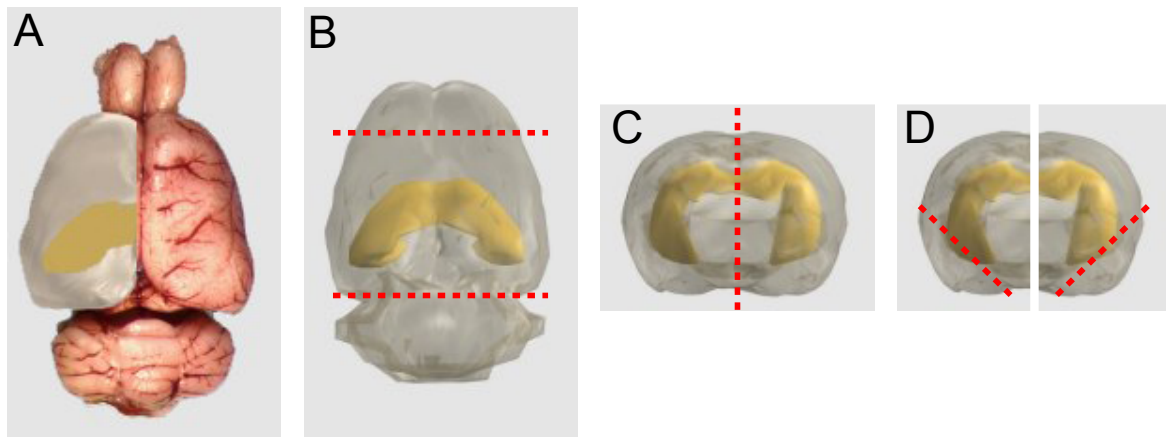


Figure 2.1 Preparing transverse hippocampal slices

(A) Photo of dorsal view of a rat brain with the location of the hippocampus indicated in yellow in the left hemisphere (B) After removing the brain from the skull of the rat, the cerebellum and front part of the cortex are removed with a scalpel (red lines indicate where the cuts were made). (C) Diagram of the caudal part of the rat brain after the cerebellum has been removed. Red line indicates the cut to separate the two hemispheres. (D) Each hemisphere is cut at a 45 °C angle to remove a part of the basal-temporal cortex. Modified with permission from Synapse Web, Kristen M. Harris, PI, <http://synapses.clm.utexas.edu/>.

2.2 Electrode preparation

Standard-wall borosilicate tubing was used to make the electrode pipettes. Pipettes were pulled with a vertical patch pipette puller (Narishige, PP-830, Tokyo, Japan). Pipettes were filled either with ACSF for extracellular recordings in interface conditions or with current-clamp or voltage-clamp intracellular solution for whole cell recordings in submerged conditions. Pipettes for extracellular and somatic whole cell recordings had a final resistance of 4 – 6 M Ω , whereas pipettes for dendritic recordings had a final resistance of 16 – 24 M Ω .

2.3 Multi-electrode array preparation

Planar multi-electrode arrays containing an 8 x 8 array of planar multi-electrodes (electrode size, 50 μm x 50 μm ; interpolar distance, 150 μm ; Panasonic MED-P2105, Tensor Biosciences, Irvine, CA) were prepared the day before usage by covering them with 0.25 M borate buffer, pH 8.4 containing 0.1 % polyethylenimine, and leaving them for at least 8 hours at room temperature to coat.

2.4 Field Recording

2.4.1 Interface recording

In a subset of early experiments slices were kept in an “Oslo” style interface chamber (Fine Science Tools, Heidelberg, Germany), on a netting and lens paper (Kodak), in the interface between humidified carbogen gas and ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4 , 1 MgSO_4 , 3 CaCl_2 , 26 NaHCO_3 , and 10 glucose, pH 7.2-7.4. and kept at 32 °C. After at least 1 hour rest, recordings were made in interface conditions by using an Axoclamp-2A amplifier (Axon Instruments), filtered at 2 kHz and acquired at 10 kHz using a CED 1401 analog/digital converter (Cambridge Electronic Design, Cambridge, UK) using Signal software (Cambridge Electronic Design, Cambridge, UK).

The results from these experiments are reported in Figures 3.1 – 3.6, 3.9D and 4.4. There were no significant differences in the properties of recorded sharp wave–ripples between the interface and submerged conditions, except for a smaller amplitude when recorded with a planar multi-electrode array in submerged conditions (see Figure 3.7D, page 54). Most of the data shown were recorded using a single recording electrode in the stratum pyramidale or the stratum radiatum of CA3 or CA1. Only to investigate the spatial extent of sharp waves were three recording electrodes used, which were placed in the CA3c, CA3b and CA3a stratum pyramidale (Figure 3.9D, page 58).

LTP experiments were performed by placing a monopolar stainless steel stimulation electrode (A-M systems, Carlsborg, WA, USA) in the Schaffer collateral pathway going towards CA1, and a glass recording pipette in the stratum radiatum of CA1. Synaptic potentials were evoked via the stimulation electrode by stimulating with single, rectangular 50 μs current pulses, at stimulation strength sufficient to induce half of the maximum possible field excitatory postsynaptic potential (fEPSP), at a frequency of 0.2 Hz. Following stable synaptic transmission, with no more than 15% change in fEPSP slope over 15 minutes, the Schaffer collateral pathway was stimulated via a tetanic LTP induction paradigm or a theta-burst LTP induction paradigm. The tetanic paradigm involves giving a train of pulses (50 μs) at 100 Hz for 1 second. The theta-burst paradigm involves giving five bursts of five pulses

(50 μ s) at 100 Hz delivered at theta (5 Hz) frequency. After a single LTP induction paradigm I reverted back to stimulating at 0.2 Hz for at least 45 minutes.

2.4.2 Multi-electrode array recording

Slices were, immediately after slicing and trimming of most extrahippocampal areas, transferred to an interface chamber on lens paper (Kodak) and maintained at room temperature for one hour between humidified carbogen gas and ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4 , 1 MgSO_4 , 3 CaCl_2 , 26 NaHCO_3 , and 10 glucose, pH 7.2-7.4.

Planar multi-electrode arrays were rinsed once with distilled water and once with ACSF. Finally, they were filled with 400 μ l ACSF and were ready to receive the hippocampal slices. Slices were transferred to planar multi-electrode arrays using a paintbrush and flipped over, so that the side contacting the lens paper was facing upwards. Slices were positioned so that most of the CA3 region (both somatic and dendritic regions) was above the 8 x 8 array of electrodes. This was done using an inverted microscope and a 10x magnification objective. The 400 μ l ACSF was carefully removed with a Pasteur pipette and any remaining liquid around the slice removed with filter paper. This was done to facilitate the adhesion of the hippocampal slice to the substrate. Quickly and carefully 400 μ l ACSF was added again and the probe was stored in a custom built holding chamber containing humidified carbogen gas at 32 – 37 °C for at least 1 hour and until used for recording.

For recording the probe was removed from the humidified chamber and the outside edges were dried of liquid thoroughly to avoid water contamination of the recording setup, after which it was placed in the recording setup. Slices were perfused at 6 ml/min with ACSF containing (in mM): 124 NaCl, 3 KCl, 1.25 KH_2PO_4 , 1 MgSO_4 , 3 CaCl_2 , 26 NaHCO_3 , and 10 glucose, pH 7.2-7.4. at 32 °C. In order to reduce the volume of the chamber and direct the superfusion fluid over the slice, an inert plastic insert was used. Spontaneous field potentials from 62 – 64 recording electrodes of the planar multi-electrode arrays were acquired at 5 kHz, using the Panasonic MED64 system (Tensor Biosciences, Irvine, CA).

The effect of different ionic conditions on sharp wave incidence, amplitude and spatial extent was investigated by wash-in of ACSF containing various concentrations of

[K⁺], [Mg²⁺] or [Ca²⁺] for more than 5 minutes after which sharp waves were recorded on the planar multi-electrode array.

The effect of different pharmacological agents on sharp wave incidence and amplitude was investigated by wash-in of various compounds for at least 10 minutes after which sharp waves were recorded on the planar multi-electrode array.

2.5 Electrophysiological recording

Cell-attached recordings, whole-cell current-clamp recordings, whole-cell voltage-clamp recordings and dendritic recordings from CA3 pyramidal neurons and interneurons were performed with glass pipettes, pulled from standard wall borosilicate glass capillaries. Neurons were visualized using infrared differential interference contrast (IR-DIC) microscopy (Zeiss Axioskop, Jena, Germany) and healthy looking neurons were patched in the top 50 – 150 μ m of the slice (Figure 2.2A). Recordings were made using a Multiclamp 700B amplifier (Axon Instruments), lowpass filtered at 2 kHz using the built-in Bessel filter, and acquired using 2 channels of the Panasonic MED64 system at 5 kHz. Following intracellular recording, the slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) (see section 2.6). Recordings were analysed off-line using custom-made procedures in IGOR Pro 5.0 software (Wavemetrics, Lake Oswego, OR).

2.5.1 Cell-attached recording

Spiking activity of individual pyramidal neurons was recorded from healthy pyramidal neurons in cell-attached mode with a >1 G Ω seal between the pipette and the neuron. The pipette was filled with current-clamp intracellular solution. Recordings were made approximately one minute after formation of a seal.

2.5.2 Whole-cell current-clamp recording

Whole-cell current-clamp recordings were made with glass capillaries containing (in mM):

110 potassium gluconate, 40 HEPES, 2 ATP-Mg, 0.3 Na-GTP, 4 NaCl and 4 mg/ml biocytin (pH 7.2-7.3; osmolality, 290-300 mosmol/l). Seal resistance before whole-cell access was $> 1 \text{ G}\Omega$. Fast and slow capacitive currents were cancelled using the automated function of the amplifier. Neurons were broken in with sharp negative pressure. Neurons were held in current-clamp mode in bridge balance. Membrane voltage was measured and if more depolarised than -45 mV the neuron was not used for the experiment or analysis. No holding current was applied when recording from pyramidal neurons and interneurons in current-clamp mode. Nominal resting membrane potentials were, for pyramidal neurons, $-57 \pm 1 \text{ mV}$ ($n = 51$); for interneurons, $-59 \pm 1 \text{ mV}$ ($n = 59$).

2.5.3 Whole-cell voltage-clamp recording

Whole-cell voltage-clamp recordings were made with glass capillaries containing (in mM): 120 cesium gluconate, 40 HEPES, 4 NaCl, 2 ATP-Mg, 0.3 Na-GTP, 0.2 QX-314 and 4 mg/ml biocytin (pH 7.2-7.3; osmolality, 290-300 mosmol/l). Seal resistance before whole-cell access was $> 1 \text{ G}\Omega$. Fast and slow capacitive currents were cancelled using the automated function of the amplifier. Neurons were broken in with sharp negative pressure and held in whole-cell voltage-clamp mode. Whole-cell series resistance was in the range of $5 - 15 \text{ M}\Omega$. No series resistance compensation was applied during the recording. Voltage measurements were not corrected for liquid-liquid junction potential. To record excitatory postsynaptic currents

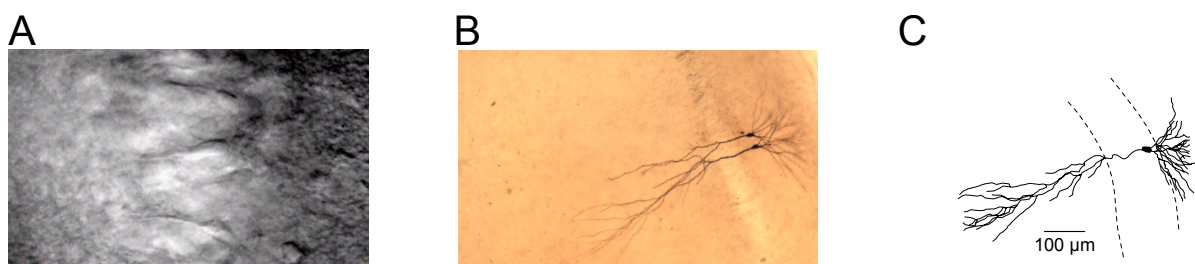


Figure 2.2 Different views of a CA3 pyramidal neuron

(A) View of the stratum pyramidale and several pyramidal neurons as visualized with IR-DIC microscopy. (B) View of the same pyramidal neuron after the cell has been filled with biocytin which has been reacted with 3,3'-diaminobenzidine (DAB). Notice that a second pyramidal neuron has been patched, filled and reacted as well. (C) A neuroLucida trace of the bottom pyramidal neuron in (B).

(EPSCs) and avoid interference from inhibitory events, neurons were voltage clamped at a nominal holding potential of the estimated reversal potential for inhibitory postsynaptic currents (IPSCs) (-65 to -60 mV of the uncorrected potential). Similarly, IPSCs were recorded at a nominal holding potential of the estimated EPSC reversal potential (0 to 20 mV of the uncorrected potential).

2.5.4 Dendritic recordings

Dendritic recordings were made using two glass capillaries containing intracellular current-clamp solution. One was used for somatic recording (4 – 6 MΩ resistance) and the other for dendritic recording (16 – 24 MΩ resistance). A superficial CA3 pyramidal neuron with clear apical dendrite was located in the slice and both electrodes were placed either close to the soma or dendrite (50 – 100 μm from the soma). First a somatic cell-attached configuration was established after which dendrite-attached configuration was established. Both membranes were ruptured using negative pressure (Davie *et al.*, 2006). Recordings were made in current-clamp mode as described above.

2.5.5 Stimulation and recording protocols

Stimulation of neurons

To investigate the effect of firing of individual neurons, a 500 ms long depolarising current step, sufficient for spike generation of approximately half maximum spike frequency, was repeatedly injected at 0.1 Hz for at least 10 minutes. Simultaneously sharp waves were recorded using the planar multi-electrode array. In a subset of experiments this protocol was combined with another neuron recorded in voltage-clamp mode. This neuron was held at -65 mV and 0 mV to record EPSCs and IPSCs respectively, for at least 10 minutes at each holding voltage. Neurons were held, in arbitrary order, first at -65 mV and subsequently at 0 mV, or vice versa.

Titration of increasing concentration of SR 95331

The effect of different concentrations of SR95331 on GABA_A receptor-mediated currents

in CA3 pyramidal neurons was investigated by extracellular stimulation using a monopolar electrode placed in the stratum pyramidale. Series resistance was measured throughout the recording and the recording was discarded if it changed by more than 10%. Evoked IPSCs were recorded from pyramidal neurons held in whole-cell voltage-clamp at a holding potential of 0 mV. The slices were perfused with standard ACSF containing 20 μ M NBQX and 50 μ M D-AP5 to block excitatory transmission.

2.6 Histochemical techniques

Biocytin-filled cells were visualised following standard procedures. Hippocampal slices containing filled cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). Slices were stored in the refrigerator until processed. Slices were washed four times for 10 minutes in 0.01 M PBS (pH 7.4). They were then incubated in freshly made 0.5% H_2O_2 in PBS for 30 minutes to block endogenous peroxidase activity. Subsequently slices were washed four times in PBS for 10 minutes. Slices were then incubated (with shaking) in PBS containing 0.3% Triton X-100 and avidin-biotinylated antibody (ABC Vector kit P6100 *Elite*: Vector laboratories, Burlingham, CA) at room temperature overnight. The following day the slices were washed twice in PBS for 10 minutes. Next they were washed twice in 0.05 M Tris buffer (pH 8.0) for 10 minutes. Slices were reacted with 3, 3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 in Tris buffer (pH 8.0) (Sigma Aldrich) for 3 to 10 minutes until the neuron was sufficiently stained, but before background staining became too dark. The reaction was stopped by washing the slices twice for 10 minutes in 0.05 M Tris buffer (pH 8.0) and subsequently twice for 10 minutes in PBS. Slices were taken through glycerine steps (25%, 50%, 75%, 100% glycerol in ddH₂O) and cover-slipped immediately with 100% glycerol. Stained neurons were visualized using a light microscope (Figure 2.2B, page 29) and reconstructed using Neurolucida and Neuroexplorer software (MBF Bioscience, Williston, USA) (see Figure 2.2C, page 29). Neurolucida software enables the reconstruction of the entire stained cell in 3-dimensions by drawing short (5 – 10 μ m) straight lines to indicate the different components (axon, dendrite and soma) of the cell. After a cell was

traced the outline of the pyramidal cell layer was also marked. The Neurolucida generated file was exported to Neuroexplorer software which collapses the image to 2 dimensions and enables export as an image for further processing.

2.7 Measurement of oxygen saturation

2.7.1 Measurement of oxygen saturation of superfusate

The local oxygen saturation of the ACSF was measured 50-100 μm vertically above the CA3 region of the slice with an optode (tip diameter $\sim 50 \mu\text{m}$; Microx TX3, PreSens GmbH, Germany). Vertical adjustment of the optode between 50 and 100 μm above the slice did not cause any substantial change in measured values at a given flow rate. The sensor was calibrated as follows: 2-3 mM sodium sulfite (Na_2SO_3) was used to eliminate dissolved oxygen from non-bubbled ACSF, defining 0% of oxygen, while 95% was set in ACSF, bubbled for one hour with 95% O_2 / 5% CO_2 . Thus, the maximal Po_2 in ACSF at room temperature was estimated to be ~ 720 Torr. In a subset of experiments ACSF bubbled with a mixture of 95% N_2 / 5% CO_2 was used to change the oxygen level in the chamber while retaining a constant flow rate.

2.7.2 Measurement of oxygen saturation inside tissue

Oxygen concentrations were detected in submerged hippocampal slices using fast-scan cyclic voltammetry (FCV) at carbon-fibre microelectrodes (fibre diameter 7 μm , tip length 20-30 μm , fabricated in-house) and a Millar Voltammeter (Paul Summers, UK) using methods similar to those described previously for the detection of other electroactive biological substances e.g. dopamine (Cragg *et al.*, 1997, Cragg, 2003, Exley *et al.*, 2008). For the detection of oxygen specifically, the applied voltage was a triphasic waveform scanning from 0.0 V to +0.8 V to -1.4 V and back to 0.0 V (vs. Ag/AgCl) as described previously (Venton *et al.*, 2003) with a scan rate of 880 V/sec and a sampling frequency of 8 Hz. The peak reduction current for oxygen was detected between -1.3 and -1.4 V. Bath temperature was 32 $^{\circ}\text{C}$. Currents

due to oxygen were determined after subtraction of the steady-state background current that results from the charging of the electrode as well as exposure to the brain tissue environment. Currents were normalised to the current observed in solution at a flow-rate of 6 ml/min which was subsequently set to 80% saturation as measured with the optode at this perfusion speed.

Measurements were made outside of the tissue (50 μm – 100 μm above the surface) as well as 50 and 150 μm below the tissue surface in the CA3 pyramidal cell layer. Perfusion speeds were 6, 3 or 1.8 ml/min. For a given flow rate and electrode depth, a steady-state background measurement was first obtained in oxygen-free ACSF (bubbled with 95% N_2 / 5% CO_2), oxygen-saturated buffer (bubbled with 95% O_2 / 5% CO_2) was then applied and the increase in detected oxygen recorded. The slice was thoroughly superfused with oxygen-free ACSF between each change in flow rate or change in electrode placement.

2.8 Data analysis

Data were analysed off-line using custom-made procedures in IGOR Pro 5.0 software (Wavemetrics, Lake Oswego, OR). Recording from both Signal (Cambridge Electronic Design, Cambridge) and Panasonic MED64 system (Tensor Biosciences, Irvine, CA) were exported as tab delimited text files and imported into IGOR Pro 5.0 software. Before analysis all imported extracellular traces were scaled and highpass filtered at 0.1 Hz.

2.8.1 Field recording

Sharp wave – ripple detection *in vitro*

For sharp wave detection, extracellular recordings were filtered between 0.1 Hz and 20 Hz and sharp waves were detected as voltage fluctuations of more than 2 standard deviations (SD) above the baseline value, with a duration of more than 40 ms. Baseline is taken as 0 mV.

The start and end of a sharp wave was taken as the time points when the sharp wave crosses the baseline. The duration of the sharp wave was taken as the time between the start and end of the sharp wave.

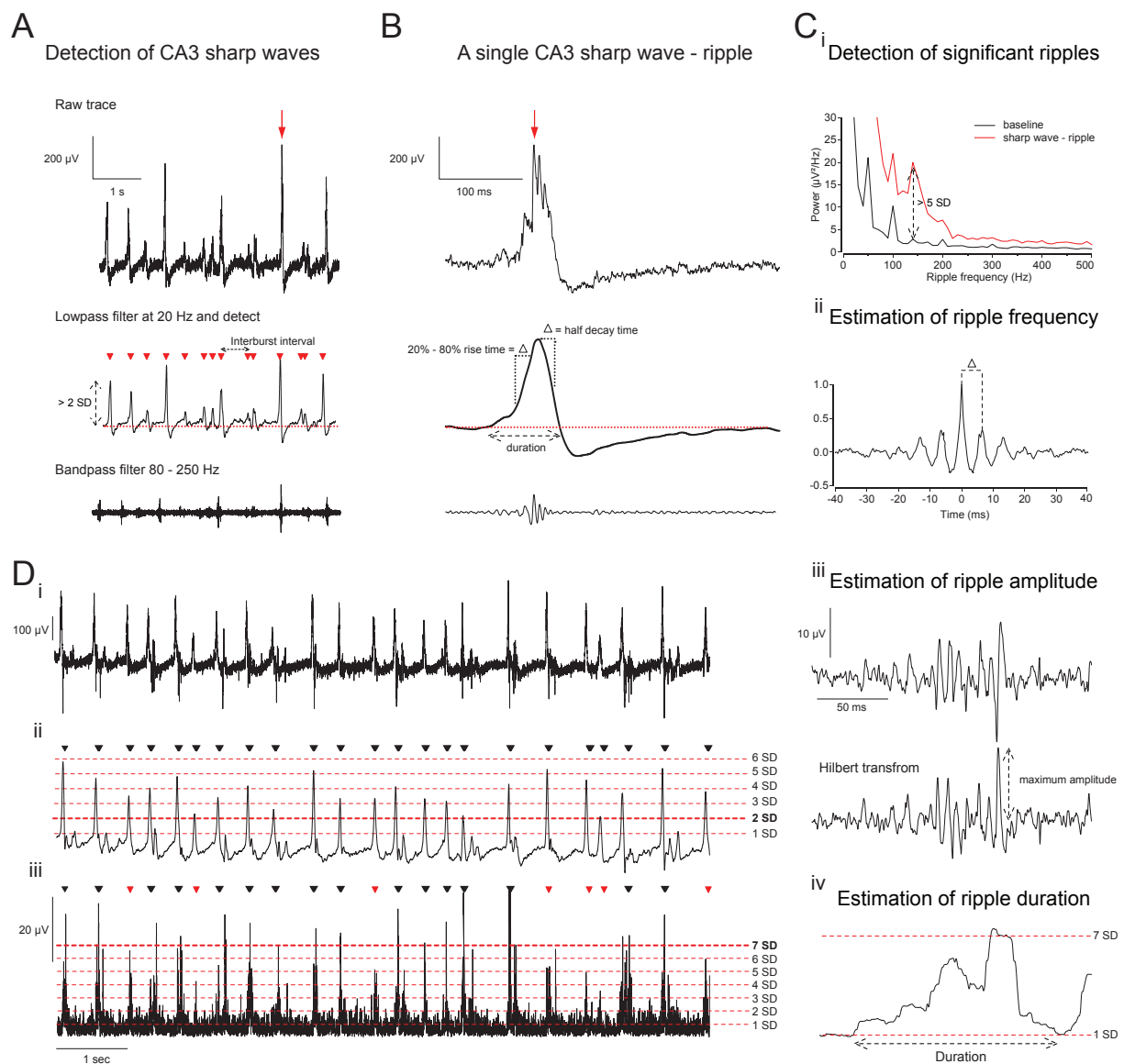


Figure 2.3 Detection of sharp wave – ripples and their characteristics

(A) Transverse hippocampal slices (400 μ m) exhibited spontaneous sharp wave – ripples with a frequency between 0.35 and 2.3 Hz. Traces were lowpass filtered at 20 Hz and sharp waves were detected as events of more than 2 SD above baseline. Bandpass filtering of recordings between 80 Hz and 250 Hz revealed the fast frequency oscillations. These traces are from interface conditions.

(B) Equivalent for a single CA3 sharp wave – ripple showing the variables estimated for individual events.

(C) i. Fast frequency components were calculated on detected events with significant peaks between 60 and 250 Hz in the power spectrum (more than 5 SD above baseline). ii. The frequency of significant ripple oscillations was taken as the inverse of the interval (Δ) between the central and adjacent peak in the autocorrelation function. iii. Ripple amplitude was estimated from the maximum peak of the Hilbert transform. iv. Ripple duration was estimated from the RMS trace as the period which the RMS remained 1 SD above baseline (Csicsvari et al., 1999).

(D) i. Unfiltered trace of sharp wave – ripples. ii. Traces were lowpass-filtered at 20 Hz and sharp waves were detected as events of more than 2 SD above baseline (black triangles). iii. Sharp wave – ripples *in vivo* are commonly detected as voltage deflections >7 SD above baseline in the RMS of 80 – 250 Hz bandpass-filtered traces (Csicsvari et al., 1999; O'Neill et al., 2006). Using this method of analysis on data shown in i. >7 SD leads to 75.2% detection (325/432 events, 5 slices), >6 SD leads to 97.2% detection (420/432 events, 5 slices). Red triangles are events detected by method in ii., but not with method in iii using >7 SD threshold.

The amplitude of the sharp wave was taken as the difference in voltage between the maximum amplitude of the sharp wave and the baseline.

The inter-burst interval was taken as the time between the maximum amplitudes of two detected sharp waves.

The rise time was taken as either the time between the baseline and 50% of the maximum amplitude of the sharp wave or the time between 20% of the maximum amplitude and 80% of the maximum amplitude of the sharp wave.

The decay time was taken as the time between the maximum amplitude of the sharp wave and 50% of the maximum amplitude going towards baseline.

Sharp wave detection *in vivo*

Sharp wave – ripples *in vivo* are commonly detected from the ripple component as events $>2-7$ SD above the root mean square (RMS) of the 80 – 250 Hz bandpass-filtered signal (Csicsvari et al., 1999). I investigated if this detection method would alter the number of detected events. A threshold of >6 SD detected 97.2% of events as detected by using the 2 SD amplitude threshold. A threshold of >7 SD detected 75.2% of events as detected using the 2 SD amplitude threshold (Figure 2.3).

Estimating spatial extent of sharp waves in interface conditions

The spatial extent of sharp waves in interface conditions was estimated by calculating the cross correlation of detected events (grouped in 50 ms bins) from pairs of electrodes placed in the CA3c and CA3a, CA3c and CA3b and CA3b and CA3a of the stratum pyramidale.

Estimating spatial extent of sharp waves in submerged conditions

The spatial extent of sharp waves in submerged conditions was estimated from the electrodes along the pyramidal cell layer with a deflection of more than 1 SD above the baseline value. Baseline was taken as the average voltage over all 62 - 64 electrodes of the multi-electrode array.

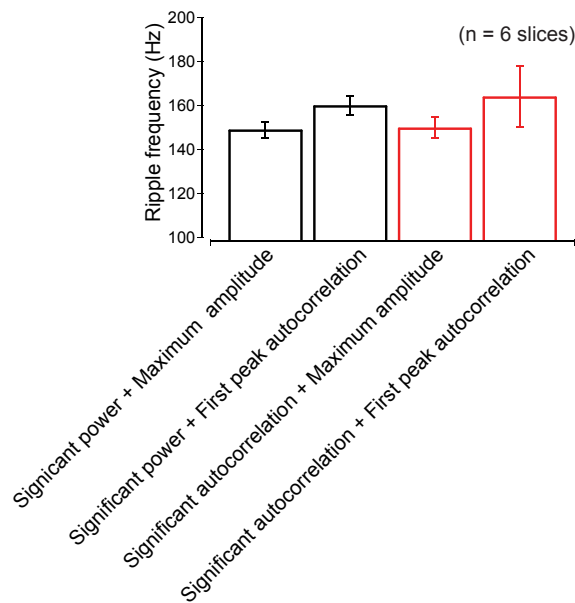


Figure 2.4 Estimated ripple frequency is not dependent on detection method

Four different methods were used to estimate the ripple frequency of detected events using traces filtered 60 and 250 Hz.

A significant ripple was either detected as a peak in the power spectrum (between 60 – 250 Hz) of more than 5 S.D. above baseline, or as a significant peak (not central but subsequent peak) in the autocorrelation of more than 5 S.D. above baseline.

The ripple frequency was then estimated from the inverse of the interval between the central and first subsequent or maximum subsequent peak in the autocorrelation function.

Estimating frequency of ripples

Fast frequency components were determined from detected events with significant peaks in the power spectrum between 60 and 250 Hz of more than 5 SD above baseline, with subsequent frequency of significant events estimated from the interval between the central and maximum of the adjacent peak of the autocorrelation (see Figure 2.3).

To investigate if this method of analysis had a significant influence on the estimation of ripple frequency three other methods were used as well. Firstly, instead of estimating the frequency from the interval between the central and maximum of adjacent peak of the autocorrelation the frequency was estimated from the interval between the central and first adjacent peak of the autocorrelation. Secondly, fast frequency components were determined from detected events with significant second peak in the autocorrelation function more than 5 SD above baseline with subsequent frequency of significant events estimated from the interval between the central and maximum of the adjacent peak of the autocorrelation. Lastly, fast frequency components were determined from detected events with a significant second peak in the autocorrelation function more than 5 SD above baseline with subsequent frequency of significant events estimated from the interval between the central and first adjacent peak of the autocorrelation.

No significant difference in ripple frequency was found between the different methods ($p > 0.05$; paired-sample t test; Figure 2.4).

Estimation of ripple amplitude

Ripple amplitude was estimated as the maximum amplitude of the Hilbert transform on 80–250 Hz bandpass filtered traces (Foffani *et al.*, 2007).

Estimation of ripple duration

Ripple duration was estimated from the RMS of 80–250 Hz bandpass filtered events as the period during which the RMS remained 1 SD above baseline (Csicsvari *et al.*, 1999) (see Figure 2.3, page 34).

2-D current-source density

2-D current-source density (CSD) analysis was performed on single sharp wave – ripples as previously described (Shimono *et al.*, 2000). In brief, after lowpass filtering at 100 Hz of all 64 traces acquired with the planar multi-electrode array, the data were spatially smoothed by a 3 x 3-weighted average kernel (0 1/8 0, 1/8 1/2 1/8, and 0 1/8 0), and the result was convolved with a 3 x 3 Laplacian kernel (0 1 0, 1 24 1, and 0 1 0) to produce a discrete approximation of the second spatial derivative. The medium was considered ohmic with a homogeneous conductance. The full correlation matrix was computed for all 64 channels for the duration of the sharp wave – ripple including ~10 ms on either side. Current-versus-time plots in the slice were obtained by computing the 8 x 8 current source densities for each time step and calculating the value at the desired location via bilinear interpolation. CSD plots are shown using cold and warm colours for sinks and sources, respectively.

Wavelet transform

Wavelet analysis (Le Van Quyen *et al.*, 2001) was used to examine the magnitude and frequency of the field potential oscillation of detected sharp wave – ripples in 0.1 Hz highpass filtered traces. The Morlet wavelet function was used as the basis for the wavelet transform,

$$\Psi_0(\eta) = \pi^{-\frac{1}{4}} e^{i\omega_0\eta} e^{-\frac{\eta^2}{2}}$$

where η is the non-dimensional time parameter, and ω_0 is the non-dimensional frequency, set equal to 6 to ensure that the mean of the basis functions was non-zero, and that the functions

were localised. The wavelet transform of the field recording was examined between 10 and 250 Hz. As the power of the sharp wave component is so much larger than the ripple or high frequency oscillatory component the log of the wavelet transform was taken.

Spike detection

Spikes were detected from cell-attached recordings as voltage deflections of more than 5 SD above baseline. Spikes and sharp waves were deemed correlated if the spike occurred within the duration of the sharp wave event. Spike times were binned in 5 ms bins and the total number of spikes for each bin plotted relative to the maximum peak of the detected sharp wave. Single cell plots were averaged to obtain the spike probability plot.

2.8.2 Intracellular recording

EPSP/C and IPSP/C detection

For intracellular event detection, recordings were filtered between 0.1 and 20 Hz and intracellular events were detected as voltage or current fluctuations of more than 1 SD above baseline, with a duration for voltage fluctuation of more than 20 ms and for current fluctuation more than 8 ms. A sharp wave and intracellular event were deemed correlated if they occurred within 5 ms of each other.

The intracellular response of a pyramidal neuron (EPSP, IPSP, Biphasic and no response) was calculated by dividing the number of responses to a sharp wave at a specific distance (0, 150, 300, 450, and 600 μm) with the total number of sharp waves originating at that distance. Pyramidal neurons were assigned the location of their nearest probe on the multi-electrode array, corresponding to a probe at the stratum pyramidale. Sharp wave maximum amplitudes were detected from specific probes. The distance between the pyramidal neuron location and a sharp wave location was estimated by taking 150 μm steps left or right until the corresponding sharp wave probe, or the column of the multi-electrode array to which this probe belonged, was reached.

Estimation of charge transfer

Charge transfer was estimated as the integral of detected intracellular events, within or outside sharp waves.

Estimation of membrane voltage prior to sharp wave initiation

The membrane voltage prior to sharp wave initiation was determined for individual neurons by averaging the voltage fluctuations 300 ms prior to a sharp wave for all detected sharp waves. Whole-cell current-clamp traces bandpass filtered between 0.1 and 20 Hz were used. The traces were then sorted according to class of neuron and averaged.

Estimation of the frequency of intracellular events

Frequency of intracellular events was calculated by dividing the number of detected events by the time interval over which these events were detected. For Figure 6.9C (page 108) this was done in 100 ms bins and overall frequency was estimated as the average frequency of a 400 ms segment centred between the start and end of a time interval. 'Baseline' was the time between the end of a sharp wave and start of a subsequent sharp wave. '1st interval' was the time between the end of stimulation until the start of the subsequent sharp wave. '2nd interval' and '3rd interval' was the time between the end of a sharp wave until the start of the subsequent sharp wave. For Figure 6.9B, D and F total time was used. 'Before' represents the time between the end of a sharp wave until the start of stimulation. 'During' was the time of stimulation. 'After' was the time between the end of stimulation until the start of the subsequent sharp wave.

2.9 Statistics

All data are presented as means $\pm$ SEM, except where stated. Student's *t* tests, ANOVA, least-significant difference (LSD) post hoc test, and linear regression were done in SPSS 16.0 (* $p < 0.05$, ** $p < 0.01$).

2.10 Drugs and Chemicals

The following is a list of the reagents and compounds used with their supplier and product code, where available.

2.10.1 Anaesthetic

Isoflurane (Animalcare Ltd.)

2.10.2 ACSF compounds

Carbogen gas (95% CO₂/5% O₂) (BOC Gases)
 D-(+)-glucose (BDH Chemicals)
 KCl (BDH Chemicals)
 MgCl₂ (BDH Chemicals)
 MgSO₄ * 7H₂O (Sigma-Aldrich)
 NaCl (BDH Chemicals)
 CaCl₂ * 2H₂O (Sigma-Aldrich)
 KH₂PO₄ * 2H₂O (BDH Chemicals)
 NaHCO₃ (BDH Chemicals)

2.10.3 Intracellular fluid compounds

Current-clamp solution

Kgluconate (2,3,4,5,6-Pentahydroxycaproic acid potassium salt) (Sigma-Aldrich)
 HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Sigma-Aldrich)
 ATP-Mg (Sigma-Aldrich)
 Na-GTP (Guanosine 5' triphosphate) (Sigma-Aldrich)
 NaCl (BDH Chemicals)
 Biocytin (Sigma-Aldrich)

Voltage-clamp solution

CsOH (Sigma-Aldrich)
 Gluconic acid (45% - 50%) (Sigma-Aldrich)
 HEPES (Sigma-Aldrich)
 ATP-Mg (Sigma-Aldrich)
 Na-GTP (Sigma-Aldrich)
 NaCl (BDH Chemicals)
 QX-314(Lidocaine N-ethyl bromide; N-(2,6-Dimethylphenyl carbamoylmethyl)tri-thylammonium bromide) (Sigma-Aldrich)
 Biocytin (Sigma-Aldrich)

2.10.4 Pharmacological compounds

All drugs were obtained from Tocris Biosciences (Bristol) and Sigma-Aldrich (Poole, Dorset, UK), except NVP AAM077, which was a kind gift of Novartis.

DMSO

Dimethyl sulfoxide

SR 95531 hydrobromide ‘Gabazine’

6-imino-3-(4-methoxyphenyl)-1(6 H) - pyrida-zinebutanoic acid hydrobromide

NBQX

1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide

GYKI 52466

1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepinehydrochloride

Philanthrotoxin-433 tris(trifluoroacetate)

(S)-N-[4-[[[3-[(3-aminopropyl)amino]propyl]amino]butyl]-4-hydroxy- α -(1-oxobutyl)amino]benzenepropanamide tris(trifluoroacetate)

D-AP5

D (-)-2-Amino-5-phosphonopentanoic acid

NVP AAM077

(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid

Ro 256981 hydrochloride

(aR,bS)-a-(4-Hydroxyphenyl)-b-methyl-4-(phenylmethyl)-1 -piperidinepropanol hydrochloride

4-CGP

(S)-4-carboxyphenylglycine

CPCCOEt

7-(Hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester

MPEP hydrochloride

2-Methyl-6-(phenylethynyl)pyridine hydrochloride

CGP 52432

3-[[[(3,4-Dichlorophenyl)methyl]amino]propyl] diethoxymethyl)phosphinic acid

Pentobarbital

5-Ethyl-5-(1-methylbutyl)-2,4,6(1H,3H,5H)-pyrimidinetrione

Diazepam

7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one

Zolpidem

N,N,6-trimethyl-2-(4-methylphenyl)-imidazo(1,2-a)pyridine-3-acetamide

L-655,708

11,12,13,13a-Tetrahydro-7-methoxy-9-oxo-9H-imidazo[1,5- a]pyrrolo[2,1- c][1,4]benzodiazepine-1-carboxylic acid, ethyl ester

Carbenoxolone

(3 β)-3-[(3-carboxypropanoyl)oxy]-11-oxoolean-12-en-30-oic acid

Mefloquine

2,8-bis(trifluoromethyl)quinolin-4-yl]-(2-piperidyl)methanol

Cycloheximide

4-[(2*R*)-2-[(1*S*,3*S*,5*S*)-3,5-Dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2,6-dione

Actinomycin D

2-amino-*N,N'*- bis[(6*S*,9*R*,10*S*,13*R*,18*aS*)- 6,13-diisopropyl- 2,5,9-trimethyl- 1,4,7,11,14-pentaoxohexadecahydro- 1*H*-pyrrolo[2,1-*i*] [1,4,7,10,13] oxatetraazacyclohexadecin- 10-yl]- 4,6-dimethyl- 3-oxo- 3*H*-phenoxazine- 1,9-dicarboxamide

2.10.5 Anatomical identification

Avidin-biotinylated horseradish peroxidase complex reaction	(Vector Laboratories)
Hydrogen peroxide	(Sigma-Aldrich)
Nickel-intensified 3,3'-diaminobenzidine (Ni-DAB)	(Sigma-Aldrich)
Paraformaldehyde	(Sigma-Aldrich)
Phosphate buffered saline	(Sigma-Aldrich)
Glycerol	(Sigma-Aldrich)

Sharp wave – ripples *in vitro*

3.1 Introduction

Sharp waves (0.1 – 3 Hz) and associated ripples (~200 Hz), which occur during phases of rest and slow-wave sleep, can be observed in the hippocampal electroencephalogram (EEG) in freely moving rats (O'Keefe & Nadel, 1978; Buzsaki *et al.*, 1983; Buzsaki, 1986). Although synaptic excitation, synaptic inhibition and electrical coupling have all been implicated (Maier *et al.*, 2002; Maier *et al.*, 2003; Nimmrich *et al.*, 2005), their relative importance, the details of their involvement as well as their role in the initiation of sharp wave – ripple are unknown.

I set out to study the cellular mechanisms underlying the initiation of these population bursts using a hippocampal slice model which was recently published and which produces this activity spontaneously (Kubota *et al.*, 2003). Firstly, the exact conditions for the emergence of these population bursts were explored. Secondly, both the temporal and spatial characteristics of sharp wave – ripples *in vitro* were analyzed and compared to those observed *in vivo* to investigate if this model is relevant to address the aims. Lastly, the conditions were explored under which this activity pattern could be obtained in submerged slices, which would enable a more detailed investigation of this activity pattern using e.g. IR-DIC imaging and whole-cell patch-clamp recording.

3.2 Sharp wave – ripples in interface conditions

3.2.1 Conditions for sharp wave – ripples

The initial model developed by Kubota *et al.* (2003) consisted of transverse hippocampal slices kept in an interface-style recording chamber in altered ACSF (4.25 mM $[K^+]$, 1 mM $[Mg^{2+}]$ and 3 mM $[Ca^{2+}]$). In an interface-style recording chamber the slices are kept at the interface between ACSF and a humidified gas mixture composed of 95% O_2 / 5% CO_2 . Under these conditions transverse hippocampal slices were shown to produce spontaneous sharp wave – ripple population bursts in area CA3.

I started out investigating the conditions under which sharp waves *in vitro* could be obtained in further detail to find the optimal conditions to use this model. Firstly, it was investigated if only a subset of slices taken from the rat hippocampus show sharp wave – ripple activity. The initial slice model (Kubota *et al.*, 2003) was established using 400 μm thick transverse slices, taken only from the ventral region of the hippocampus, which was suggested to retain as much of the recurrent network of CA3 as possible (Ishizuka *et al.*, 1990). It was important to determine if, and to what extent, slices taken from other parts of the hippocampus also showed sharp waves. Firstly, this would enable the use of many more slices from one animal, secondly there are reported differences in functionality between septal and temporal hippocampus which would impact on our findings in case only a particular region would show sharp waves (Moser & Moser, 1998; Witter *et al.*, 2000), and, finally, *in vivo* recordings are generally made from dorsal hippocampus (Andersen, 2007). I found that slices taken from the entire septal – temporal (dorsal – ventral) axis of the rat hippocampus showed spontaneous sharp wave – ripples (Figure 3.1A). However, there was a quantitative difference. The more ventral slices tended to generate larger amplitude sharp waves compared to those taken from the dorsal pole, whereas the incidence stayed constant along the entire septal – temporal axis (Figure 3.1A).



(B) No sharp wave – ripples are generated in slices incubated in standard ACSF (n = 25). However, slices incubated in modified ACSF exhibit spontaneous sharp wave – ripples (n = 5). Incubating slices in modified ACSF containing either D-AP5 (50 μ M; n = 7), 4-CPG (0.5 mM; n = 16), cycloheximide (50 μ M; n = 8) or actinomycin D (50 μ M; n = 12) did not block the emergence of sharp wave – ripples.

These experiments were done in interface conditions.

Secondly, the ionic conditions under which spontaneous sharp wave – ripples were generated were explored in further detail. Superfusing slices with standard ACSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 10 glucose) did not lead to sharp wave – ripple generation (Figure 3.1B). I confirmed that the altered ACSF as published by Kubota *et al.* (2003) (in mM: 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 1 MgSO₄, 3 CaCl₂, 26 NaHCO₃, and 10 glucose) did support the generation of sharp waves (Figure 3.1B). More detailed investigation into the contribution of the altered [K⁺], [Mg²⁺] and [Ca²⁺] separately has not been done for slices kept in interface-style recording chamber, but was investigated in slices in submerged-style recording chambers (see Figure 3.10, page 60).

To investigate if the altered ionic conditions resulted in NMDA receptor-mediated plasticity or excitability changes, which could be necessary for sharp wave – ripples to emerge (Maier *et al.*, 2003), an antagonist at NMDA receptors, D-AP5 (50 μM), was applied to the slices. Incubating slices, immediately after preparation, in ACSF containing D-AP5 (50 μM) did not block the emergence of sharp wave – ripples (incidence: 1.8 ± 0.1 Hz amplitude: 129 ± 11.1 μV; Figure 3.1B). This concentration was sufficient to block the NMDA receptors as LTP induction by tetanic or theta burst stimulation at the Schaffer collateral – CA1 pyramidal neuron synapses was blocked (Figure 3.1C). Interestingly, incubating with D-AP5 (50 μM) did not lead to an increase in sharp wave amplitude as seen during wash-in of AP5 (Behrens *et al.*, 2005; Colgin *et al.*, 2005; Figure 4.1, page 70).

Besides NMDA receptor-mediated changes in plasticity or excitability it has been suggested that developmental synaptic rewiring (Le Be & Markram, 2006) and hippocampal synaptic plasticity (Bikbaev *et al.*, 2008) can be dependent on the metabotropic glutamate receptor 5 subtype (mGluR5) of the group I metabotropic glutamate receptor family. To investigate this, slices were incubated immediately after slicing with the group I metabotropic glutamate receptor antagonist 4-CPG (0.5 mM). This did not block the emergence of sharp wave – ripples (incidence: 2.0 ± 0.2 Hz amplitude: 87 ± 11.5 μV; Figure 3.1B).

As plasticity changes have been suggested to be dependent on translation (protein synthesis) (Costa-Mattioli *et al.*, 2009) and/or transcription (Greer & Greenberg, 2008), inhibitors of these processes were applied as well. Incubation with either the protein synthesis

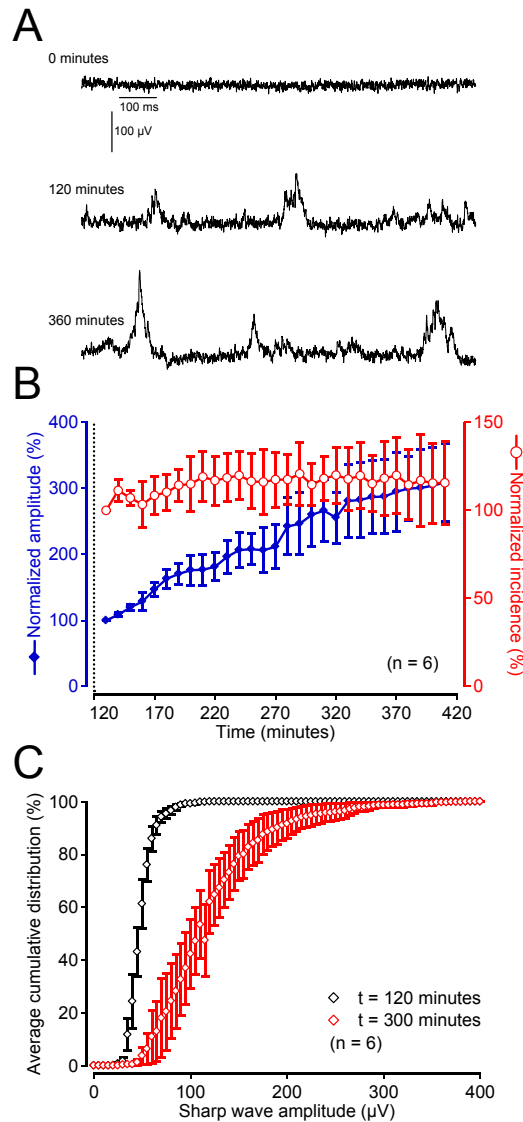


Figure 3.2 Sharp wave amplitude increases over time

(A) Transverse hippocampal slices (400 μ m), superfused with modified ACSF exhibit spontaneous sharp wave – ripples, which appeared approximately 1 - 2 hours after slicing and persisted for more than 6 hours.

(B) Sharp wave amplitude increases over time, while the incidence stays constant.

(C) Cumulative distribution of sharp wave – ripple amplitude measured at t = 120 minutes (2 hours after slicing) and t = 300 minutes (5 hours after slicing).

inhibitor cycloheximide (50 μ M) (incidence: 1.2 ± 0.2 Hz amplitude: 189 ± 31.9 μ V; Figure 3.1B) or the inhibitor of DNA transcription actinomycin D (50 μ M) (incidence: 1.3 ± 0.1 Hz, amplitude: 200.4 ± 45.5 μ V; Figure 3.1B) did not inhibit sharp wave – ripples.

These combined results show that an altered ACSF is necessary for the initiation of sharp wave – ripples *in vitro* and furthermore suggests that sharp wave – ripple emergence does not depend on plasticity or excitability changes induced by either NMDA receptors, group I metabotropic glutamate receptors, protein synthesis or transcription.

3.2.2 Development of sharp wave – ripples

I noticed that the amplitude of the recorded sharp waves appeared larger after several hours compared to the start of the experiment. I therefore investigated the development of sharp waves over time. I found that it takes approximately 60 – 120 minutes after preparation for slices to show spontaneous sharp wave activity (Figure 3.2A). This time period is similar to the time it takes for a slice to recover normal synaptic transmission (Kirov & Harris, 1999; Fiala *et al.*, 2003; Kirov *et al.*, 2004). The average amplitude and incidence of sharp waves, detected within a one minute sweep, were quantified and plotted over time. There was a clear increase in sharp wave amplitude over time (from 100% of the normalized amplitude to $309.3 \pm 59.6\%$ at $t = 420$ minutes (7 hours) after slicing; $n = 6$) while the incidence remained constant (from 100% of the normalized incidence to $115.7 \pm 23.5\%$ at $t = 420$ minutes (7 hours) after slicing; $n = 6$). Plotting the cumulative distribution of the amplitude of the sharp waves 2 hours after the preparation and 5 hours into the recording shows a shift to the right corresponding to a 3.2x average linear increase (Figure 3.2C). This could be the result of flattening of the hippocampal slice over time, but other possibilities exist, which will be discussed in more detail later.

These results suggest that transverse hippocampal slices can exhibit sharp wave – ripple population burst activity for many hours.

3.2.3 Temporal characteristics of sharp wave – ripples

For an *in vitro* model to be useful in studying network activity as recorded in the intact brain it is important that the characteristics of the *in vitro* and *in vivo* recorded events are similar. Transverse hippocampal slices perfused with modified ACSF exhibit sharp waves and associated ripple activity in both the CA3 and CA1 area as well as dentate waves in the dentate gyrus (Kubota *et al.*, 2003a; Colgin *et al.*, 2004a) (Figure 3.3 A, B) similar to that seen *in vivo* (Buzsaki *et al.*, 1983; Bragin *et al.*, 1995).

Sharp wave – ripples have been suggested to be generated intrinsically in the recurrent network of CA3 with an incidence of 0.01 – 2 Hz (Buzsaki, 1986) and subsequently propagate

to CA1 (Csicsvari *et al.*, 2000). Sharp wave – ripples *in vitro* accurately reflect these *in vivo* properties. First, with a cut between the dentate gyrus and CA3 the amplitude of sharp wave – ripples in CA3 remained unchanged (from $119 \pm 17 \mu\text{V}$ to $113 \pm 15 \mu\text{V}$; independent-samples *t* test; $p > 0.05$; $n = 26$ and $n = 12$) and there was a slight but significant increase in incidence (from $1.6 \pm 0.1 \text{ Hz}$ to $1.9 \pm 0.1 \text{ Hz}$; independent-samples *t* test; $p < 0.05$). Secondly, a cut between CA3 and CA1 ceased all sharp wave – ripple activity in CA1, whereas those in CA3 were unaltered (from $119 \pm 17 \mu\text{V}$ to $92 \pm 9 \mu\text{V}$ and from $1.6 \pm 0.1 \text{ Hz}$ to $1.6 \pm 0.1 \text{ Hz}$;

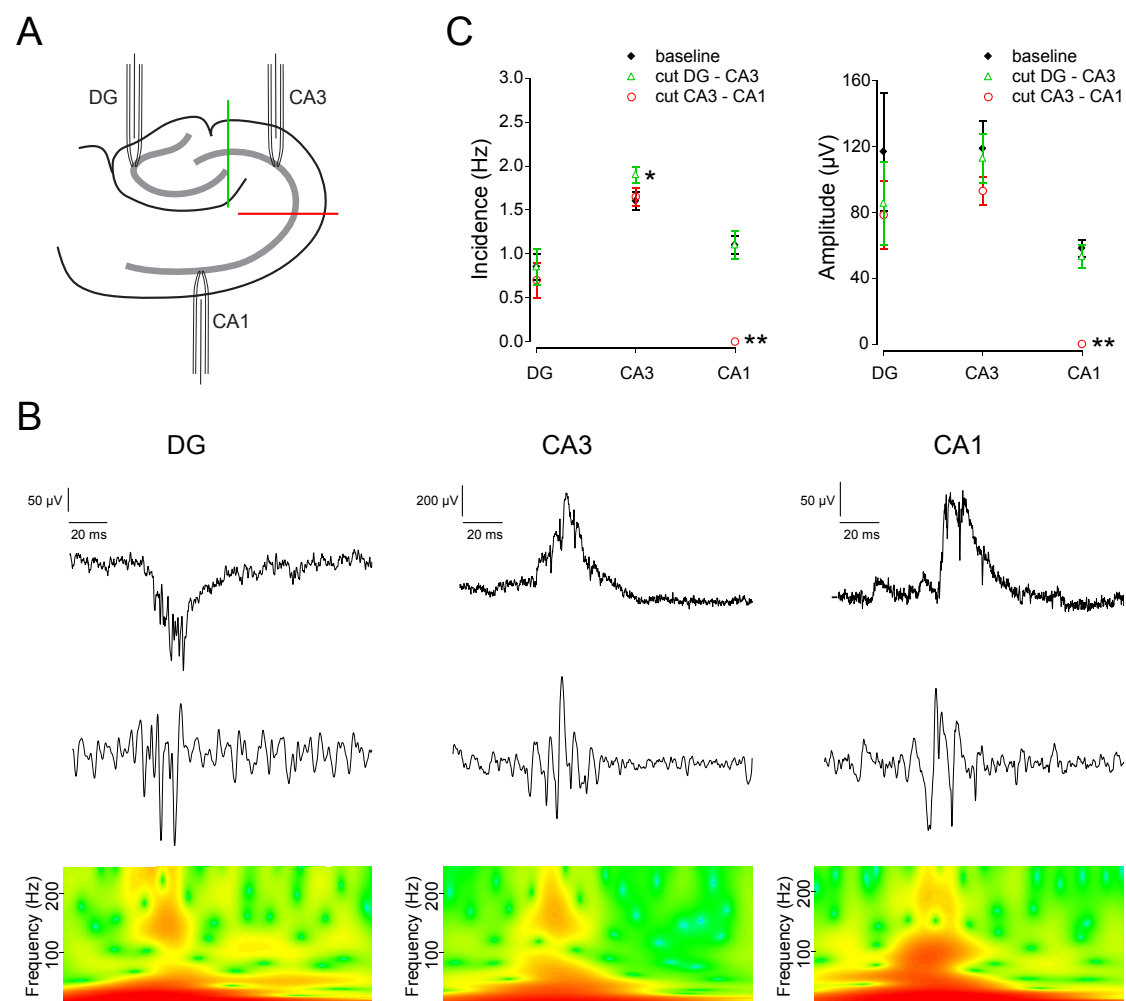


Figure 3.3 Sharp wave – ripples are independently generated in hippocampal CA3

(A) Diagram of a hippocampal slice showing the location of field recording electrodes in dentate gyrus (DG), CA3 and CA1. Colored lines represent the locations where axonal fibres were cut.

(B) Spontaneous network discharges were observed in the dentate gyrus (left), hippocampal CA3 (middle) and hippocampal CA1 (right). These population discharges (highpass filtered at 0.1 Hz; top), recorded in the principal cell body layers, were superimposed by high-frequency oscillations (bandpass filtered between 100 and 300 Hz; middle), with both components apparent in the wavelet transform (normalized Morlet wavelet; $w_0 = 6$; warmer colors representing increasing magnitude; bottom).

(C) A cut between CA3 and CA1 abolished all sharp wave – ripple activity in CA1 without affecting those in CA3. A cut between the dentate gyrus and CA3 did not alter the amplitude, but significantly increased the incidence of sharp wave – ripples in CA3.

independent-samples *t* test; $p > 0.05$; $n = 26$ and $n = 15$) (Figure 3.3 C).

A single CA3 sharp wave – ripple as recorded in the field can be divided in several temporal components; a baseline, a sharply rising phase from baseline, a peak of variable duration and a decay phase back to baseline. We estimated the amplitude (i), interburst interval (ii), rise time (iii), decay time (iv) and duration (v) of detected sharp waves (see Experimental Procedures) and plotted the distribution of these variables. I found that these variables are approximately normally distributed within a slice, but the mean varied between slices (Figure 3.4).

Dentate waves and CA3 and CA1 sharp wave – ripples *in vivo* have been shown to contain pronounced fast frequency components (O'Keefe & Nadel, 1978; Buzsaki, 1986;

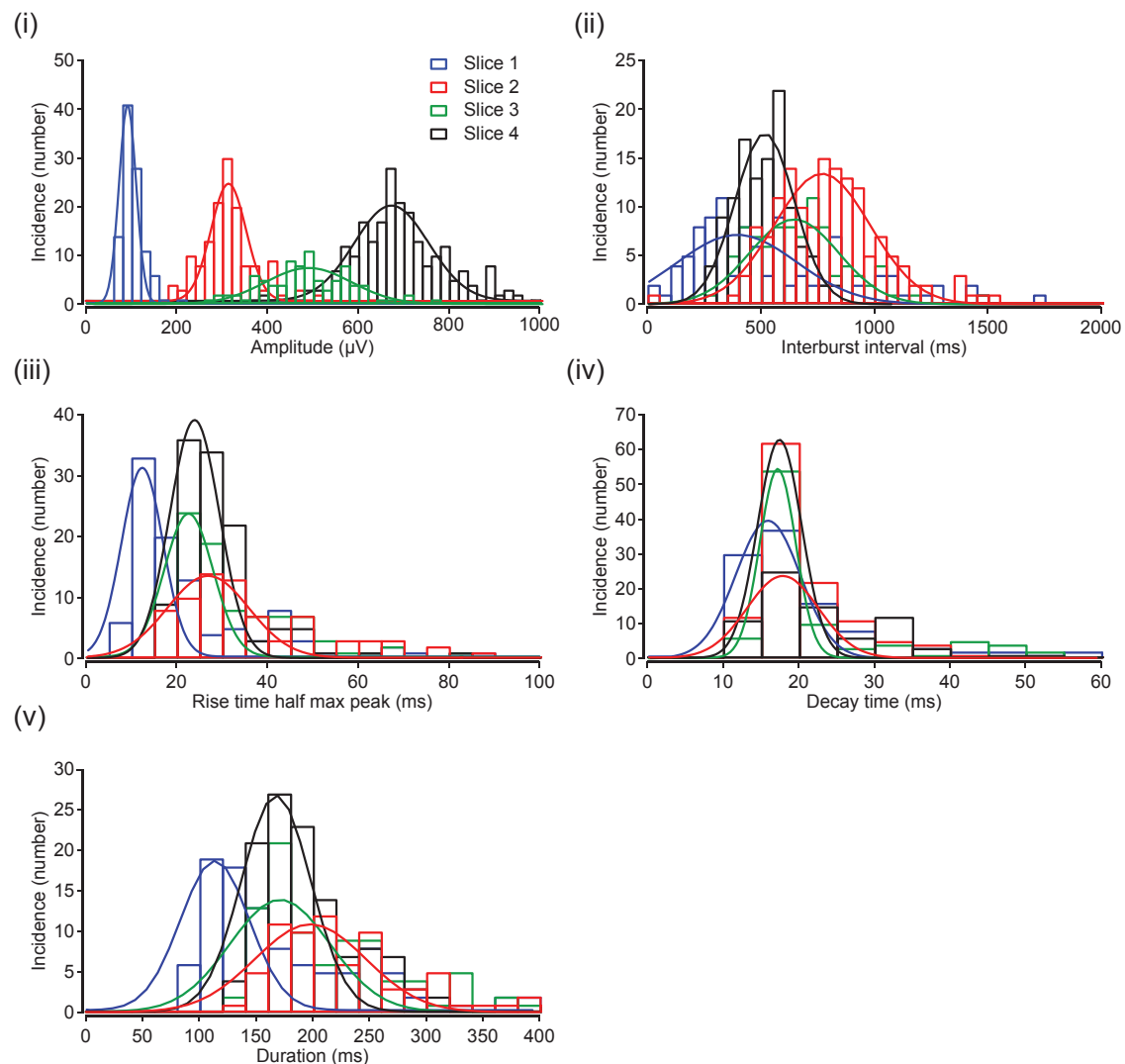


Figure 3.4 Distribution of CA3 sharp wave characteristics

Temporal characteristics of CA3 sharp waves *in vitro* are approximately normally distributed.

(i) Distribution of sharp wave amplitude (ii) Distribution of sharp wave incidence (iii) Distribution of sharp wave rise time (20% - 80%) (iv) Distribution of sharp wave decay time (v) Distribution of sharp wave duration. A gaussian curve was fitted to the histograms.

Bragin *et al.*, 1995). In agreement, both bandpass filtering (100 – 300Hz) and wavelet transform of the *in vitro* events revealed prominent fast frequency components with similar frequencies to those found *in vivo* (Figure 3.3B and Figure 3.5 for detailed analysis of CA3 ripples). Events with a significant peak in the power spectrum were used for analysis, with the ripple frequency estimated from the interval between the central and adjacent peak of the autocorrelation. Dentate waves and CA3 and CA1 sharp wave – ripples showed pronounced fast frequency components with an average frequency of 230 ± 3 Hz, 150 ± 18 Hz and 149 ± 8 Hz, respectively.

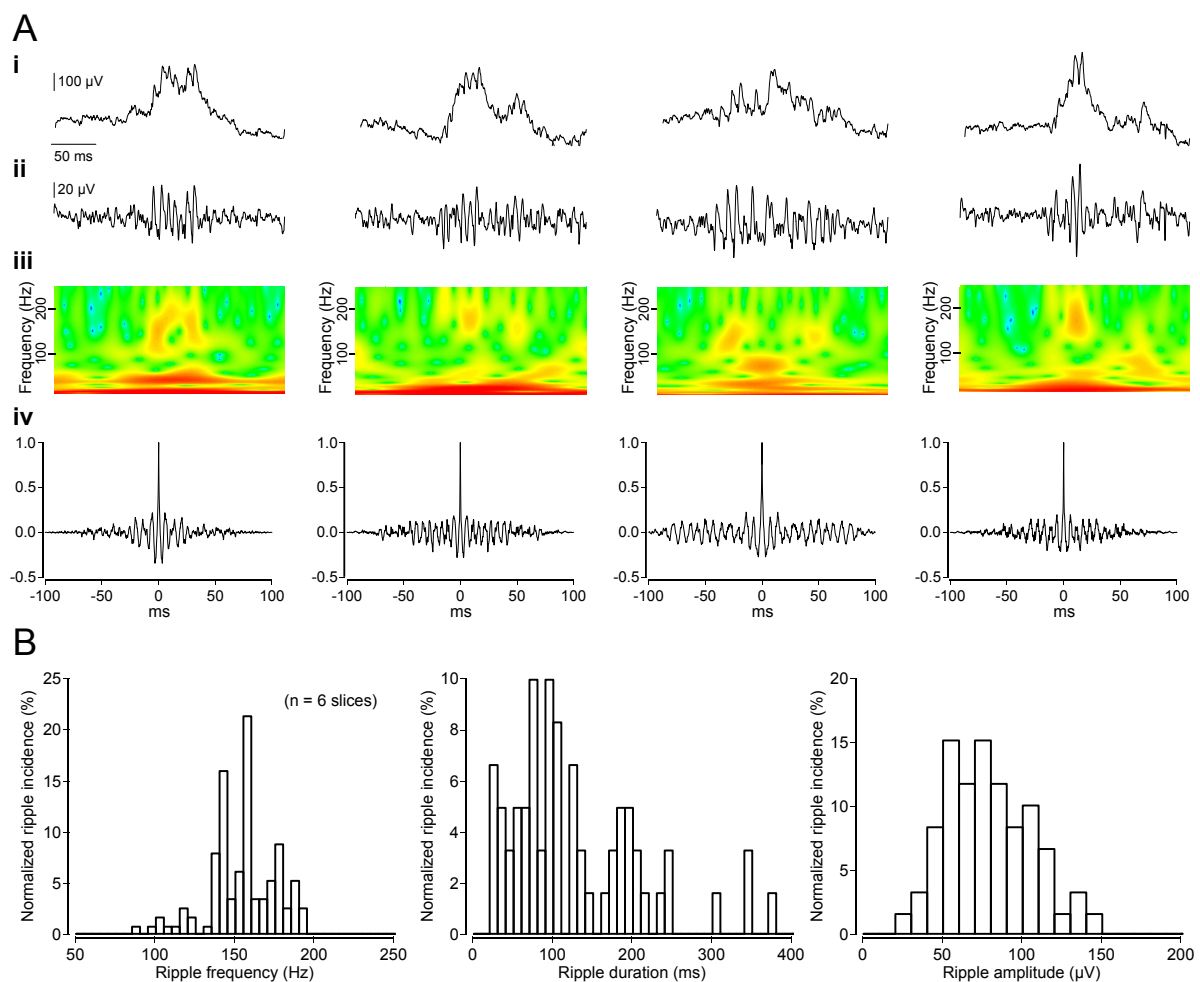


Figure 3.5 Variability in CA3 sharp wave – ripple characteristics

(A) Four individual examples of CA3 sharp wave – ripples *in vitro*. These population discharges (highpass filtered at 0.1 Hz) recorded in the principal cell body layers (i) were superimposed by high-frequency oscillations (as seen in the bandpass filtered trace between 100 and 300 Hz) (ii), with both burst and ripple components apparent in the wavelet transform (normalized Morlet wavelet; $w_0 = 6$; warmer colors representing increasing magnitude) (iii). The high frequency ripple was also apparent in the autocorrelation function (iv). Note the variability in duration of the sharp wave, duration of the ripple, as well as the different frequencies of the ripple components.

(B) Normalized distribution plots of ripple frequency, duration and amplitude.

I next investigated if the average amplitude of sharp waves was correlated with the average incidence of sharp waves, as it might be expected that larger amplitude discharges impose a limit on the incidence of these events. I did not find any significant correlation (Figure 3.6A). I also investigated if ripple frequency was correlated with the amplitude of the sharp wave, as it might be the case that a larger excitatory drive, mediated by activity of a larger group of pyramidal neurons, would drive an interneuron population to a higher firing rate and lead to a higher ripple frequency as seen in the field. I did not find any significant correlation (Figure 3.6B). Finally, I investigated if the temporal characteristics of sharp wave generation could be the result of vesicle recovery dynamics, as has been suggested for the generation of epileptiform bursts (Staley *et al.*, 1998). No correlation was seen between the duration of a sharp wave with the preceding inter-event interval ($r^2 = 0.03 \pm 0.05$, see Figure 3.6C). A correlation would be expected in systems where vesicle recovery processes

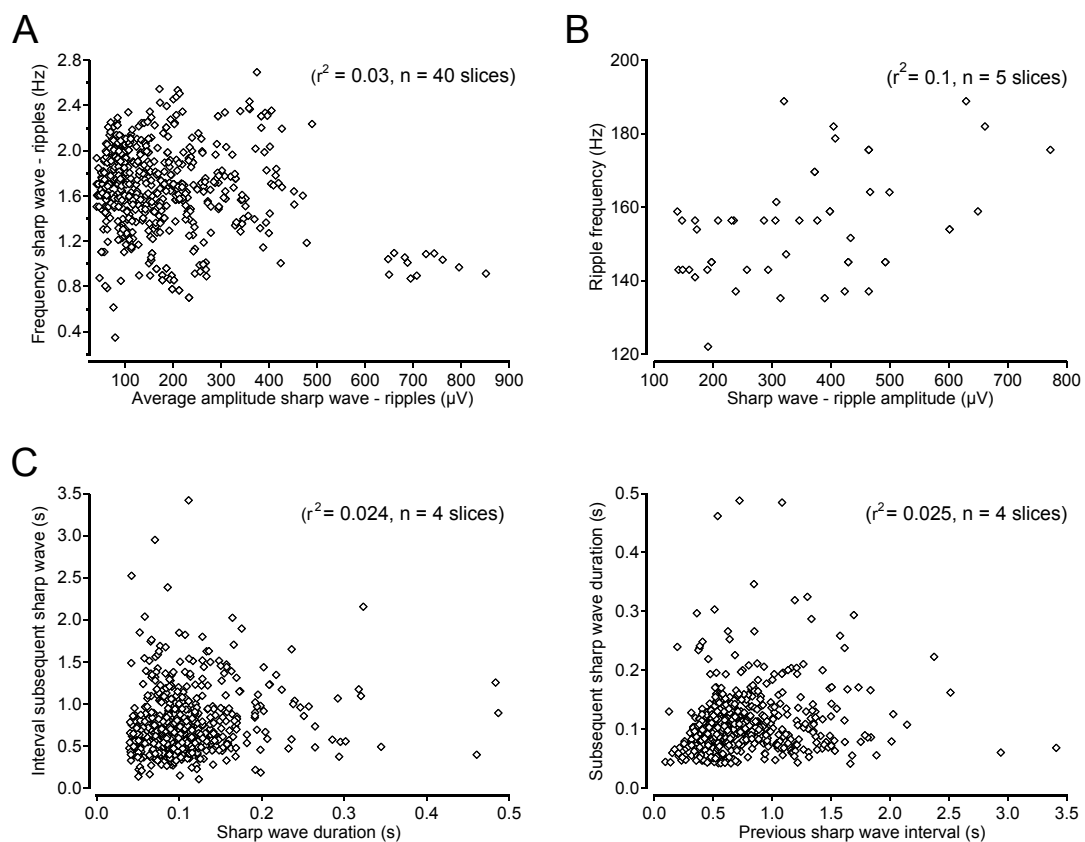


Figure 3.6 Correlations

(A) Average amplitude of sharp waves does not determine the frequency of sharp waves in an hippocampal slice.

(B) The amplitude of sharp waves is not correlated with the ripple frequency.

(C) Sharp wave initiation is not dominated by vesicle recovery processes. No correlation was seen between previous sharp wave interval and subsequent sharp wave duration ($r^2 = 0.025$; $n = 4$ slices) or between sharp wave duration and preceding inter-burst interval ($r^2 = 0.024$; $n = 4$ slices).

dominate initiation processes of population events (Staley *et al.*, 1998; Menendez de la Prida *et al.*, 2006).

Together, these results suggest that hippocampal slices can generate sharp wave – ripple population burst activity with similar characteristics to those observed *in vivo*.

3.3 Sharp wave – ripples in submerged conditions

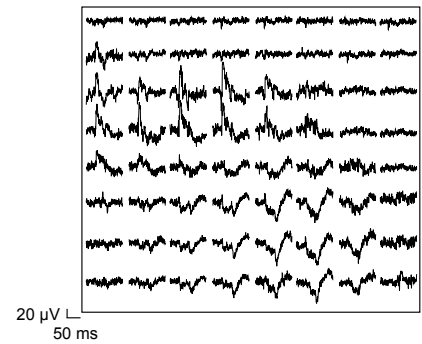
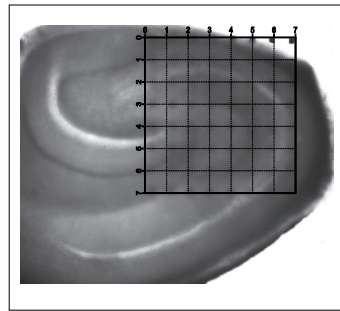
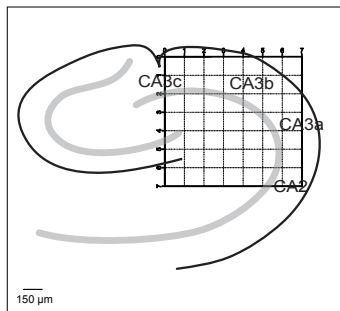
3.3.1 Conditions for sharp wave – ripples

Maintaining sharp wave – ripples in submerged conditions would offer important experimental advantages over interface conditions, including faster exchange of pharmacological agents, visually-guided patch-clamp recordings and advanced imaging techniques. I therefore investigated the conditions necessary to obtain spontaneous sharp wave – ripple activity in submerged conditions.

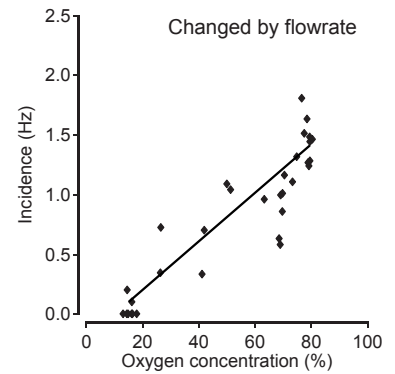
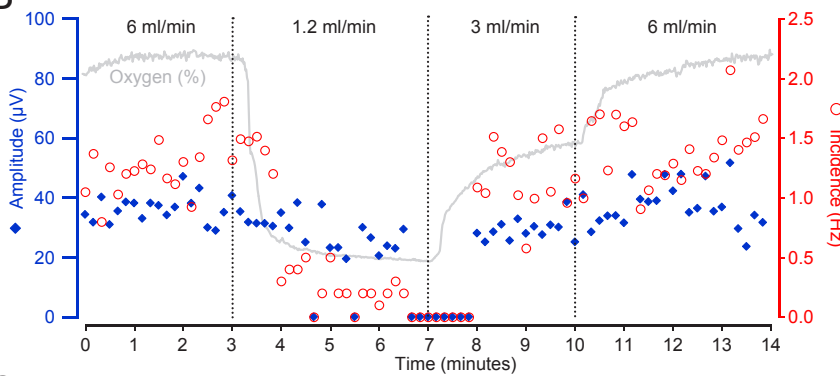
To investigate this, hippocampal slices were mounted on planar multi-electrode arrays to measure field sharp wave – ripples. I found that a high flow rate of 6 ml/min enabled spontaneous sharp waves in submerged conditions with similar incidence to interface conditions (interface: 1.5 ± 0.2 Hz, submerged 1.4 ± 0.1 Hz; independent-samples *t* test; $p > 0.05$; $n = 26$; Figure 3.7A, D). Sharp wave amplitude was significantly lower in submerged conditions, however (interface: 120.0 ± 3.0 μ V, submerged 31.3 ± 0.8 μ V; independent-samples *t* test; $p < 0.05$; $n = 250$; Figure 3.7A, D). This reduction in amplitude between interface recordings and submerged recordings from planar multi-electrode arrays was also seen for carbachol-induced gamma oscillations (Hajos *et al.*, 2004).

It was hypothesized that oxygen concentration might be the limiting factor for the emergence of sharp wave – ripples in submerged conditions. To investigate the relation between flow rate, oxygen saturation and sharp wave – ripple activity, both oxygen saturation and the incidence and amplitude of sharp waves were monitored while altering the flow rate (Figure 3.7B, left). Reducing the superfusion rate from 6 ml/min to 1.2 ml/min caused a rapid reduction in oxygen concentration as well as incidence of sharp wave – ripples, and there was a strong correlation between the incidence of sharp wave – ripples and the measured oxygen

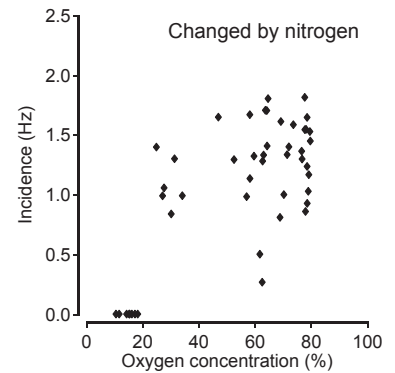
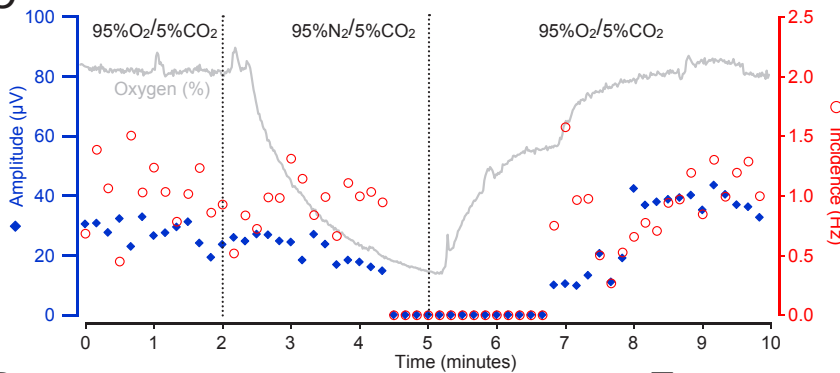
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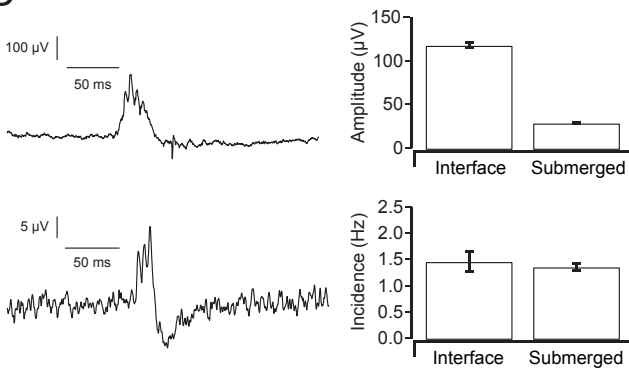
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D



E

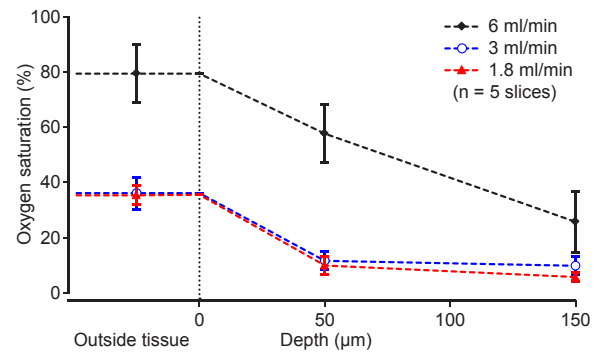


Figure 3.7 Importance of oxygen levels for sharp wave – ripples in submerged slices

(A) Position of a hippocampal slice on an 8 x 8 planar multi-electrode array, shown with schematic of the anatomy (left) and photograph of the slice (middle). A corresponding recording of a single sharp wave – ripple is shown on the right.

(B) Left: Effect of flow rate on oxygen saturation and incidence of recorded sharp wave – ripples. Grey trace, oxygen saturation (%) during experiment; Red circles, incidence of sharp wave – ripples (Hz); Blue circles, amplitude of sharp wave – ripples (μV). Note the reduction of the incidence of sharp wave – ripples with lower oxygen saturation. We estimated the values of both the average amplitude and average incidence of sharp wave – ripples at flow rates of 6 ml/min, 3 ml/min and 1.8 ml/min by averaging the last two measurements at each flow rate. This analysis led to an estimate for the average amplitude of $28.0 \pm 7.4 \mu\text{V}$, $27.0 \pm 8.2 \mu\text{V}$ and $23.3 \pm 6.3 \mu\text{V}$, respectively. The average amplitude was not significantly different with different perfusion speeds ($p > 0.05$, $n = 4$ slices). This analysis led to an estimate for the average incidence of $1.1 \pm 0.1 \text{ Hz}$, $1.3 \pm 0.1 \text{ Hz}$ and $0.4 \pm 0.14 \text{ Hz}$. The average incidence of sharp wave – ripples at 1.8 ml/min is significantly lower than either at 6 ml/min and 3 ml/min (both $p < 0.01$, Student t test, $n = 4$ slices). The average incidence is not significantly different between 6 ml/min and 3 ml/min ($p > 0.05$, t test, $n = 4$ slices). Right: Plot of incidence of sharp wave – ripples against oxygen saturation modified by reduced flow rate ($n = 4$ slices; 5-6 data points per slice). Least-squares line fit superimposed.

(C) Left: Superfusion of the slice with ACSF bubbled with 95% N_2 / 5% CO_2 abolishes sharp wave – ripples. Grey trace, oxygen saturation (%) during experiment; Red circles, incidence of sharp wave – ripples (Hz); Blue circles, amplitude of sharp wave – ripples (μV). We estimated the values of both the average amplitude and average incidence of sharp wave – ripples by averaging the last two measurements in 95% O_2 / 5% CO_2 and 95% N_2 / 5% CO_2 bubbled ACSF. This analysis led to an estimate of the average amplitude of $17.5 \pm 3.1 \mu\text{V}$ and $16.3 \pm 2.4 \mu\text{V}$ respectively which does not differ significantly ($p > 0.05$, $n = 4$ slices). This analysis led to an estimate of the average incidence of $1.3 \pm 0.1 \text{ Hz}$ and $0.3 \pm 0.2 \text{ Hz}$ which differs significantly ($p < 0.01$, t test, $n = 4$ slices). Right: Plot of incidence of sharp wave – ripples against oxygen saturation modified by bubbling with 95% N_2 / 5% CO_2 ($n = 4$ slices; 5-6 data points per slice). Tygon tubing with low permeability for O_2 was used in these experiments.

(D) Amplitude of sharp wave – ripples decreases in submerged conditions whereas incidence is the same.

(E) Recording of oxygen concentration at several depths in a hippocampal slice using carbon-fibre voltammetry.

saturation ($R = 0.92$, $n = 4$ slices; Figure 3.7B, right). To test whether oxygen saturation was a causal factor, the experiment was repeated at a constant high flow rate while the oxygen content of the superfusion solution was altered by bubbling it with a mixture of 95% N_2 / 5% CO_2 . Again, I observed a rapid decrease in oxygen saturation of the ACSF accompanied by a reduction in the incidence of sharp wave – ripples ($n = 4$ slices; Figure 3.7C).

As the oxygen saturation within the tissue differs from that of the superfusate (Foster *et al.*, 2005), I also measured the oxygen saturation above the slice and within the slice at 50 μm and 150 μm depth using carbon fibre voltammetry at flowrates of 6, 3 and 1.8 ml/min ($n = 5$ slices; Fig. 3.7E). Measurement of oxygen level immediately above the tissue showed a reduction in oxygen saturation from $79.3 \pm 10.6\%$ at 6 ml/min to $36.1 \pm 5.8\%$ and $35.5 \pm 3.5\%$ at 3 ml/min and 1.8 ml/min, respectively. Oxygen saturation decreased steeply between 50 and 150 μm inside the tissue at 6 ml/min flow rate (Figure 3.7E). At 150 μm depth within the slice the oxygen saturation was slightly hyperoxic ($25.7 \pm 11\%$) at 6 ml/min flow rate compared to air, whereas this dropped to a hypoxic conditions (9.8 ± 3.2 and $5.7 \pm 1.8\%$) at

3 and 1.8 ml/min flow rate.

These results show that it is possible to obtain sharp wave – ripples in submerged conditions. Furthermore, they suggest that a high laminar flow rate (3.9 – 6 ml/min) enables stable sharp wave – ripple activity in submerged hippocampal slices by providing sufficient oxygen supply.

3.3.2 Spatial characteristics of sharp wave – ripples

Sharp waves *in vivo* are predominantly recorded in CA1 region of the hippocampus and these recordings have shown that CA1 regions up to 4 mm apart are synchronously active during sharp wave – ripples *in vivo* (Buzsaki, 1992; Chrobak & Buzsaki, 1996; Ylinen *et al.*, 1995). Sharp waves as recorded in CA1 are thought to reflect the synchronous firing of a population of CA3 pyramidal neurons (Buzsaki *et al.*, 1986), which is preferentially initiated in CA3a/b before spreading to CA3c and CA1 (Csicsvari *et al.*, 2000). Although a strong temporal correlation between CA3 and CA1 activity has been shown (Csicsvari *et al.*, 2000; Maier *et al.*, 2003; Both *et al.*, 2008), the spatial extent of synchronization within CA3 is unknown. Now that it was possible to obtain stable sharp wave – ripples in submerged conditions, I decided to investigate the spatial characteristics of CA3 sharp waves by recording the events from hippocampal slices mounted on 8 x 8 planar multi-electrode arrays with 150 μ m spacing (Figure 3.8A, n = 36 slices from 26 animals). A high superfusion rate (6 ml/min) enabled the generation of sharp waves in these submerged conditions (see previous section and Hajos & Ellender *et al.*, 2009).

Similar to sharp waves *in vivo*, most sharp waves *in vitro* were detected as a downward deflection (sink) in the dendritic layers associated with an upward deflection (source) in the pyramidal cell layer (Figure 3.8A, B). I observed that individual sharp waves could be generated locally in, and remain confined to, single CA3 subfields (Figure 3.8B). In most slices, sharp waves were observed to originate from each of the CA3 subfields, but there was often a clear preference for a particular location (Figure 3.8C and Figure 3.9A). The location

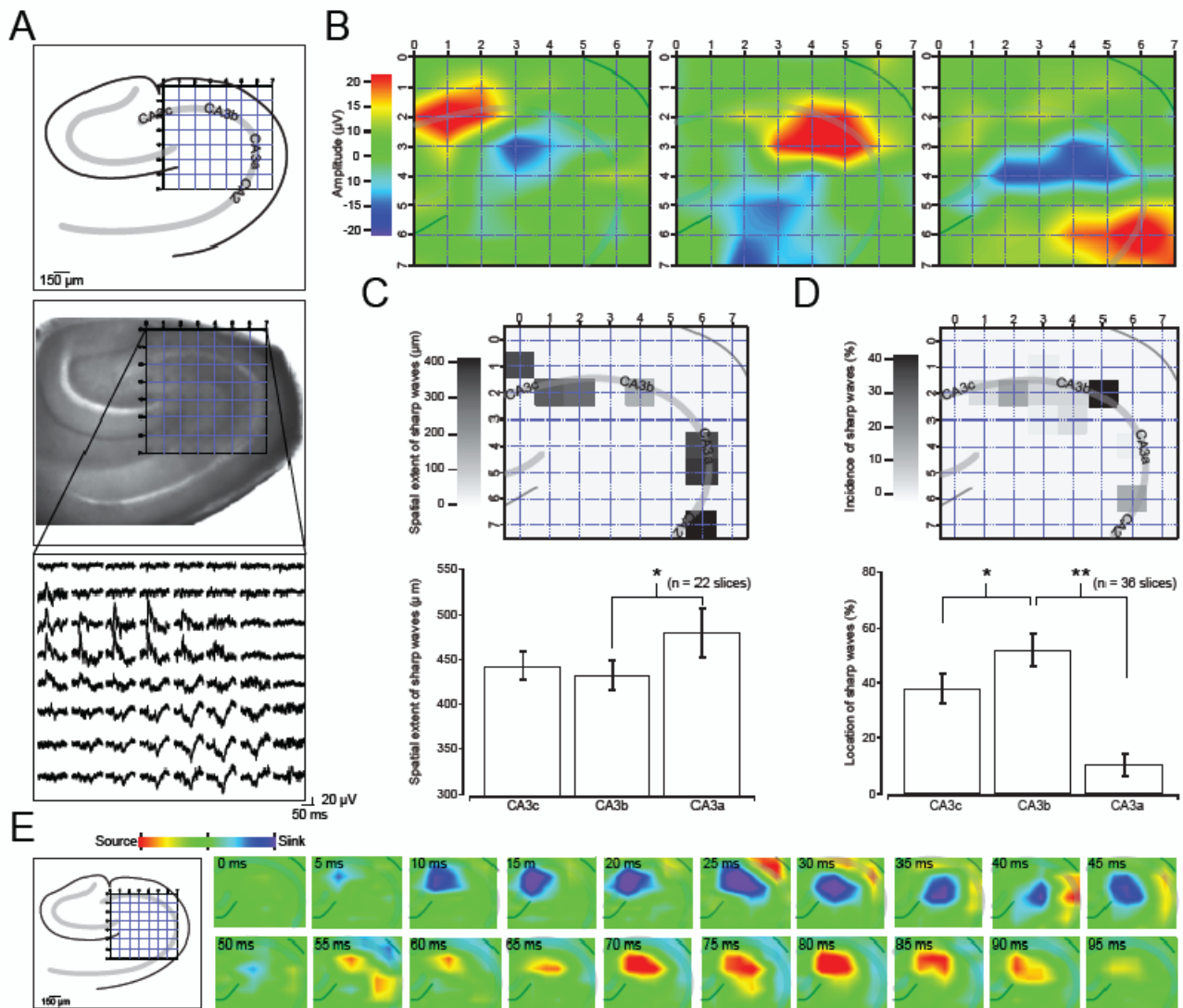


Figure 3.8 Sharp waves can arise locally and independently in all CA3 subfields

(A) Position of a hippocampal slice on an 8 x 8 planar multi-electrode array, shown with schematic of the anatomy (top) and photograph of the slice (middle). A corresponding recording of a single sharp wave shows that the event is localized (bottom).

(B) Pseudocolor representation of the voltage deflection observed during 3 separate sharp waves in the same slice, with warm colors representing positive deflections and cooler colors negative deflections. Sharp waves could originate in all subfields of CA3 in a slice.

(C) Top, Percentage of sharp waves originating in the different subfields of CA3 in a single slice, showing preference for CA3b. Bottom, Distribution of locations of sharp wave occurrence showing a significant preference for CA3b across slices (CA3c: $37.8 \pm 5.4\%$, CA3b: $52.0 \pm 5.9\%$, CA3a: $9.2 \pm 4.0\%$; $n = 36$ slices).

(D) Top, Spatial extent of sharp waves originating in the different subfields of CA3 in a single slice. Bottom, Average spatial extent of sharp waves was largest in CA3a (CA3c: 443 ± 16.2 , CA3b: 432 ± 16.9 , CA3a: 480 ± 27.6 ; ANOVA; $p < 0.05$; $n = 20$ slices; pairwise comparisons: CA3b vs. CA3a, $p < 0.05$, Student's t test).

(E) Current – source density analysis of a single sharp wave in 5 ms frames (colour scale above the CSD). The event started as a sink in the stratum radiatum with a corresponding source in the pyramidal layer followed by a somatic sink with a corresponding source in the stratum radiatum.

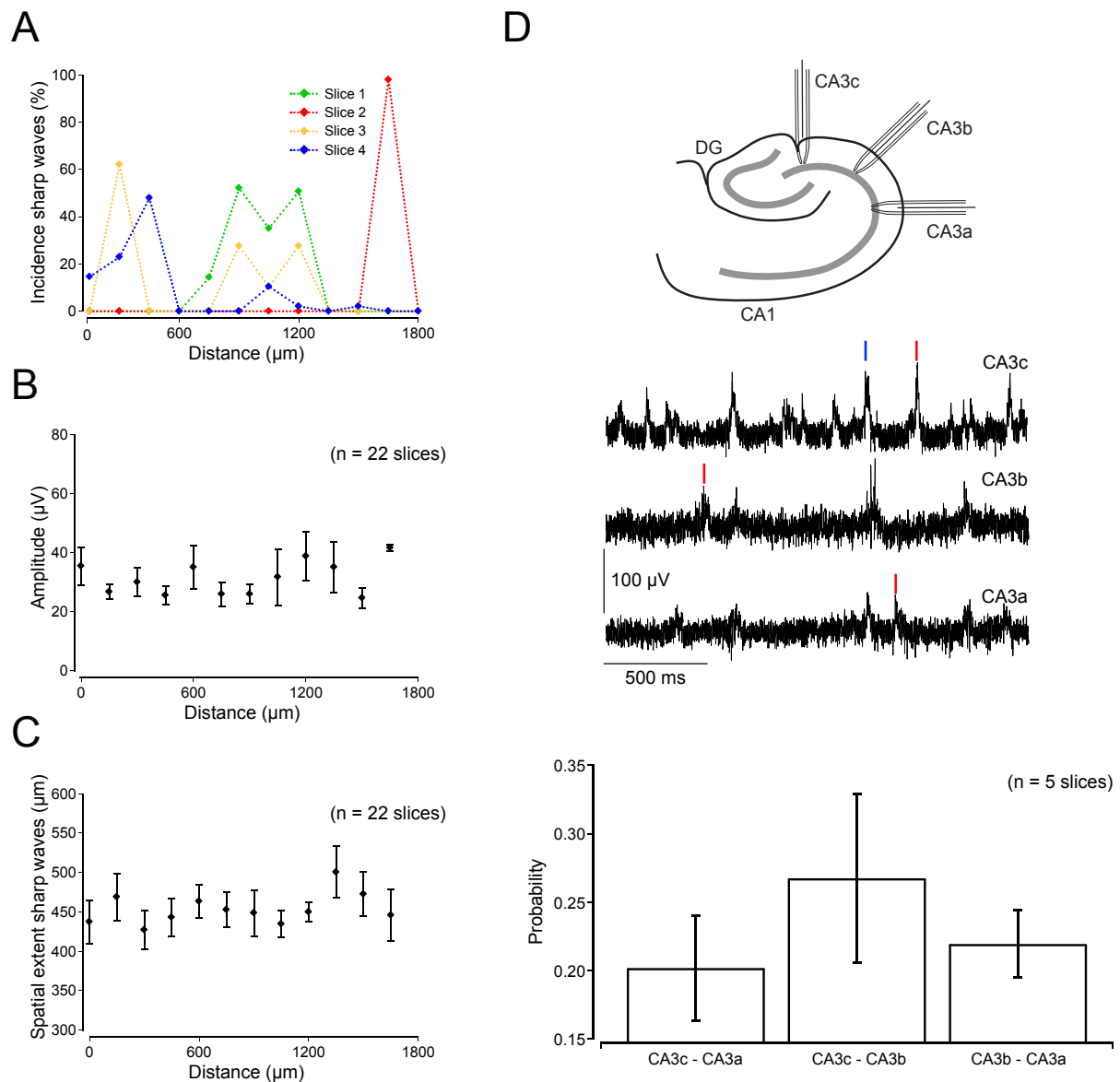


Figure 3.9 Preference and variability in location for sharp wave occurrence

(A) Incidence of sharp waves in single slices as recorded from a planar multi-electrode array, with location given as the linearised distance along the stratum pyramidale from CA3c towards CA3a. Note the difference between slices in both preferred location as well as the variability around this preferred location.

(B&C) The amplitude (B) and spatial extent (C) of sharp waves was independent of the site of generation.

(D) The spatial extent of sharp waves in interface conditions is similar to those recorded on planar multi-electrode arrays. Top, Location of field recording electrodes. Middle, 3 example traces of sharp wave activity recorded simultaneously from the CA3c, CA3b and CA3a stratum pyramidale. Note that sharp waves can be generated independently in all three subregions (red lines). In this slice the preferred location of generation is CA3c. Bottom, plot of the correlation probabilities for sharp waves generated in one subfield to be also recorded simultaneously from another subfield (an example of such a sharp wave is indicated with a blue line in the example traces).

of sharp wave occurrence over all slices showed a significant preference for CA3b ($p < 0.05$, ANOVA, $n = 36$ slices; pairwise comparisons: CA3b vs. CA3c, $p < 0.05$, and CA3b vs. CA3a, $p < 0.01$, Student's t test; Figure 3.8C). The average spatial extent of detectable sharp wave activity covered $449 \pm 12 \mu\text{m}$ (Figure 3.8D and 3.9C, $n = 22$ slices), corresponding to approximately one third of the CA3 pyramidal cell layer (Amaral & Witter, 1989). Current-source density (CSD) analysis of individual sharp wave events revealed that this activity started as a sink in the stratum radiatum (60%; Figure 3.8E) or the stratum oriens (40%) with a corresponding source in the somatic layer. Many pyramidal neurons make their recurrent connections in the stratum radiatum and the stratum oriens (Sik *et al.*, 1993), suggesting that sharp waves are generated by activity at synapses on either the apical or basal dendrites, but an active source (inhibition) at the soma may also contribute to the field event.

It could be argued that the spatial characteristics might differ with recording conditions (submerged planar multi-electrode recording array versus interface recording conditions) or specific ionic conditions. I therefore, also, investigated the spatial profile of sharp wave – ripples in interface conditions with recording electrodes placed in the CA3c, CA3b and CA3a stratum pyramidale. I found that sharp waves can be generated in all subfields of CA3, similar to the recordings taken with the planar multi-electrode array, and remain confined to that subfield, but could be recorded from all subfields in a few instances (Figure 3.9D). This suggests that the spatial characteristics found in submerged conditions using a planar multi-electrode array are similar to those found in interface conditions.

Secondly, I investigated if either the temporal or spatial characteristics were dependent on the exact ionic conditions of the ACSF used. I increased the concentration of $[\text{K}^+]$, decreased the concentration of $[\text{Mg}^{2+}]$ or decreased the concentration of $[\text{Ca}^{2+}]$ while measuring the incidence, amplitude and spatial extent of the sharp waves (Figure 3.10). A steady increase in $[\text{K}^+]$ from 4.25 mM to 7.25 mM did not alter any of these characteristics significantly. Only at the maximum concentration of 8.25 mM $[\text{K}^+]$ was there a transition in all slices from sharp wave generation to epileptiform activity which were characterised by a lower incidence, larger amplitude and larger spatial extent. Similarly, a steady decrease in $[\text{Mg}^{2+}]$

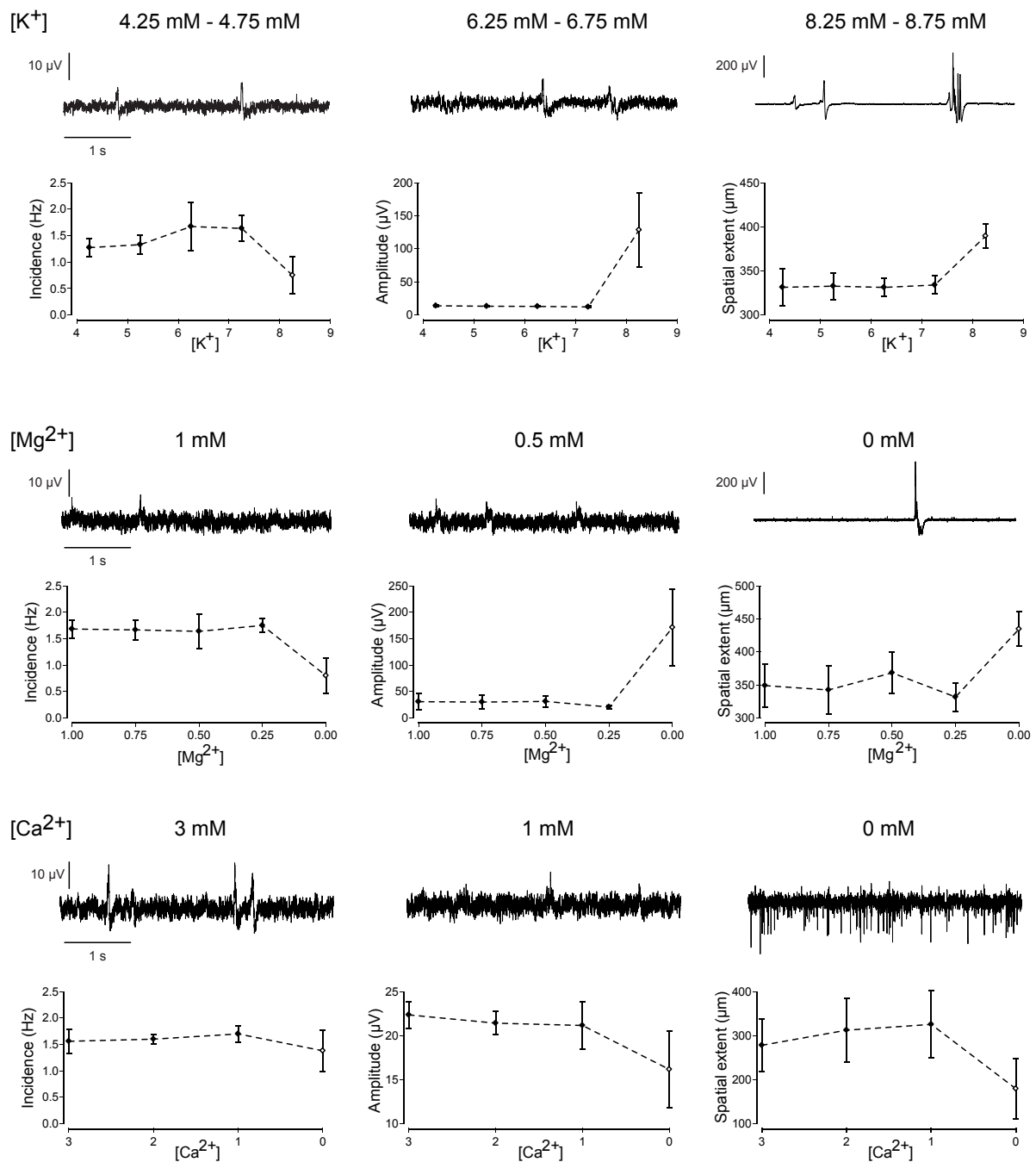


Figure 3.10 Sharp wave characteristics are not dependent on exact ionic conditions

Characteristics of sharp waves (incidence, amplitude and spatial extent) are not significantly altered by increasing the [K⁺] (top), lowering the [Mg²⁺] (middle) or lowering the [Ca²⁺] (bottom) concentrations of the ACSF. Only at 8.25 mM K⁺, 0 mM Mg²⁺ or 0 mM Ca²⁺ no sharp waves are generated and in some cases replaced by epileptiform bursting (8.25 mM K⁺ and 0 mM Mg²⁺) or aberrant field spikes (0 mM Ca²⁺) (n = 5 slices each condition).

did not alter any of these characteristics either. Again, only at the minimum of 0 mM $[Mg^{2+}]$ did most slices make a transition from sharp wave generation to epileptiform activity. A steady decrease in $[Ca^{2+}]$ did not affect any of these characteristics, neither was there a transition to epileptiform bursting. Sharp wave – ripples were replaced by aberrant field spiking at 0 mM $[Ca^{2+}]$, similar to that seen by Draguhn *et al.* (1998). These results suggest that sharp wave incidence, amplitude and spatial extent are not dependent on the exact ionic conditions.

The combined results suggest that sharp waves *in vitro* are locally generated events. Furthermore, they suggest that this observation is not dependent on the recording conditions or the exact ionic conditions, but on the properties of the CA3 network.

See Table 1 for additional sharp wave – ripple *in vitro* temporal and spatial characteristics.

Table 1. Characteristics of CA3 sharp wave — ripples *in vitro*

Incidence (Hz)	1.5 ± 0.01 (0.35 - 2.3)
Amplitude (μV)	450.4 ± 9.9 (60 - 1020)
Duration (ms)	123.7 ± 2.5 (46 - 518)
Rise time (20% - 80%) (ms)	39.9 ± 0.8
Rise time (half max peak) (ms)	33.2 ± 0.7
Time to peak (ms)	63.5 ± 1.3
Half decay time (ms)	28.6 ± 0.9
Spatial extent (μm)	449 ± 12
Ripple frequency (Hz)	150.1 ± 18.2
Ripple amplitude (μV)	78.8 ± 3.5
Ripple duration (ms)	122.2 ± 10.7
Data are given as mean ± SEM (range)	

3.4 Discussion

I set out to investigate the conditions and characteristics of the sharp wave – ripple model published by Kubota *et al.* (2003) in further detail. My main findings are: (1) Hippocampal slices can produce sharp wave – ripple population burst activity similar to that seen *in vivo* during rest and sleep. (2) Sharp waves can arise independently and intrinsically in different subfields of CA3. I also explored the conditions necessary for their emergence in submerged conditions, which revealed that besides an altered ACSF, a high laminar flow rate (3.9 – 6 ml/min) is necessary.

3.4.1 Conditions for sharp wave – ripples

I found that an altered ACSF is necessary for the emergence of sharp wave – ripples in rat hippocampal slices. It is thought that a 400 μm transverse hippocampal slice keeps sufficient recurrent connections of area CA3 intact and it has been suggested that this retained recurrent connectivity facilitates the generation of bursting activity (Ishizuka *et al.*, 1990; Kubota *et al.*, 2003a). Bursting in CA3 depends most likely on the release probability (Pr) of glutamate and the extent to which the released glutamate will evoke action potentials. The latter will be dependent on the connectivity and the state of the neighbouring cells (Bains *et al.*, 1999). It might be that the Pr and state of the pyramidal neurons are both affected by the buffer conditions used, as to facilitate the generation of action potentials. It is interesting to note that the extracellular fluid in the hippocampus *in vivo* has been estimated to be 4.3 mM $[\text{K}^+]$, 0.7 $[\text{Ca}^{2+}]$ and 0.4 $[\text{Mg}^{2+}]$ (McNay & Sherwin, 2004). Cerebrospinal fluid (CSF) $[\text{Ca}^{2+}]$ and $[\text{K}^+]$ concentration has been estimated to be approximately 2.0 mM and 3.1 mM in rats (Jones & Keep, 1987, 1988). The ACSF used to induce sharp wave – ripples contained 4.25 mM $[\text{K}^+]$, 1 mM $[\text{Mg}^{2+}]$ and 3 mM $[\text{Ca}^{2+}]$. Most likely it is the increased potassium and decreased magnesium concentration which excites the network into the generation of population bursts. It is often assumed that an increase in extracellular $[\text{Ca}^{2+}]$ will reduce the negative charge density on the outer surface of the membrane, leading to an increase the gradient of the potential field within the membrane, and hence raise the membrane potential

at which channels become opened (Aidley, 1998), in effect hyperpolarising a neuron, whereas a decrease in extracellular $[Ca^{2+}]$ leads to an increase in excitability in the neurons (Sanchez-Vives & McCormick, 2000b).

The altered ionic conditions might also lead to plasticity which would alter the connectivity in the network and has been suggested to be necessary for the emergence of sharp wave – ripples (Maier *et al.*, 2003; Behrens *et al.*, 2005). The observation that sharp wave amplitude increases over time would be consistent with this scenario. Several models of hippocampal population bursts have been proposed, which show persistent bursting after theta burst stimulation of area CA3 (Behrens *et al.*, 2005), application of the GABA_A receptor antagonist bicuculline (Stoop *et al.*, 2003) or upon application of ACSF containing high $[K^+]$ (Bains *et al.*, 1999). The induction of these bursts could be prevented by application of the *N*-methyl-*D*-aspartate (NMDA) receptor blocker 2-amino-5-phosphonopentanoic acid (AP5) suggesting that activation and NMDA receptor-dependent recruitment of CA3 pyramidal neurons is necessary for their emergence (Bains *et al.*, 1999; Stoop *et al.*, 2003; Behrens *et al.*, 2005). Persistent hippocampal population bursts, induced with ACSF containing high $[K^+]$, could not only be prevented by blocking NMDA receptors, but also by blocking mGluR5 receptors (Bains *et al.*, 1999). Metabotropic glutamate receptor 5 has also been shown to be necessary for developmental synaptic rewiring (Le Be & Markram, 2006). However, we found that the emergence of sharp wave – ripples was not blocked by either application of an NMDA or group I metabotropic glutamate receptor antagonist. Furthermore, application of the protein synthesis inhibitor cycloheximide or the transcription inhibitor actinomycin D did not block the emergence of sharp wave – ripples either (see Lipton & Raley-Susman (1999)). These results suggest that plasticity or excitability changes mediated via NMDA or group I metabotropic glutamate receptors are not necessary for the emergence of sharp wave – ripples.

To confirm that the concentration of D-AP5 used was sufficient to block NMDA receptors I attempted to induce LTP at the Schaffer collateral – CA1 synapse. In the presence of D-AP5 (50 μ M) this was not possible, but during control conditions, LTP could be readily induced via either tetanic or theta-burst stimulation with ongoing sharp wave activity. This

is different from a previous finding (Colgin *et al.*, 2004b) in which it was suggested that ongoing sharp waves inhibits the induction of LTP. It was suggested that this was the result of an increased adenosine concentration in the CA1 area. These different observations might be the result of different recording conditions and in particular differences in flow rate. My findings suggest that the sharp wave state might well be a permissive state for plasticity (Kamondi *et al.*, 1998; King *et al.*, 1999).

As plasticity or excitability changes would affect the incidence or amplitude of sharp waves these variables were measured for a prolonged period. Although incidence stayed constant over the recording period, the amplitude increased substantially. It is possible that the increase in sharp wave amplitude over time is the result of a simple flattening of the slice with a concurrent increase in resistance, which according to Ohm's law ($V = R \times I$, where V is voltage, I is current and R is resistance) would lead to a larger voltage deflection for the same current. Of course, it could also be influenced by any of the following other factors: (1) an increase in excitability, (2) an increase in synaptic efficacy, (3) an increase in release sites (n) or probability of release (Pr) or (4) an increase in connectivity. The increase in amplitude which we observe, although interesting, probably does not have a physiological relevance for the study of sharp wave – ripples as such, as this increase has not been observed *in vivo* and an episode of slow-wave sleep, at least in humans, does not last longer than ~60 minutes (Stickgold, 2005). It is however important to keep in mind when doing long-term experiments in which amplitude is monitored as a readout.

3.4.2 Temporal characteristics of sharp wave – ripples

Transverse hippocampal slices superfused with modified ACSF exhibited spontaneous sharp wave – ripples in CA3 with similar characteristics to those observed *in vivo*. The average frequency of sharp waves *in vitro* (1.50 ± 0.01 Hz) was within the range of those observed *in vivo* (0.01 – 2 Hz) but their amplitude was smaller, in the microvolt range instead of millivolt range normally seen in CA1 *in vivo* (Buzsaki, 1986). This could be explained by the significantly reduced hippocampal network present in a slice, although it must be noted that up till now no detailed characterisation has been made of CA3 sharp wave – ripples *in vivo*.

The packing density of CA3 pyramidal neurons increases from septal (dorsal) to temporal (ventral) hippocampus and this could explain the increase in amplitude (Amaral & Lavenex, 2007). The observed CA3 ripple frequency was slightly lower than that observed *in vivo* for CA1, most likely because slices were kept at slightly below physiological temperatures (~32 °C), and markedly lower than those found during pathological epileptiform burst activity (Foffani *et al.*, 2007). It has been shown that CA3 can generate fast frequency ripples *in vivo* with a frequency of approximately between 80 – 140 Hz which fits these observations well (Buzsaki *et al.*, 1992; Ylinen *et al.*, 1995). The observation that CA3 can generate sharp wave – ripples independently from input from the dentate gyrus or the entorhinal input is consistent with previous publications which showed an increase in incidence upon bilateral removal of entorhinal cortex (Bragin *et al.*, 1995). Although one must be aware that connections between the dentate gyrus, entorhinal cortex and the CA3 region might not follow the precise angle of cutting in the preparation of the slice and that because of this, the modulatory aspects of the dentate gyrus and entorhinal cortex on sharp wave – ripple generation (Colgin *et al.*, 2004a) might be lost in a slice preparation.

It is important to distinguish between sharp wave – ripple population bursts and other bursts which can be generated in CA3. Firstly, it has been shown that CA3 is capable of producing epileptiform bursts when disinhibited (Miles & Wong, 1987; Menendez de la Prida *et al.*, 2006), or superfused with ACSF containing a high concentration of [K⁺] (Staley *et al.*, 1998). These bursts are often much larger in amplitude and have a lower incidence (Miles & Wong, 1987; Staley *et al.*, 1998; Menendez de la Prida *et al.*, 2006). The ripples they contain are also of a higher frequency (200 – 500 Hz) than those observed physiologically. This has been shown in rodents *in vivo* (Bragin *et al.*, 1999b) and *in vitro* (Foffani *et al.*, 2007) as well as in humans (Bragin *et al.*, 1999a; Staba *et al.*, 2007; Urrestarazu *et al.*, 2007; Le Van Quyen *et al.*, 2008).

Secondly, during development the neonatal brain produces giant depolarizing potentials (GDPs) which are suggested to be the result of depolarising GABA and the neonatal equivalent of adult sharp wave – ripples (Ben-Ari, 2001; Allene *et al.*, 2008). The depolarizing

effect of GABA is the result of an active $\text{Na}^+\text{-K}^+\text{-Cl}^-$ -cotransporter isoform 1 (NKCC1) which sets up the Cl^- gradient responsible for the depolarising effect (Ben-Ari *et al.*, 2004; Sipila *et al.*, 2006). The ontogenetic shift to hyperpolarising GABA action is caused by a concomitant developmental down-regulation of NKCC1 and an up-regulation of the $\text{K}^+\text{-Cl}^-$ -cotransporter isoform 2 (KCC2) (Rivera *et al.*, 1999). However, the depolarising effect of GABA might be present much longer in the distal apical dendrites than elsewhere (Romo-Parra *et al.*, 2008). These GDPs are, similar to epileptiform bursts, lower in frequency and larger in amplitude than sharp wave – ripples (Allene *et al.*, 2008).

These results suggest that sharp wave – ripples *in vitro* have most in common with their counterparts *in vivo* and not with epileptiform bursts or GDPs.

3.4.3 Sharp wave – ripples in submerged conditions

Obtaining sharp wave – ripples in submerged conditions enables more detailed study of the network pattern using IR-DIC imaging and whole-cell patch-clamp. I show that it is possible to obtain stable sharp wave – ripples in submerged conditions if a sufficiently high laminar flow rate (6 ml/min) is achieved. The high flow rate enables a sufficient oxygen supply which is necessary for the emergence of sharp wave – ripples *in vitro*. The high sensitivity of hippocampal network activity to oxygen tension is consistent with other recent studies (Wu *et al.*, 2005; Huchzermeyer *et al.*, 2008). Using this high flowrate it is possible to maintain stable sharp wave – ripples for many hours. Earlier investigations into the cellular mechanisms underlying network oscillations have been hampered by the transient nature of the induced activity in submerged conditions (McMahon *et al.*, 1998; Kawaguchi, 2001; Gloveli *et al.*, 2005) compared to interface conditions (e.g. (Whittington *et al.*, 1995; Fisahn *et al.*, 1998; Sanchez-Vives & McCormick, 2000a; Kubota *et al.*, 2003b).

Having identified conditions for obtaining sharp wave – ripples in submerged slices offers experimental advantages (e.g. fast exchange of pharmacological agents and visually-guided patch-clamp recordings) which will be used in the rest of this thesis.

3.4.4 Spatial characteristics of sharp wave – ripples

Hippocampal slices mounted on 8 x 8 arrays of planar multi-electrodes revealed that sharp wave – ripples *in vitro* are locally generated events which can arise in each of the different subfields of CA3 independently. Similar to sharp waves described *in vivo* they consist of a downward deflection (sink) in the dendritic layer with a simultaneous positive deflection (source) in the pyramidal cell layer (Buzsaki *et al.*, 1983). A single sharp wave covers approximately 450 μm or one third of the pyramidal cell layer. Most *in vivo* recordings are done in the CA1 region of the hippocampus and it has been shown that CA1 regions up to 4 mm apart are synchronously active *in vivo* (Buzsaki, 1992; Chrobak & Buzsaki, 1996; Ylinen *et al.*, 1995). It is at present unknown what the spatial characteristics of CA3 sharp wave – ripples *in vivo* are. It is possible that these are larger than described here as slices contain a vastly reduced recurrent network.

I find that the majority of sharp waves are generated in CA3b. This is consistent with observations *in vivo* that sharp waves are preferentially initiated in CA3a/b before propagating to CA3c and CA1 (Csicsvari *et al.*, 2000).

We did not investigate if there was an underlying regularity or pattern to generation of sharp waves in the different subfields. In the future it would be interesting to see if they were generated in a deterministic, chaotic or random way.

3.5 Summary

The combined data from this chapter suggest that transverse hippocampal slices exhibit sharp wave – ripples with similar characteristics to those observed *in vivo*. I show how it is possible to obtain this network pattern in submerged conditions which enables the study of sharp wave – ripples in further detail using fast exchange of pharmacological agents (Chapter 4) and visually-guided patch-clamp recordings (Chapter 5 and Chapter 6). I show that sharp wave – ripples are locally generated events and, furthermore, I suggest that there is no need for synaptic plasticity or excitability changes of the CA3 circuitry for this network pattern to emerge in a hippocampal slice.

Synaptic receptors

4.1 Introduction

The observation that sharp waves are small, localised events as recorded in the field suggests that they are the result of firing of a localized population of pyramidal neurons. To investigate which synaptic currents might underlie the synchronisation of the select group of pyramidal neurons taking part in a sharp wave event, I applied antagonists at six major receptor types. Firstly, I applied antagonists at the two major ionotropic glutamate receptors; α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and *N*-methyl-*D*-aspartic acid (NMDA) receptors. Secondly, I applied antagonists at the group I metabotropic glutamate receptor family and its two subtypes; mGluR1 and mGluR5. Thirdly, I applied antagonists at the two major inhibitory receptors; the gamma-aminobutyric acid A (GABA_A) receptor and gamma-aminobutyric acid B (GABA_B) receptor. Lastly, I investigated the role of gap junction connections in the generation of sharp waves.

4.2 Experimental setup

For the subsequent experiments I obtained stable sharp wave – ripples, as recorded from planar multi-electrode arrays and recorded their baseline incidence and amplitude before washing in antagonists at various glutamatergic and GABAergic receptors, for at least 10 minutes, before recording incidence and amplitude of sharp wave – ripples again. As the solvent DMSO was used to solubilise several of the antagonists (namely NBQX, CPCCOEt, MPEP and diazepam), the effect of DMSO on sharp wave – ripple incidence and amplitude was investigated first. The solvent DMSO (0.1%) did not have a significant effect on sharp wave incidence or amplitude ($101.7 \pm 15.7\%$ and $92.6 \pm 7.3\%$; paired-samples *t* test; $p > 0.05$; $n = 6$; Figure 4.2, page 72).

4.3 AMPA receptors

Sharp wave – ripples are thought to be the result of synchronous firing of a population of CA3 pyramidal neurons. The postsynaptic receptors of synapses made by the pyramidal neurons include, among others, AMPA receptors, NMDA receptors and group I metabotropic glutamate receptors. We found that fast synaptic transmission through AMPA receptors is obligatory, as application of both the AMPA / kainate receptor antagonist NBQX (40 μM) and the selective AMPA receptor antagonist GYKI 52466 (100 μM) completely abolished sharp wave activity (Figure 4.1 A, B; $p < 0.01$; paired-samples *t* test; $n = 5$ and $n = 6$). To investigate if it is a particular type of AMPA receptor that is obligatory for sharp wave generation we applied the specific Ca^{2+} -permeable AMPA receptor blocker PhTx 433 (1 μM). Application of this antagonist significantly reduced, but did not abolish, sharp wave incidence ($86.8 \pm 2.4\%$; paired-samples *t* test; $p < 0.05$; $n = 5$; Figure 4.2) suggesting that this type of AMPA receptor is involved in sharp wave generation. These combined results suggest that synchronized bursting by pyramidal neurons depends on AMPA receptor-mediated currents.

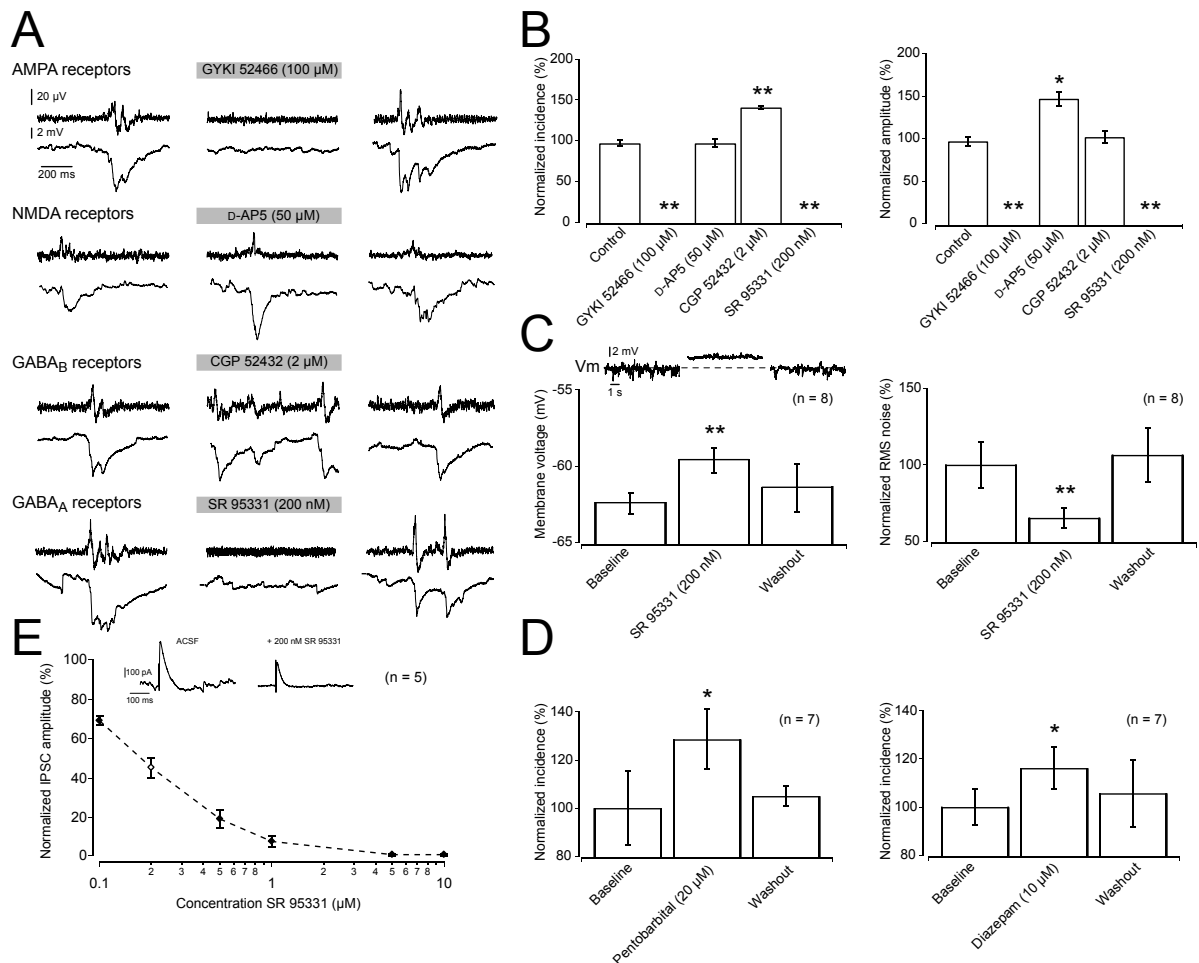


Figure 4.1 GABA_A receptor-mediated inhibition is necessary for sharp wave generation

(A) Measurement of sharp waves on planar multi-electrode array while simultaneously recording from a pyramidal neuron in whole-cell current-clamp mode. After 10 minutes of baseline recording, drugs acting on AMPA, NMDA, GABA_B or GABA_A receptors were washed in (for at least 10 minutes), and their effect on both incidence and amplitude of ongoing sharp waves was assessed.

(B) Effect of block of AMPA, NMDA, GABA_B, or GABA_A receptors on sharp wave amplitude and incidence, averaged over several slices. AMPA receptor antagonist GYKI 52466 (100 μ M) abolished all sharp wave activity from both field and intracellular recordings. NMDA receptor antagonist D-AP5 (50 μ M) significantly increased the amplitude of sharp waves with no effect on their incidence. GABA_B receptor antagonist CGP 52432 (2 μ M) significantly and reversibly increased sharp wave incidence while not affecting amplitude. GABA_A receptor antagonist SR 95331 (200 nM) abolished all sharp wave activity from both field and intracellular recordings.

(C) Application of SR 95331 (200 nM) significantly depolarized pyramidal neurons and decreased the intracellular noise, measured as the RMS of the membrane potential fluctuations outside of sharp wave events, from 0.9 ± 0.1 mV to 0.5 ± 0.05 mV and back to 0.9 ± 0.2 mV upon washout (n = 8). Inset shows an intracellular recording of pyramidal neuron in current-clamp mode. Dashed line indicates resting membrane voltage of -62.4 mV.

(D) Enhancing GABA_A receptor-mediated currents by application of pentobarbital (20 μ M) or diazepam (10 μ M) led to a significant and reversible increase in sharp wave incidence.

(E) Dose dependent reduction in IPSC amplitude. Application of 200 nM SR 95331 resulted in a $54.7 \pm 5.0\%$ reduction in IPSC amplitude.

4.4 NMDA receptors

In addition to AMPA receptors, NMDA receptors can also be found postsynaptically to pyramidal neuron boutons and I therefore examined if these receptors are involved in sharp wave generation as well. In contrast to AMPA receptors, I found that NMDA receptors are not necessary for sharp wave generation. In fact, application of D-AP5 (50 μ M) led to a significant increase in their amplitude (140 ± 10 %; paired-samples *t* test; $p < 0.05$; $n = 11$) with no change in incidence (100 ± 5 %; paired-samples *t* test; $p > 0.05$, Figure 4.1 A, B), consistent with a previous report (Colgin *et al.*, 2005). The increase in amplitude observed upon wash-in of D-AP5 (50 μ M) was also seen when using the NMDA receptor NR2A subunit-preferring antagonist NVP-AAM077 (200 nM) (136.3 ± 15.1 %; paired-samples *t* test; $p < 0.05$; $n = 3$), while superfusion of the NMDA receptor NR2B subunit-specific antagonist Ro 256981 (0.5 μ M) did not lead to a significant change (108 ± 3.5 %; paired-samples *t* test; $p > 0.05$; $n = 6$) (Figure 4.2).

These results combined suggest that synchronized bursting by pyramidal neurons does not depend on NMDA receptor-mediated currents, but suggest that such bursting might actually be modulated by these receptors and in particular the NR2A subunit-containing NMDA receptors.

4.5 Metabotropic glutamate receptors

To investigate if metabotropic glutamate receptors are necessary for the synchronised bursting of pyramidal neurons during sharp wave – ripples, antagonists at the group I mGluR family were applied. I have previously shown that group I mGluRs are not necessary for sharp wave induction (Chapter 3, Figure 3.1B, page 44). However, I did observe an increased sharp wave incidence and reduced amplitude. To investigate if this was the result of altering either the development or expression of sharp waves, I applied the group I mGluR antagonist 4-CPG (0.5 mM), the mGluR1 antagonist CPCCOEt (100 μ M) or the mGluR5 antagonist MPEP

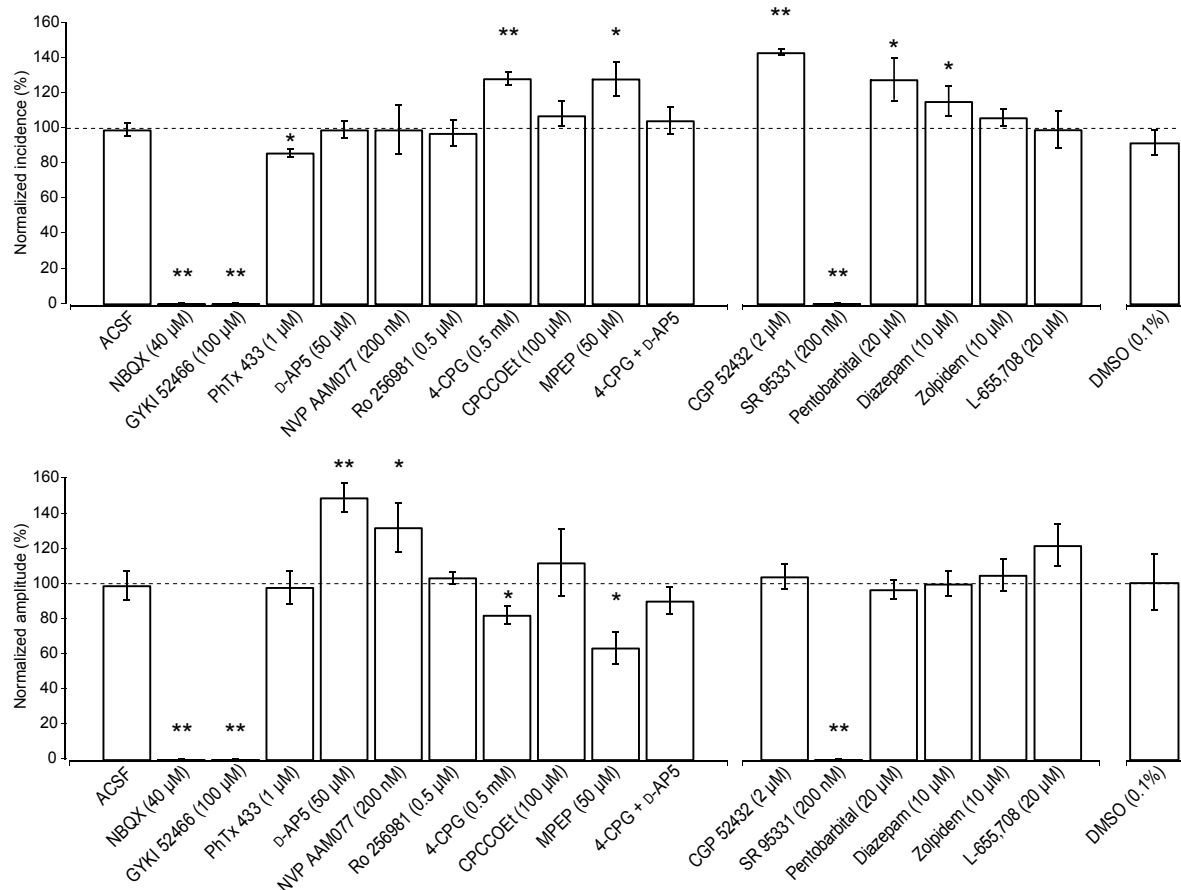


Figure 4.2 Role of ionotropic and metabotropic excitatory and inhibitory receptors on sharp wave generation AMPA receptors are necessary for sharp wave generation as application of both NBQX (40 μM) and GYKI 52466 (100 μM) blocked sharp waves completely ($p < 0.01$; paired-samples t test; $n = 5$ and $n = 6$). The Ca^{2+} permeable AMPA receptor blocker PhTx 433 (1 μM) significantly reduced sharp wave incidence ($86.8 \pm 2.4\%$; paired-samples t test; $p < 0.05$; $n = 5$), suggesting a role for CA3 interneurons in the generation of sharp waves (Toth & McBain, 1998; McBain et al., 1999).

NMDA receptors are not necessary for sharp wave generation as washin of D-AP5 (50 μM) did not block them but significantly increases their amplitude. The reported increase in amplitude is most likely mediated by NMDA receptors containing the NR2A subunit, as superfusion with NVP AAM077 (200 nM) led to a significant increase in amplitude ($136.3 \pm 15.1\%$; paired-samples t test; $p < 0.05$; $n = 3$), while superfusion of the NR2B blocker Ro 256981 (0.5 μM) did not ($108 \pm 3.5\%$; paired-samples t test; $p > 0.05$; $n = 6$).

Application of the group I mGluR blocker 4-CPG (0.5 mM) led to a significant reduction in amplitude ($85.3 \pm 6.2\%$; paired-samples t test; $p < 0.05$; $n = 7$) and increase in incidence ($128 \pm 3.3\%$; paired-samples t test; $p < 0.01$), which is most likely mediated by mGluR5 as the mGluR5 blocker MPEP (50 μM) but not the mGluR1 blocker CPCCOEt (100 μM) mimicked these effects (MPEP; amplitude: $64.5 \pm 15.8\%$, incidence: $129.2 \pm 17.1\%$; paired-samples t test; $p < 0.05$, $n = 6$, CPCCOEt; amplitude: $113 \pm 19.6\%$, incidence: $114.5 \pm 7.6\%$; paired-samples t test; $p > 0.05$; $n = 9$). Interestingly, co-application of both D-AP5 (50 μM) and 4-CPG (0.5 mM) occluded these effects (amplitude: 91.2 ± 7.7 and incidence: 105.2 ± 7.6 ; paired-samples t test; $p > 0.05$; $n = 15$), suggesting a possible interaction of these receptors in modulating sharp wave activity.

The GABA_A receptor antagonist SR 95331 (200 nM) abolished all sharp wave activity from both field and intracellular recordings. GABA_B receptor antagonist CGP 52432 (2 μM) significantly increased sharp wave incidence while not affecting amplitude. Enhancing GABA_A receptor-mediated currents by application of diazepam (10 μM) significantly enhanced sharp wave incidence. Application of the GABA_A receptor $\alpha 1$ -subunit preferring benzodiazepine, zolpidem (10 μM), mimicked these effects, but not significantly (incidence: $106.7 \pm 6.1\%$, amplitude: 90.2 ± 14.1 ; paired-samples t test; $p > 0.05$; $n = 10$). The GABA_A receptor $\alpha 5$ -subunit receptor-specific blocker L655,708 (20 μM) did not alter sharp wave amplitude or incidence (amplitude: $122.8 \pm 12.2\%$ and incidence: $94.3 \pm 6.1\%$; paired-samples t test; $p > 0.05$; $n = 5$).

The solvent DMSO (0.1%) did not have an effect on sharp wave amplitude or incidence ($101.7 \pm 15.7\%$ and $92.6 \pm 7.3\%$; paired-samples t test; $p > 0.05$; $n = 6$). DMSO was used to dissolve NBQX, CPCCOEt, MPEP, diazepam and L655,708.

(50 μ M) to slices with already established sharp waves. Application of the group I mGluR antagonist 4-CPG (0.5 mM) led to a significant reduction in amplitude ($85.3 \pm 6.2\%$; paired-samples *t* test; $p < 0.05$; $n = 7$) and increase in incidence ($128 \pm 3.3\%$; paired-samples *t* test; $p < 0.01$; Figure 4.2) similar to the previous observation when incubating slices in 4-CPG (0.5 mM) immediately after slicing (Chapter 3, Figure 3.1B, page 45). These changes are most likely mediated by the mGluR5 subtype as the mGluR5 antagonist MPEP (50 μ M), but not the mGluR1 antagonist CPCCOEt (100 μ M), mimicked these effects (MPEP; amplitude: $64.5 \pm 15.8\%$, incidence: $129.2 \pm 17.1\%$; paired-samples *t* test; $p < 0.05$, $n = 6$, CPCCOEt; amplitude: $113 \pm 19.6\%$ incidence: $114.5 \pm 7.6\%$; paired-samples *t* test; $p > 0.05$; $n = 9$; Figure 4.2). Interestingly, co-application of both D-AP5 (50 μ M) and 4-CPG (0.5 mM) occluded these effects (amplitude: 91.2 ± 7.7 and incidence: 105.2 ± 7.6 ; paired-samples *t* test; $p > 0.05$; $n = 15$), suggesting a possible interaction of these receptors in modulating sharp wave activity (Figure 4.2).

These results suggest that group I metabotropic glutamate receptors do not modulate the induction, but rather the expression of sharp wave – ripples, and this modulation is likely mediated by the mGluR5 subtype.

4.6 GABA_B receptors

GABA receptor-mediated inhibition has been shown to play a major role in shaping network activity (Mann & Paulsen, 2007). To investigate the role of synaptic inhibition on sharp wave activity, I applied antagonists at each of the two major inhibitory GABA receptor subtypes (GABA_A and GABA_B). Block of metabotropic GABA_B receptor-mediated transmission, by application of the antagonist CGP 52432 (2 μ M), led to a significant increase in sharp wave incidence ($144 \pm 1\%$; paired-samples *t* test; $p < 0.01$, $n = 6$ slices) with no change in amplitude ($105 \pm 7.3\%$; paired-samples *t* test; $p > 0.05$) (Figure 4.1 A, B).

These results suggest that sharp wave expression is modulated by GABA_B receptor-mediated events.

4.7 GABA_A receptors

The other member of GABAergic receptor is the ionotropic GABA_A receptor. This receptor has been shown to play a major role in shaping gamma activity in CA3 (Mann *et al.*, 2005), and it is generally thought that a reduction in GABA_A receptor-mediated inhibition is responsible for the transition from physiological to pathological population burst generation (Menendez de la Prida *et al.*, 2006). It was therefore expected that wash-in of an antagonist at this receptor would lead to an enhancement of burst generation. Instead, block of the phasic component of the GABA_A receptor-mediated current with SR 95531 (200 nM) abolished all sharp wave activity ($n = 10$ slices) and this effect was reversible (Figure 4.1 A, B). This concentration blocked only $54.7 \pm 5.0\%$ of the GABA_A receptor-mediated current (Figure 4.1 E). Pyramidal neurons slightly but significantly depolarized upon application of SR 95331 (200 nM) (from -62.4 ± 0.7 mV to -59.6 ± 0.8 mV; paired-samples *t* test; $p < 0.01$; $n = 8$) and this effect was also reversible (-61.4 ± 1.5 mV) (Figure 4.1 C). Moreover, the application of 200 nM SR 95331 led to a significant reduction in the intracellular noise, measured as the root mean square (RMS) of membrane potential fluctuations ($65.2 \pm 6.7\%$; paired-samples *t* test; $p < 0.01$; $n = 8$) (Figure 4.1 C).

Conversely, enhancing GABA_A receptor-mediated currents by application of pentobarbital (20 μ M) or diazepam (10 μ M) significantly increased the incidence of sharp waves by $28.6 \pm 12.0\%$ and $16.2 \pm 8.6\%$, respectively (each $p < 0.05$; paired-samples *t* test; $n = 7$ slices) (Figure 4.1 D) (see Buzsaki, 1986; Suzuki & Smith, 1988 for the effect of systemic application of these drugs). Application of the GABA_A receptor $\alpha 1$ -subunit preferring benzodiazepine, zolpidem (10 μ M; Palhalmi *et al.*, 2004), mimicked the diazepam effects, but not significantly (incidence: $106.7 \pm 6.1\%$, amplitude: 90.2 ± 14.1 ; paired-samples *t* test; $p > 0.05$; $n = 10$; Figure 4.2). The GABA_A receptor $\alpha 5$ -subunit receptor-specific inverse agonist L655,708 (20 μ M; Atack *et al.*, 2006; Bonin *et al.*, 2007) did not alter sharp wave amplitude or incidence (amplitude: $122.8 \pm 12.2\%$ and incidence: $94.3 \pm 6.1\%$; paired-samples *t* test; $p > 0.05$; $n = 5$; Figure 4.2).

Block of both phasic and tonic GABA_A receptor-mediated currents with a higher concentration of SR 95531 (1 μ M) led to epileptiform burst generation (Figure 4.3).

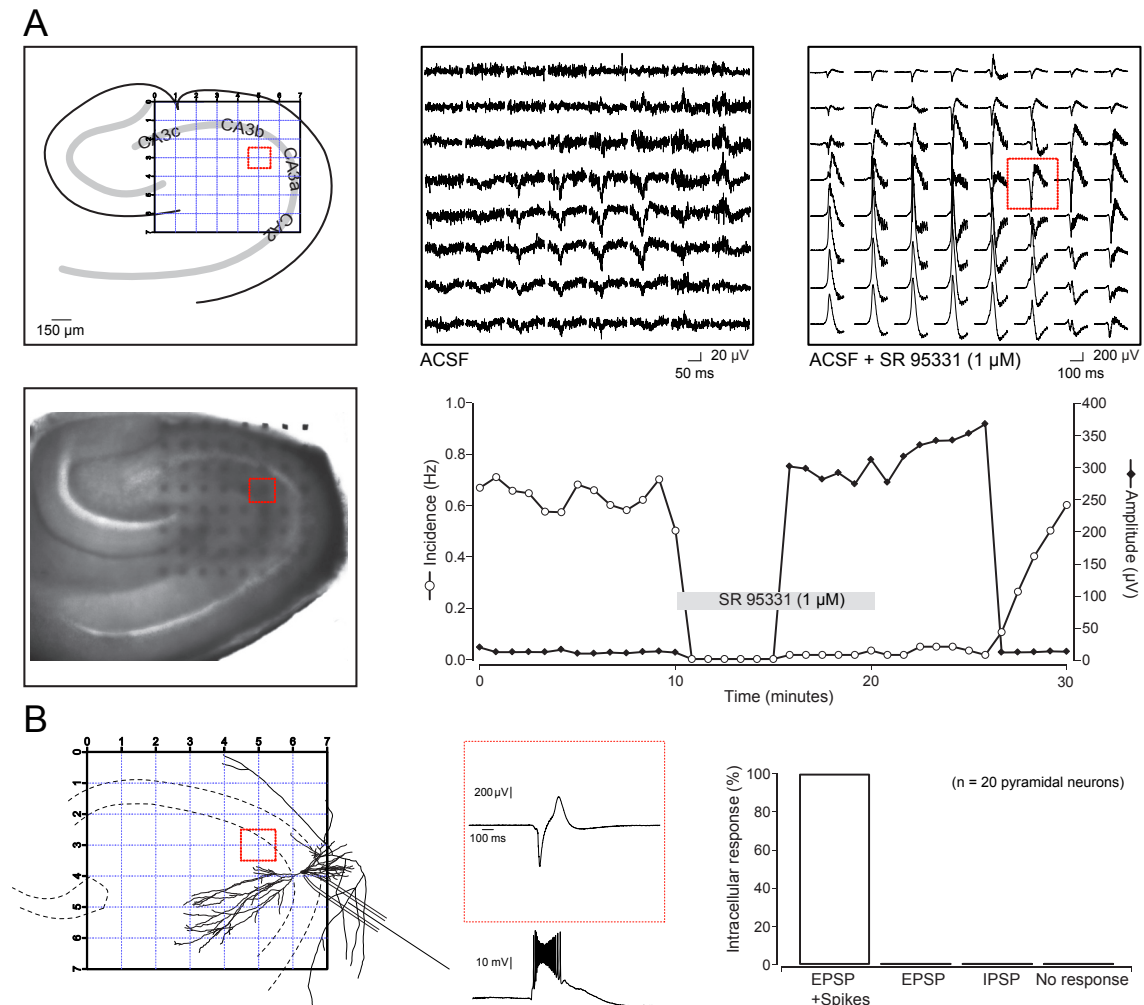


Figure 4.3 Recurrent connectivity in CA3 is retained in 400 μm slice

(A) Left: Diagram of a hippocampal slice on an 8 x 8 planar multi-electrode array and photograph of slice on an 8 x 8 planar multi-electrode array. Right: Blocking all GABA_A receptor-mediated inhibition by application of SR 95331 (1 μM) led to the generation of epileptiform bursts in area CA3. Shown is a recording of a sharp wave (ACSF) and an epileptiform burst (ACSF containing 1 μM SR 95331). Note the difference in incidence and amplitude of both events over time.

(B) Left, Diagram of a hippocampal slice and reconstructed CA3 pyramidal neuron on an 8 x 8 planar multi-electrode array. Middle, Trace of epileptiform activity as seen on a single electrode of the planar multi-electrode array (red box) with synchronous multiple spike discharges of a patched pyramidal neuron. Right, All pyramidal neurons recorded (n = 20) took part in the epileptiform bursts even those that received inhibitory or no input during sharp wave activity.

Sharp wave – ripples recorded with planar multi-electrode arrays are small local events whereas induced epileptiform bursts were recorded from the entire multi-electrode array and were an order of magnitude larger in spatial extent as well as in amplitude (Figure 4.3A, right; sharp waves; 0.6 Hz, 20 μ V and $\sim$ 450 μ m and epileptiform bursts; 0.05 Hz, 350 μ V and $\sim$ 1200 μ m).

These combined results show that sharp wave – ripple generation depend on GABA_A receptor-mediated events and suggest that GABA_A receptor-mediated currents keep the sharp wave – ripple population burst spatially confined.

4.8 Gap junctions

It has been suggested that the ripple component of a sharp wave – ripple depends on gap junction connections between pairs of pyramidal neurons (Draguhn *et al.*, 1998) or interneurons (Katsumaru *et al.*, 1988). This is very much a matter of debate and other mechanisms e.g. GABA_A receptor-mediated inhibition (Ylinen *et al.*, 1995; Buhl & Buzsaki, 2005) and/or population spiking (Engel Jr *et al.*, 2008) (and see¹) have also been proposed. I therefore attempted to investigate the role of gap junctions in the generation of sharp wave – ripples.

Sharp wave – ripple incidence and amplitude were recorded from planar multi-electrode arrays before washing in either of two gap junction blockers for at least 60 minutes before recording incidence and amplitude of sharp wave – ripples again. Application of the gap junction blocker carbenoxolone (200 μ M) led to a large reduction in incidence and amplitude of sharp waves (Figure 4.4Ai). The remaining sharp wave-like voltage deflections did not contain significant ripples. This suggests that gap junctions are necessary for ripple generation, but the lack of ripples might be the result of the lack of sharp waves. Electrical stimulation with a monopolar electrode was used to try to induce population burst activity and see if those induced population events contained a ripple component. Electrical stimulation did not produce a convincing sharp wave – ripple (Figure 4.4Aii). Further application of SR 95331 (200 nM) completely abolished all voltage deflections (Figure 4.4Aiii).

1 [http://www.cell.com/neuron/viewComment/S0896-6273\(07\)00587-9](http://www.cell.com/neuron/viewComment/S0896-6273(07)00587-9)

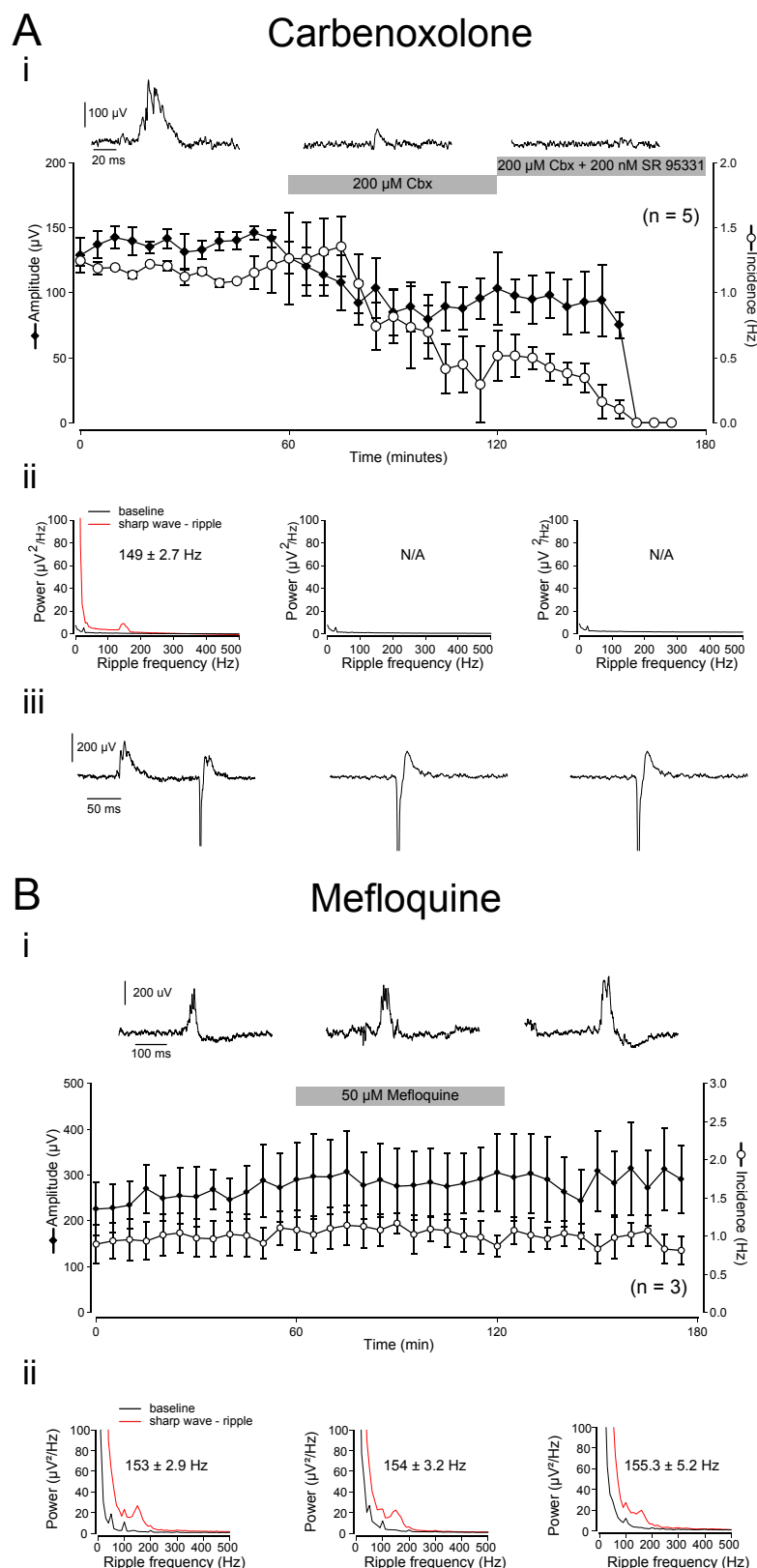


Figure 4.4 Gap junctions

(A) i, Example traces of sharp wave – ripples and plot of sharp wave amplitude and incidence during baseline, carbenoxolone (200 μ M) and combination of carbenoxolone (200 μ M) and SR 95331 (200 nM) conditions. ii, Example power spectra of significant ripple events (if present) and average ripple frequency during baseline and drug conditions. iii, Example traces of induced sharp wave – ripples (using monopolar stimulating electrode in the stratum pyramidale of CA3). Stimulation was used in cases where sharp waves were affected by the drug used. (B) Washin of mefloquine (50 μ M) did not affect sharp wave incidence, amplitude or ripple frequency. i, Example traces of sharp wave – ripples and plot of sharp wave amplitude and incidence during baseline, mefloquine (50 μ M) and washout conditions. ii, Example power spectra of significant ripple events and average ripple frequency during baseline, drug conditions and washout.

Application of the specific gap junction blocker mefloquine (50 μ M) did not have any effect on sharp wave amplitude, incidence (Figure 4.4Bi) or ripple frequency (Figure 4.4Bii). The experiments with mefloquine (50 μ M) were done in interface conditions, but were repeated in submerged conditions to investigate if the lack of an effect of mefloquine on sharp wave – ripples is dependent on the recording setup (as was the case with GYKI 52466). Wash-in of mefloquine (50 μ M) in submerged slices did not have any effect on sharp wave – ripples either ($n = 4$; data not shown).

As both blockers have been proposed to have the same targets, but their effects are different, these combined results are inconclusive as to the necessity for gap junctions in the generation of sharp wave – ripples.

4.9 Discussion

I investigated the role of several major excitatory and inhibitory receptors, as well as gap junctions, in generating sharp wave – ripples *in vitro*.

4.9.1 AMPA receptors

The AMPA / kainate receptor blocker NBQX and the specific AMPA receptor blocker GYKI 52466 both completely abolished all sharp wave – ripple activity, suggesting that AMPA receptor-mediated currents are necessary for sharp wave generation. This is not surprising as AMPA receptors are necessary for fast synaptic transmission between pyramidal neurons.

I found that the Ca^{2+} -permeable AMPA receptor blocker PhTx 433 significantly reduced the incidence of sharp waves. Ca^{2+} -permeable AMPA receptors have been shown to be highly expressed in interneurons whereas excitatory neurons predominantly express Ca^{2+} -impermeable AMPA receptors. Furthermore, it has been shown that input onto CA3 interneurons from the mossy fibres is mediated by Ca^{2+} -permeable AMPA receptors whereas input from CA3 pyramidal neurons is mediated by Ca^{2+} -impermeable AMPA receptors (Toth & McBain, 1998). The observation that the Ca^{2+} -permeable AMPA receptor blocker PhTx

433 (1 μM) significantly reduced sharp wave incidence suggests, perhaps, a role for CA3 interneurons in the generation of sharp waves (Toth & McBain, 1998; McBain *et al.*, 1999) and suggests that input from dentate gyrus might activate these interneurons. The idea that interneurons are involved in the generation of sharp wave – ripples will be further explored in Chapter 6 of this thesis.

4.9.2 NMDA receptors

My results show that NMDA receptors are not necessary for sharp wave generation. In fact, sharp wave amplitude was enhanced upon wash-in of the NMDA receptor antagonist D-AP5 (50 μM). This is consistent with a previous observation (Colgin *et al.*, 2005). The action of D-AP5 (50 μM) is counterintuitive; an antagonist of excitatory transmission which leads to an increased amplitude of an excitatory population burst. It was suggested that a reduction in the conductance of Ca^{2+} -dependent K^{+} channels by a reduced inflow of Ca^{2+} through NMDA receptors would lead to a more depolarized membrane potential of CA3 neurons. This depolarization of CA3 pyramidal neurons would result in a larger population of pyramidal neurons being recruited to a sharp wave (Colgin *et al.*, 2005). A larger population of pyramidal neurons taking part in a sharp wave would lead to larger amplitude sharp waves as recorded in the field. An alternative possibility is that block of NMDA receptors reduced interneuron activity leading to reduced inhibition and resulting in participation of a larger population of pyramidal neurons in a sharp wave. Recently, it has been shown that genetic ablation of NMDA receptors in CA3 pyramidal neurons as well as pharmacological blockade of NMDA receptors in the CA3 region made CA3 pyramidal neurons more excitable and made mice more susceptible to kainate induced seizures (Fukushima *et al.*, 2009). The authors suggest that NMDA receptors may suppress the excitability of the hippocampal CA3 recurrent network as a whole *in vivo* by restricting synchronous firing of CA3 neurons.

4.9.3 Metabotropic glutamate receptors

Sharp wave amplitude and incidence were modulated similarly by wash-in of 4-CPG (0.5 mM) as by the presence of this antagonist immediately after slicing, suggesting that group I mGluRs are not necessary for sharp wave induction, but rather modulate its expression. As this effect was mimicked by the mGluR5 antagonist MPEP (50 μ M), but not the mGluR1 antagonist CPCCOEt (100 μ M), it suggests that this modulation of sharp wave amplitude and incidence is mediated by mGluR5 receptors. Interestingly, when antagonists at both group I mGluRs and NMDA receptors were co-applied, I did not observe any significant modulation suggesting a possible interaction between these two receptor types (Alagarsamy *et al.*, 1999).

I did not continue the investigation into the precise mechanism of action of the group I mGluRs on sharp wave generation. Both the expression and proposed functions of these receptors are numerous. Firstly, these receptors can be found on both CA3 pyramidal neurons (Lujan *et al.*, 1996) and interneurons (van Hooft *et al.*, 2000). Secondly, their proposed functions range from switching CA3 neurons to burst mode of firing (Young *et al.*, 2004), to regulating intracellular $[Ca^{2+}]$ (Bianchi *et al.*, 1999), changing K^+ channel conductance (Guerineau *et al.*, 1995) and regulating AMPA/kainate receptor-mediated conductance (Taylor *et al.*, 1995) as well as changing gene expression (Lindemeyer *et al.*, 2006).

4.9.4 GABA_B receptors

As inhibition has been shown to be important in a variety of network oscillations (Mann & Paulsen, 2007) I set out to investigate if either GABA_B or GABA_A receptor-mediated inhibition plays a role in sharp wave generation. Wash-in of the GABA_B receptor antagonist CGP 52432 (2 μ M) led to a significant increase in sharp wave incidence. This finding is reminiscent of the observation that block of GABA_B receptor-mediated signalling increases the incidence of epileptiform population bursts (Menendez de la Prida *et al.*, 2006). It was suggested that a delayed GABA_B receptor-mediated conductance curtails the initiation of population bursts by opposing the excitatory drive in the network. Blocking GABA_B receptors would take this inhibitory opposing force away, thereby facilitating the excitatory drive in the network to

reach a threshold for burst initiation. Whether this mechanism holds for sharp wave – ripples as well requires more detailed investigation.

4.9.5 GABA_A receptors

To our surprise block of the phasic component of the GABA_A receptor-mediated current completely abolished all sharp waves. An enhancement in incidence or amplitude was expected, as intuitively blocking inhibition would enhance an excitatory population burst. Only when blocking both the phasic and tonic component of the GABA_A receptor-mediated current was an enhancement of bursting observed. The observation that enhancing inhibition, by application of pentobarbital or diazepam, led to an increased incidence of sharp waves further suggests that inhibition can be facilitating in the generation of sharp waves. The mechanism of action of pentobarbital and diazepam are different. Barbiturates (e.g. pentobarbital) increase the open time of the GABA_A receptor by acting at a distinct barbiturate binding site separate from the GABA binding site, resulting in acute potentiation of inhibitory GABAergic tone (Davies, 1995). In contrast, the benzodiazepine diazepam acts at the interface of the α - γ subunits of GABA_A receptor and increases the probability of opening (Korpi *et al.*, 1997).

Hippocampal pyramidal neurons express mostly $\alpha 1$, $\alpha 2$ and $\alpha 5$ subunit-containing GABA_A receptors (along with $\beta 1$ -3 and $\gamma 5$ -subunits). $\alpha 1$ subunit-containing GABA_A receptors are highly expressed in postsynaptic somato-dendritic synapses, $\alpha 2$ -subunit containing GABA_A receptors are highly expressed in the axon initial segment and $\alpha 5$ -subunit containing GABA_A receptors are highly expressed extra-synaptically (i.e. a diffuse expression with no aggregation at the postsynaptic sites) (Farrant & Nusser, 2005; Ali & Thomson, 2008). It has been shown for the soma of pyramidal neurons that $\alpha 1$ and $\alpha 2$ subunits can be segregated to synapses made by respectively fast-spiking parvalbumin-positive basket cells and regular-spiking cholecystokinin-positive basket cells (Fritschy & Brunig, 2003; Ali & Thomson, 2008). The observation that zolpidem (a GABA_A $\alpha 1$ agonist), but not L655,708 (a GABA_A $\alpha 5$ inverse agonist) influences sharp wave incidence suggests that at least part of the diazepam induced increase in sharp wave incidence is mediated by the GABA_A $\alpha 1$ subunit containing GABA_A receptors. The GABA_A $\alpha 2$ subunit containing GABA_A receptors might play a role

as well, but at present there is no selective agonist or antagonist commercially available for this subunit and it is therefore difficult to study. The GABA_AR $\alpha 5$ subunit-containing GABA_A receptors are most likely not involved in the diazepam induced increase in sharp wave incidence as the GABA_AR $\alpha 5$ inverse agonist L655,708 does not affect sharp wave incidence. Moreover, it has been shown that hippocampal slices taken from mice in which the GABA_AR $\alpha 5$ subunit has been knocked out still generate bursting activity similar to the sharp waves, including 200 Hz ripple oscillations (Glykys & Mody, 2006).

There are several possible explanations why GABA_A receptors are necessary for sharp wave generation. First, it is possible that GABA could have a directly depolarising, excitatory effect, as can be seen in young animals, as well as in certain neuronal types and cortical states in adult brain (Cohen *et al.*, 2002; Gullledge & Stuart, 2003; Wozny *et al.*, 2003; Banke & McBain, 2006; Szabadics *et al.*, 2006). However, there is no good evidence that GABA is excitatory under our conditions. We used mature rats in which pyramidal cells most likely exhibit a GABA_A receptor reversal potential which is negative to resting membrane potential (Luhmann & Prince, 1991; Owens *et al.*, 1996). This is consistent with the observation that, under our conditions, blocking GABA_A receptors significantly depolarised pyramidal neurons. Nevertheless, none of these observations exclude the possibility that GABA might be excitatory in a subset of pyramidal neurons that were not recorded by us, or that GABA could be depolarising in specific subregions of the pyramidal neuron (e.g. axon initial segment; Szabadics *et al.*, (2006); but see Glickfeld *et al.*, (2009)). These possibilities are difficult to address directly experimentally with currently available techniques (Howard *et al.*, 2005). Hyperpolarising GABAergic events could also promote population bursting, either by lowering the spike threshold in individual pyramidal neurons (Bean, 2007), or by producing co-ordinated membrane potential fluctuations in neurons, seen as RMS noise, which would facilitate spike synchrony within a population of pyramidal neurons (Aradi & Maccaferri, 2004; Ermentrout *et al.*, 2008). In these ways phasic GABA_A receptor-mediated inhibition could facilitate mutual synaptic excitation.

These results suggest that GABA_A receptor-mediated inhibition is necessary for sharp wave generation although the precise mechanism of action is unknown. We will explore the mechanisms underlying the inhibitory control on sharp wave initiation further in Chapter 6.

4.9.6 Gap junctions

Gap junctions between interneurons have been shown to exist (Katsumaru *et al.*, 1988; Venance *et al.*, 2000; Zsiros *et al.*, 2007). Gap junctions between pyramidal neurons have been suggested to exist as well, as occasionally spikelets and dye coupling can be observed between neighbouring principal neurons (Schmitz *et al.*, 2001). If they are functional (for network oscillations in particular) is matter of debate (Buzsaki, 2001), although modelling studies have suggested their involvement in both burst and ripple generation (Draguhn *et al.*, 1998; Tseng *et al.*, 2008).

Gap junctions are made up of two connected connexons, each connexon is located in the membrane of two apposing cells. The connexons are homo- or heterohexamers of connexin (Cx) proteins. If two connexons are not connected they do not form a gap junction, but can exist as hemichannels which can be fully functional in their own right e.g. in cell migration (Elias *et al.*, 2007). Gap junctions or hemichannels could well be the remnants of young new neurons (Lledo *et al.*, 2006) or the result of pathological changes (Rouach *et al.*, 2002). Recently, it was shown that a hemichannel formed by pannexin-1 subunits could augment aberrant bursting (Thompson *et al.*, 2008).

To investigate the role of gap junctions in the generation of sharp wave – ripples, I applied blockers of these junctions. The first blocker I used was carbenoxolone (200 μ M) which blocked most of the sharp wave – ripples. The drawback of using this drug is that it has several unspecific effects, one of which is increasing the threshold for action potential firing (Rouach *et al.*, 2003). The observation that sharp wave – ripples disappear upon wash-in of carbenoxolone might well be due to these unspecific effects. Therefore, the more specific (especially for Cx36 and Cx50) gap junction blocker mefloquine (50 μ M) was used. This blocker had no effect on sharp wave incidence, amplitude or ripple frequency. It has been shown that this drug results in a slightly increased spontaneous activity, but it is hard to get into the tissue and slices have to be incubated with the drug for more than 1 hour, which I did (Cruikshank *et al.*, 2004; Urbano *et al.*, 2007).

It has been shown that slices from the rat hippocampus can generate fast network oscillations (125 – 333 Hz) in CA1 which have been suggested to depend on gap junctions,

as they could be blocked by octanol (1 mM), halothane (5 mM) and carbenoxolone (100 μ M), but not by the glutamate receptor blockers NBQX (20 μ M) and AP5 (40 μ M) nor by the GABA_A receptor antagonist bicuculline (50 μ M) or changing to Ca²⁺ free medium. Furthermore, enhancing gap junction conductance by intracellular alkalization enhanced the fast network oscillation. Finally, modelling showed that fast coupling, most likely by axo-axonic connections between principal cells, could generate these oscillations (Draguhn *et al.*, 1998). However, more recently, using mouse hippocampal slices, the same group showed that the AMPA receptor blocker CNQX (20 μ M) and the GABA receptor blockers bicuculline (20 μ M) or SR 95331 (3 μ M) were able to abolish both CA3 sharp waves and their associated ripples, suggesting a role for synaptic transmission after all. The gap junction blockers carbenoxolone (200 μ M) and 1-octanol reduced, but did not abolish sharp waves and associated ripples. The concentrations of bicuculline (20 μ M) and SR 95331 (3 μ M) were sufficient to induce epileptiform bursting however, most likely by affecting tonic inhibition, and therefore the detailed role of phasic inhibition on ripple generation in CA3 remains unknown (Maier *et al.*, 2003). This was partly addressed by the subsequent publication by this group (Nimmrich *et al.* 2005) which studied sharp wave – ripple generation in CA1 mini-slices (CA1 mini-slices do not contain area CA3) of mice. The authors showed that 300 nM SR 95331 blocked spontaneous sharp wave – ripples in CA1 of these slices, suggesting that phasic GABAergic transmission is necessary for sharp wave – ripple generation. However, they also showed that during superfusion with 1 μ M SR 95332 (which blocks all phasic GABA_A receptor-mediated inhibition, see Figure 4.1E, page 70), brief puffs of KCl (1M) in the stratum pyramidale could induce both sharp waves and their associated ripples. This suggested again that ripples are not the result of phasic GABAergic transmission (synchronous IPSCs) on pyramidal neurons, but were the result of gap junctions, as further application of glutamate receptor blockers did not block this induced network pattern (Nimmrich *et al.*, 2005). In conclusion it was suggested that a combination of excitatory and inhibitory synaptic transmission as well as gap junction coupling was necessary for sharp wave – ripple generation.

The observation that synchronous activity can persist without synaptic transmission does not necessarily imply gap junctions, as ephaptic interactions could also synchronize

population activity. Furthermore, modelling studies have suggested that interneuron - interneuron synaptic interactions can lead to 200 Hz oscillations as recorded in the field without the need for gap junctions (Brunel & Wang, 2003). Moreover, a knockout mouse for connexin 36 (Cx36), the major neuronal gap junction protein (Katsumaru *et al.*, 1988; Rash *et al.*, 2000; Venance *et al.*, 2000; Deans *et al.*, 2001) had normal sharp waves and associated ripples (Hormuzdi *et al.*, 2001; Maier *et al.*, 2002; Buhl *et al.*, 2003). Of course, it could be argued that the role of Cx36 has been taken over by another subunit (Maier *et al.*, 2002). However, there is supporting evidence that phasic synaptic inhibition could play a role in generating ripple oscillations: firstly, basket cells fire at ripple frequency (Ylinen *et al.*, 1995), secondly, firing of pyramidal neurons is phase related with the field ripple (Ylinen *et al.*, 1995), thirdly, ripples emerge when GABA_A receptor-mediated currents change from being excitatory to inhibitory during development (Buhl & Buzsaki, 2005).

Thus, my results, and the scientific literature, are not conclusive as to whether gap junctions are necessary for sharp wave – ripple population bursts. This state of affairs will probably remain thus until efficient and selective gap junction blockers have been developed.

4.10 Summary

These results show that both AMPA receptor-mediated events and GABA_A receptor-mediated events are necessary for sharp wave initiation *in vitro*, but they remain inconclusive as to whether gap junctions are involved. In the next chapter I will explore the excitatory and inhibitory currents involved in sharp wave generation in further detail.

Neuronal input and participation

5.1 Introduction

Field recordings of sharp wave – ripples, from both planar multi-electrode array as well as interface recordings, revealed them to be small and local events. Pharmacological study of the receptor types involved in sharp wave generation revealed that AMPA receptor-mediated events are obligatory for sharp wave generation. These observations suggested that sharp waves are the result of synchronous firing of a small localized group of pyramidal neurons. To investigate the role of pyramidal neurons in generating a sharp wave event further, planar multi-electrode array recordings of sharp wave activity were combined with cell-attached recording and subsequent whole-cell current-clamp recording of individual CA3 pyramidal neurons.

Pharmacological study not only revealed that AMPA receptor-mediated events are obligatory for sharp wave generation, but GABA_A receptor-mediated events as well. This suggested that interneurons have a hitherto unidentified role in sharp wave generation. To investigate the role of both phasic excitation and inhibition, not only in pyramidal neurons, but in all classes of CA3 neuron, we recorded in whole-cell voltage-clamp mode from anatomically identified CA3 pyramidal neurons and CA3 interneurons.

5.2 Participating pyramidal neurons

To investigate the role of pyramidal neurons in generating sharp wave – ripples I recorded from a total of 53 anatomically-identified pyramidal neurons distributed over the entire CA3 area (Figure 5.1A). I recorded the spiking behaviour of the pyramidal neurons to ongoing sharp wave activity in cell-attached mode and the subthreshold events in whole-cell current-clamp mode (Figure 5.1B). Most of the pyramidal neurons recorded remained silent throughout the entire recording period, even when the sharp waves were occurring local to the area of the pyramidal neuron. Only 7 out of 53 pyramidal neurons recorded (13.2%) fired action potentials. These 7 cells fired single action potentials in phase with a sharp wave event, but only in $6.4 \pm 3.0\%$ of all observed sharp waves (Figure 5.2B). The remaining 86.8% of pyramidal neurons (46 out of 53) were silent during all sharp wave events. Subsequent whole-cell recordings showed that, during sharp wave activity, on average, a pyramidal neuron received strong inhibitory input in $34.4 \pm 4.0\%$ and no detectable synaptic input in $50.4 \pm 4.2\%$ of sharp waves. Only a small number of sharp waves were associated with subthreshold ($15.1 \pm 2.0\%$) or suprathreshold ($0.2 \pm 0.1\%$) excitation (Figure 5.2C, D). Not only was inhibition the dominant input, but it was also strongly correlated between pairs of pyramidal neurons (Figure 5.2E).

As I observed strong inhibition of pyramidal neurons during local sharp wave activity I asked whether a population of non-participating pyramidal neurons might be actively silenced during a sharp wave event. I therefore calculated the ratio of excitation to inhibition that a pyramidal neuron received (see Chapter 2, page 38), and plotted this ratio against the distance between the pyramidal neuron and the location of the sharp wave (Figure 5.2F, $n = 53$ cells). I observed that, when sharp waves originated at a distance of 150 μm or more, a pyramidal neuron predominantly received inhibition (ratio of excitation to inhibition, 0.8 ± 0.07 , 0.6 ± 0.14 , 0.6 ± 0.11 and 0.4 ± 0.10 at 150, 300, 450 and 600 μm distance respectively, for 150 μm ; $p < 0.01$; one-sample t test; $n = 21$ and for 450 and 600 μm $p < 0.05$; one-sample t test; $n = 7$ and $n = 12$). Only when sharp waves occurred less than 150 μm from the patched pyramidal neuron did it receive more excitation (1.5 ± 0.2 ; $p < 0.05$; one-sample t test; $n = 20$).

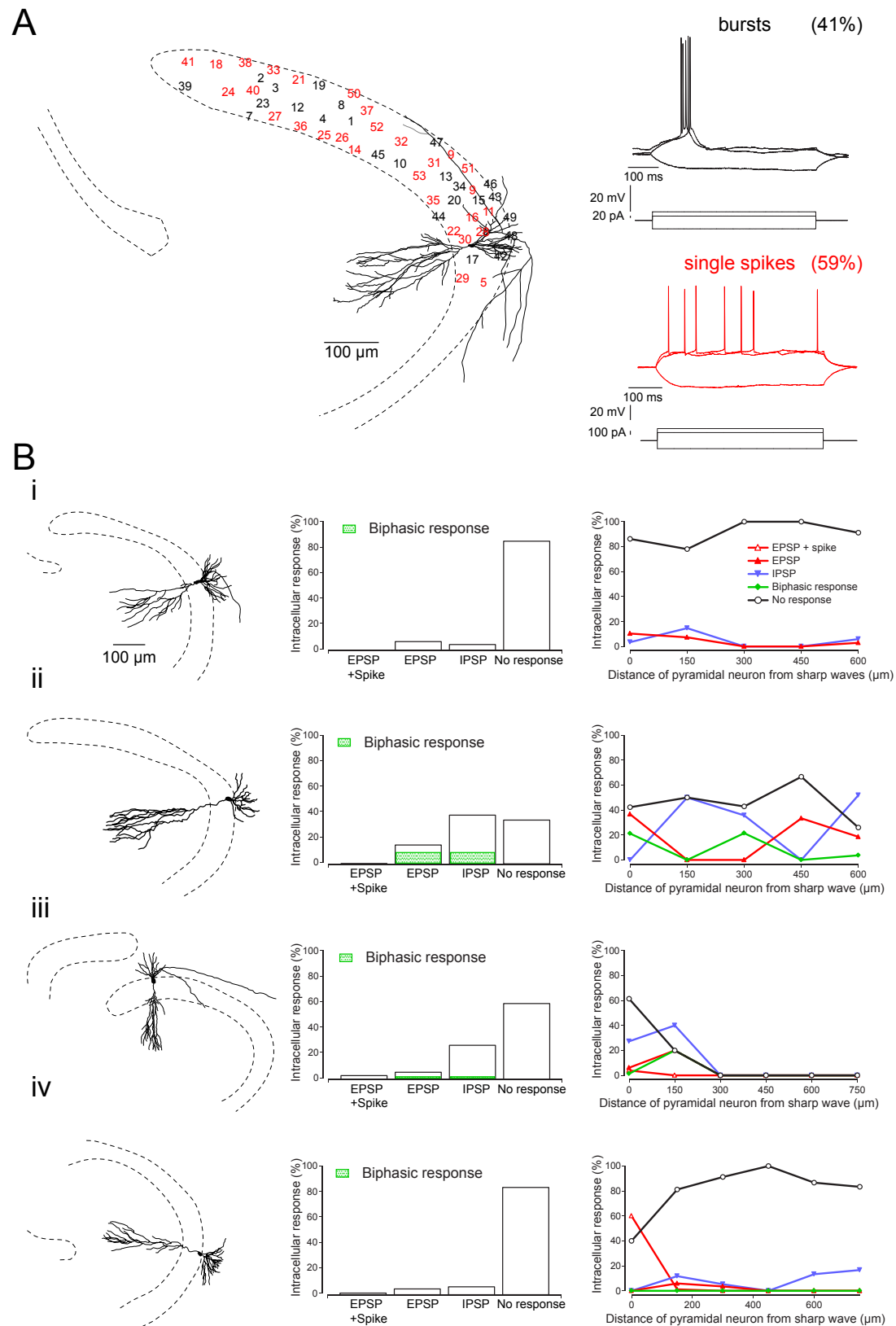


Figure 5.1 Location and response of patched pyramidal neurons to sharp waves

(A) Location of the patched pyramidal neurons color coded by their response to a depolarising current injection. 41% of pyramidal neurons responded with a burst (black), with the remaining 59% producing single spikes (red). The response was not correlated to location within CA3.

(B) Pyramidal neurons mostly received inhibition during sharp waves or were silent. Four examples (i - iv) of individual pyramidal neurons and their input during sharp waves. Left, Single pyramidal neuron as visualized with biocytin labeling. Middle, Distribution of sharp wave associated intracellular activity in pyramidal neurons revealed most events to be inhibitory or associated with no detectable input. Right, Distribution of sharp wave associated intracellular activity in pyramidal neurons as a function of distance from sharp wave events.

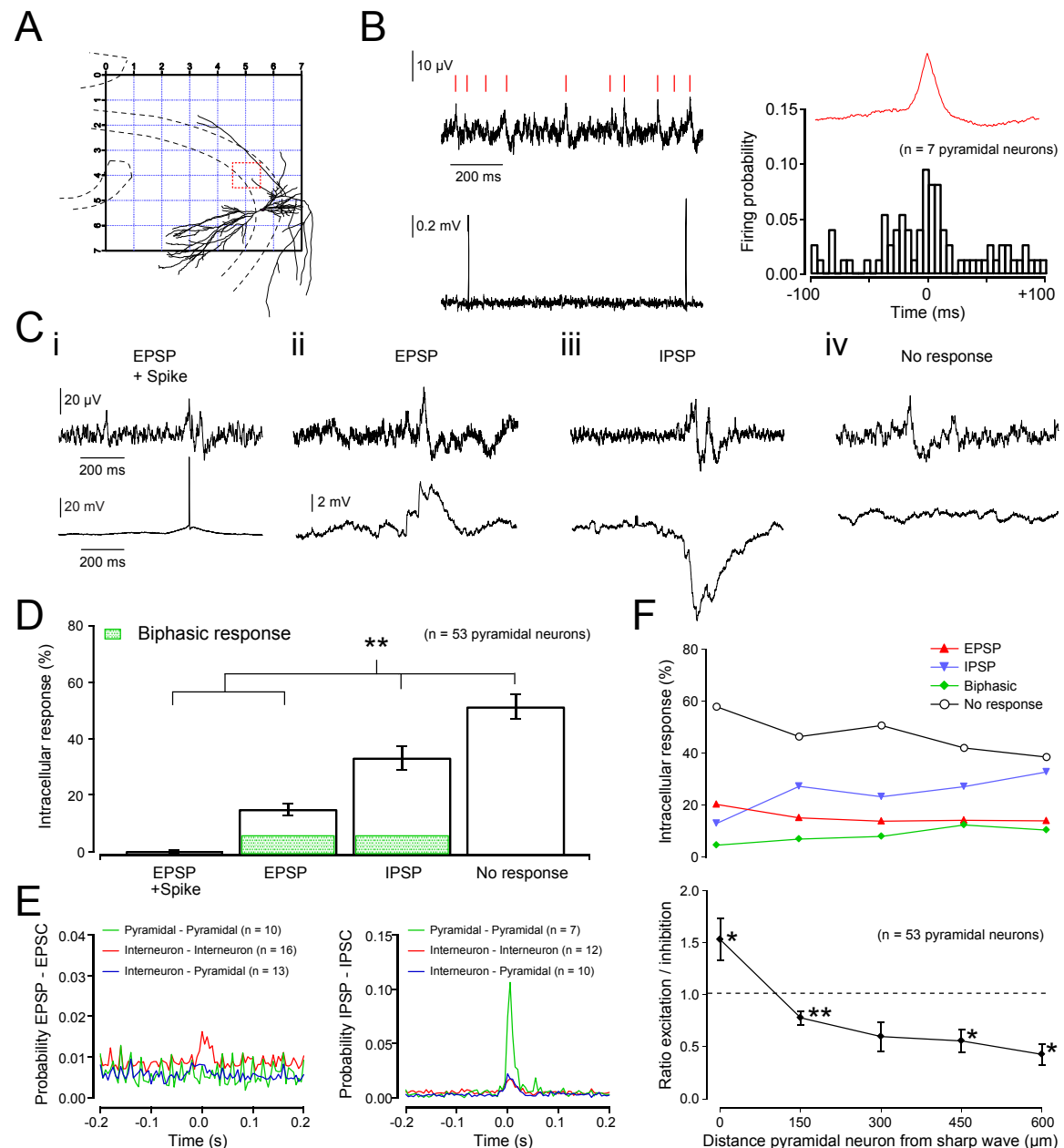


Figure 5.2 Pyramidal neurons mostly receive inhibition during sharp waves

(A) Diagram of an 8 x 8 planar multi-electrode array with the location of a single pyramidal neuron as visualized with biocytin labeling.

(B) Left, Trace with sharp waves as recorded from red boxed electrode in A (top) and spiking of pyramidal neuron recorded in cell-attached configuration (bottom). Right, Plot of average firing probability for 7 pyramidal neurons centered on the peak of a sharp wave. Mean firing rate of participating pyramidal neurons was 0.3 Hz.

(C) Example traces of whole-cell current-clamp recordings demonstrating sharp wave-associated events. Pyramidal neurons could show spikes (i) or EPSPs (ii), but mostly hyperpolarised (iii) or did not respond (iv) during sharp waves.

(D) Distribution of sharp wave-associated intracellular activity in pyramidal neurons (spikes, $0.16 \pm 0.1\%$; EPSPs, $15.1 \pm 2.0\%$; IPSPs, $34.4 \pm 4.0\%$ and no response, $50.4 \pm 4.2\%$; ANOVA; $p < 0.05$; $n = 53$; pairwise comparisons: IPSP vs. EPSP & EPSP+spike $p < 0.01$ and No input vs. EPSP & EPSP+spike $p < 0.01$, Student's t test). Of all subthreshold events, 9.1 % were biphasic responses consisting of an IPSP – EPSP or EPSP – IPSP sequence.

(E) Cross correlation probability plots of synchronous excitatory (left) and inhibitory (right) input between pairs of neurons. Note the largest correlation (0.11) for inhibitory inputs between pairs of pyramidal neurons.

(F) Top, Distribution of sharp wave associated intracellular activity in pyramidal neurons as a function of distance from sharp wave events. Bottom, Ratio of total excitatory and total inhibitory input of pyramidal neurons as a function of the distance from sharp wave events.

The fact that many pyramidal neurons did not receive detectable synaptic input during sharp wave activity could be the result of severing the recurrent connections during the slicing procedure. To exclude this possibility, phasic and tonic GABA_A receptor-mediated inhibition was blocked with SR 95531 (1 μ M). This led to the generation of epileptiform bursting in CA3, with all patched pyramidal neurons ($n = 20$) taking part in the epileptiform event and firing at high frequency, including those that received no input or inhibition during sharp waves (see Figure 4.3, page 75) (Menendez de la Prida *et al.*, 2006; Wittner & Miles, 2007). This result suggests that excitatory synapses onto the recorded pyramidal neurons were present and that connections between pyramidal neurons are retained, supporting the conclusion that only a small group of pyramidal neurons fire during any individual sharp wave event while the remaining population of pyramidal neurons is kept silent though GABA_A receptor-mediated inhibition.

5.3 Dendritic response of pyramidal neurons

Somatic recordings of pyramidal neurons revealed that pyramidal neurons fire single action potentials during sharp wave activity. It has been suggested, however, that during sharp wave – ripple activity, dendritic regenerative events might also occur, as has been shown for CA1 pyramidal neurons *in vivo* (Kamondi *et al.*, 1998). Mammalian dendrites are active structures capable of regenerative electrical activity (Euler & Denk, 2001; London & Hausser, 2005) and besides sodium-based action potentials, which can back propagate throughout the dendritic tree, cortical pyramidal neurons can also sustain local dendritic Na⁺ and Ca²⁺ spikes. The presence of local regenerative events would have implications for how we think the sharp wave field in CA3 is generated, as well as the rules for synaptic plasticity during this activity.

I therefore patched the apical dendrites of anatomically identified CA3 pyramidal neurons to investigate if local dendritic spikes can be observed concurrent with sharp wave activity. Patching of the apical dendrite of CA3 pyramidal neurons at a distance of 50 – 100 μ m from the soma revealed that the field sharp wave activity was associated with the dendritic

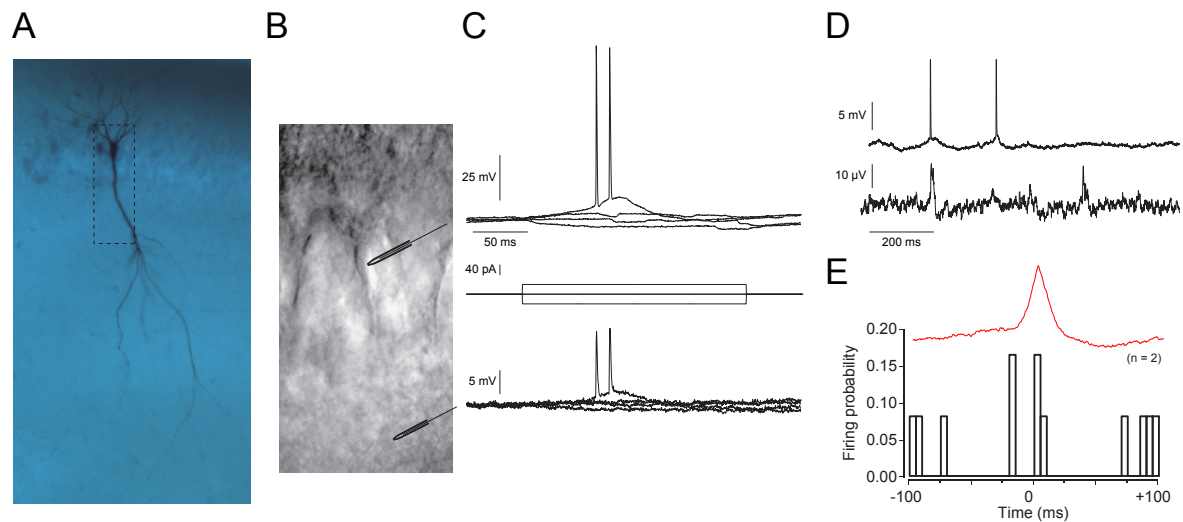


Figure 5.3 Sharp waves are associated with dendritic backpropagation of fast Na⁺ spikes

(A) CA3 pyramidal neuron as visualised after biocytin labeling. (B) Same pyramidal neuron as visualised with IR-DIC with locations of patch electrodes (average distance between the somatic and dendritic recording site was 50 – 100 μm). (C) Recording of neuronal response to current steps injected at the soma. (D) Occasionally sharp waves were associated with fast Na⁺ spikes in the dendrites (spike width was ~1.2 ms; delay between somatic and dendritic spike was ~0.3 ms). (E) 2 out of 8 patched pyramidal cell apical dendrites showed single spikes concurrent with field sharp waves.

back propagation of fast Na⁺ spikes in some pyramidal neurons (2 out of 8 pyramidal neurons). The remaining pyramidal neurons were silent during sharp wave activity. I did not observe any dendritic Ca²⁺ spikes associated with sharp wave activity (Figure 5.3). Although I can not exclude the possibility that Ca²⁺ spikes might occur in pyramidal neurons not patched, these results suggest that Ca²⁺ spikes are not a regular feature during sharp wave – ripple activity.

5.4 Synaptic input on CA3 neurons

Pharmacological study of the receptor types involved in sharp wave generation revealed that both AMPA and GABA_A receptor-mediated events are obligatory for sharp wave generation. To investigate the involvement of both phasic excitation and inhibition for sharp wave generation in further detail, whole-cell patch-clamp recordings in voltage-clamp mode from anatomically-identified pyramidal neurons, perisomatic-targeting interneurons (PTI), dendritic-targeting interneurons (DTI) and putative interneuron-selective interneurons (ISI) in hippocampal CA3 were combined with simultaneous recording of sharp wave activity with

planar multi-electrode arrays (5.4 A, B).

I found that all four major cell types received both excitatory and inhibitory synaptic input during sharp wave activity (Figure 5.4 A, B). The excitatory and inhibitory synaptic conductance was quantified by estimating the average excitatory and inhibitory synaptic charge transfer during baseline and per sharp wave event (see Chapter 2). This analysis revealed that pyramidal neurons on average received a significantly larger inhibitory than excitatory charge transfer per sharp wave event (0.75 ± 0.1 pC versus 0.16 ± 0.03 pC; independent-samples t test; $p < 0.01$; $n = 10$ and $n = 18$). Interneurons in CA3 received a more balanced input of excitation and inhibition during sharp waves (PTI, 0.27 ± 0.05 pC and 0.30 ± 0.06 pC; DTI, 0.18 ± 0.02 pC and 0.12 ± 0.01 pC; and ISI, 0.30 ± 0.16 pC and 0.46 ± 0.2 pC; Figure 5.4 C).

I also plotted the frequencies of both EPSCs and IPSCs (in 10 ms bins) relative to the peak of a sharp wave event. This analysis showed that all neuron types received an increased synaptic input during a sharp wave event with the average frequency of EPSCs increasing from 2.8 ± 0.3 Hz at baseline to 9.7 ± 1.9 Hz during sharp waves and IPSCs from 2.5 ± 0.3 Hz at baseline to 15.4 ± 3.8 Hz during sharp waves (Figure 5.4 D). The detailed time course of the EPSC and IPSC frequency (in 100 ms bins and normalized to the frequency at the start of a sharp wave), showed a reduction just before sharp wave initiation of both excitatory and inhibitory synaptic inputs in all cell types and in the majority of cells. Both the average EPSC and IPSC frequency showed a minimum ~ 200 ms prior to the initiation of a sharp wave event (respectively 85.6 ± 3.4 % and 88.5 ± 4.9 %; $n = 40$ and $n = 31$) (Figure 5.4 E). In current-clamp, a trend towards hyperpolarisation was seen prior to sharp wave initiation in the majority of neurons (Figure 5.4 F). We did not observe a plateau and build-up period as seen prior to epileptiform bursts (Menendez de la Prida *et al.*, 2006).

These results show a temporary reduction in synaptic activity ~ 200 ms prior to sharp wave initiation and an increased synaptic input during sharp wave activity in all neuron types in CA3.

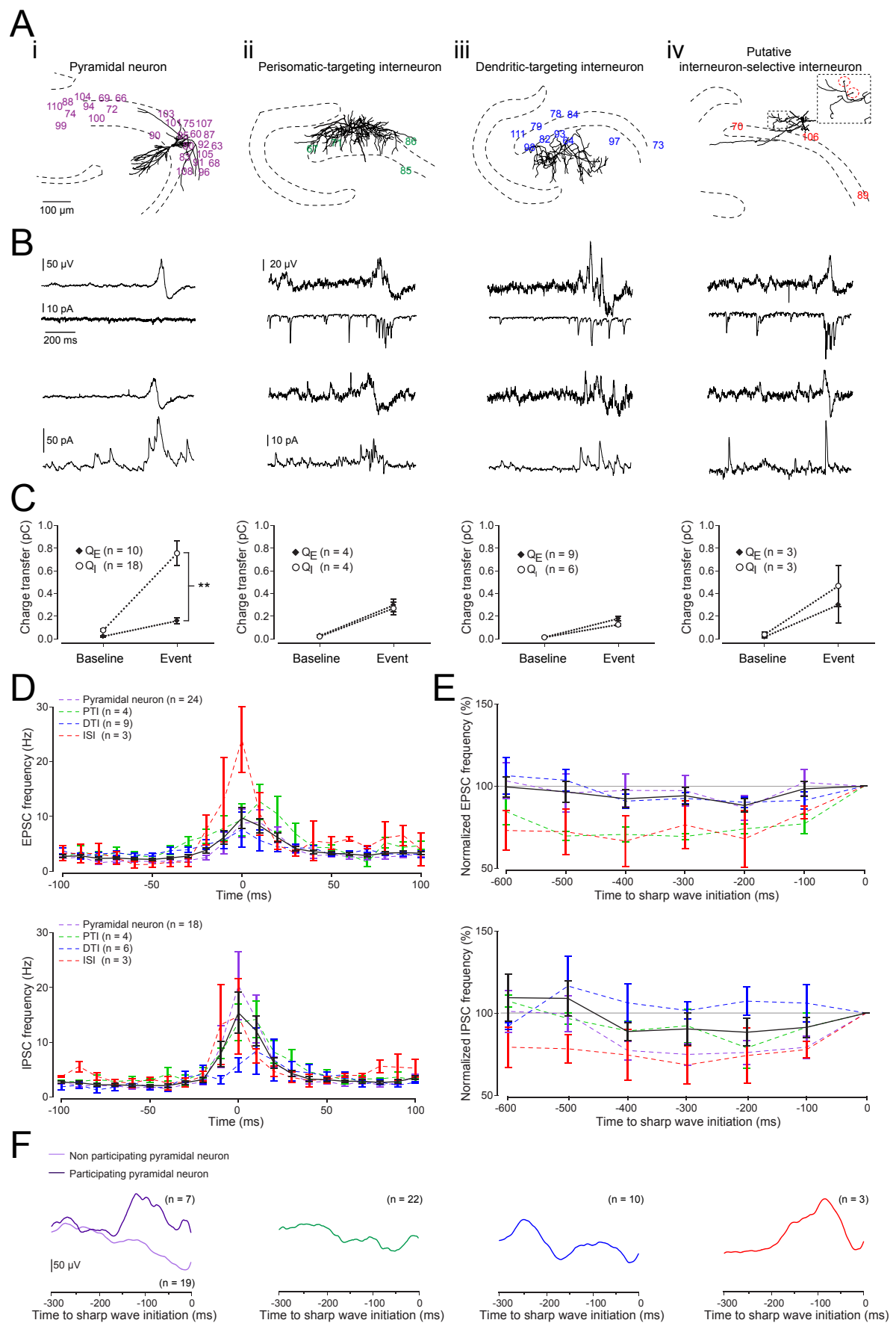


Figure 5.4 Both CA3 pyramidal neurons and interneurons show a significant reduction in synaptic input prior to a sharp wave

(A, B) Voltage-clamp recordings from anatomically-identified pyramidal neuron (i), perisomatic-targeting interneuron (ii), dendritic-targeting interneuron (iii), putative interneuron-selective interneuron (iv), showing (B) EPSCs ($V_{\text{hold}} = -65$ mV; top traces) and IPSCs ($V_{\text{hold}} = 0$ mV; bottom traces) during sharp wave events. Coloured numbers indicate locations of whole-cell voltage-clamped neurons. Inset for iv. shows magnified reconstruction of putative interneuron-selective interneuron and circled in red the varicose axon collaterals and drumsticklike axon terminals (top)

(C) Charge transfer during baseline and sharp wave events in the different neuron types of CA3. Note that charge transfer increases in all neuron types during sharp waves relative to baseline.

(D) The frequency of excitatory (top) and inhibitory events (bottom) increased during a sharp wave event. Different coloured lines indicate different cell types with the black line indicating the average response over all cell types. 0 ms refers to the peak of the sharp wave.

(E) Plot of the normalised frequency of excitatory (top) and inhibitory (bottom) events prior to the start of a sharp wave. Note a general reduction in EPSC and IPSC frequency, with a minimum for both at 200 ms prior to sharp wave initiation. 0 ms refers to the start of a sharp wave.

(F) Membrane voltage of anatomically identified neurons reveal that there is a trend for them to be hyperpolarised prior to sharp wave initiation. See Chapter 6 for locations of whole-cell current-clamped neurons.

5.5 Discussion

5.5.1 Participating pyramidal neurons

Only a small number of pyramidal neurons (13.2%) fired action potentials concurrently with field sharp waves and only in 6.4% of sharp waves. This compares to the observations *in vivo* that the majority of CA1 hippocampal neurons only fire with less than 15% of sharp wave events (Nadasdy *et al.*, 1999) and that during most ripple events in CA1 10% of detected pyramidal neurons take part (Ylinen *et al.*, 1995).

The approximate number of CA3 pyramidal neurons present in a 400 μm brain slice was estimated. Assuming the CA3 pyramidal layer consists of 3 rows of pyramidal cells (each 20 μm in diameter) and the total length of the CA3 pyramidal cell layer is 1.2 mm (1200 μm) then the total number of pyramidal neurons would be $(1200/20 \times 3) \times 400/20 = 3600$ pyramidal neurons. Assuming the top and bottom layers were damaged during the slicing conditions this would suggest that there are approximately 3000 pyramidal neurons alive. Our observation that sharp waves covered approximately 450 μm or one third of the pyramidal cell layer suggests that a maximum of 1000 pyramidal neurons are in the area of sharp wave generation *in vitro*. If the 53 pyramidal neurons we patched are a representative sample of the total pool of pyramidal neurons taking part we would expect that approximately 400 pyramidal neurons (13.2% of 3000) took part in any sharp wave. If one assumes that pyramidal neurons fire in

only 1 out of 15 sharp waves (6.4%) then the total number of pyramidal neurons that take part in any sharp wave might be as low as $400/14 = 27$ neurons. The percentage of the total population of pyramidal neurons taking part in a sharp wave, as well as the percentage of sharp waves they take part in, is equal to or less than has been reported *in vivo* (Ylinen *et al.*, 1995; Nadasdy *et al.*, 1999; Csicsvari *et al.*, 2000). This could be explained by the reduced connectivity present in a hippocampal slice relative to an intact hippocampus. Also it must be noted that observations *in vivo* only observe active units. Units which are silent throughout the experiment are never included in the total population.

The study by Foffani *et al.* (2007) suggested a higher percentage of participating pyramidal neurons during sharp wave – ripples *in vitro*. They find that 31% (11 out of 36 pyramidal neurons) take part during sharp wave – ripples. This difference might be attributed to differences in ionic conditions of the ACSF (4 mM $[K^+]$, 1 mM $[Ca^{2+}]$ and 1 mM $[Mg^{2+}]$). Although we did not find any significant changes in the field sharp wave characteristics upon changing the buffer conditions (Chapter 3, Figure 3.10, page 60), we did not test the exact buffer conditions used by this group. It might also be that the way they sampled their population of pyramidal neurons; by blind patching, would involve the sample of pyramidal neurons deep inside the tissue with increased connectivity or excitability.

The majority of patched pyramidal neurons fired single spikes during current injection to rheobase. It is commonly assumed that the majority of CA3 pyramidal neurons (unlike CA1 pyramidal neurons) tend to fire bursts of action potentials (Shepherd, 2004) instead of single spikes. However, recent evidence suggests that the burst preference of CA3 pyramidal neuron is lower than previously thought, and that bursts of spikes only occur when the neurons are very depolarized (Hemond *et al.*, 2008). Sharp waves, consisting of the synchronous firing of a population of pyramidal neurons, could well reach this high threshold assuming sufficient conversion onto its target cells, but this high conversion might not be retained in slices.

Although a subset of pyramidal neurons fired, the majority of pyramidal neurons were silent during sharp wave activity. Plotting the ratio of excitatory input to inhibitory input against the distance of the pyramidal neuron relative to the sharp wave, suggests that during sharp wave activity the surrounding non-participating pyramidal neurons are actively inhibited.

This is further corroborated by the observation that application of a high concentration of the GABA_A receptor blocker SR 95331 leads to an increased participation of pyramidal neurons, such that previously silent or inhibited neurons took part during all events. The source of this lateral inhibition is unknown and could be mediated by perisomatic-targeting interneurons, dendritic-targeting interneurons or another type of inhibitory neuron. The idea that a large group of non-participating neurons are actively silenced by inhibition is further corroborated by the observation that only inhibition seems to be highly correlated between CA3 pyramidal neurons.

These combined results suggest that CA3 sharp waves are the result of action potential firing in a small population of CA3 pyramidal neurons whilst the surrounding non-participating pyramidal neurons are actively inhibited.

5.5.2 Dendritic patching

It has been suggested that the synchronous firing of a population of pyramidal neurons (e.g. during a sharp wave) would be able to induce dendritic Na⁺ or Ca²⁺ regenerative events (Gasparini *et al.*, 2004). It was shown *in vivo* that large-amplitude fast spikes in CA1 pyramidal neuron apical dendrites occurred during population discharges of CA3 – CA1 pyramidal neurons concurrent with field sharp waves (Kamondi *et al.*, 1998). These large amplitude fast spikes were often followed by bursts of smaller amplitude spikes and putative Ca²⁺ spikes, and it was suggested that these Ca²⁺ spikes were the result of activation of high threshold Ca²⁺ channels by the large amplitude fast spikes (Kamondi *et al.*, 1998). The function and induction of these Ca²⁺ spikes is still a matter of debate. The dendritic spikes and associated Ca²⁺ inflow have been suggested to facilitate LTP (Kamondi *et al.*, 1998; Mehta, 2004) and LTD induction (Holthoff *et al.*, 2004) and may represent a cellular mechanism for rapid information storage. They might be generated by back propagation of somatically generated spikes. It has been shown that somatically generated spikes back propagate to the dendrites (Stuart & Sakmann, 1994) but this back propagation deteriorates very rapidly, especially at dendritic branching points (Spruston *et al.*, 1995b) and at more distal parts of CA1 pyramidal neurons (Jaffe *et al.*, 1992). The other possibility would be that these spikes are generated

locally, e.g. some dendritic Na^+ spikes occur before axonal action potential initiation and occasionally occur without somatic action potentials (Golding & Spruston, 1998).

To investigate if CA3 sharp wave – ripples *in vitro* were associated with regenerative events, dendritic patch-clamp recordings in current-clamp mode were made from the apical dendrite of CA3 pyramidal neurons. We observed that sharp waves were associated with back propagating Na^+ spikes in 2 out of 8 dendrites. The remaining dendrites were silent during sharp wave activity. We did not observe dendritic Ca^{2+} spikes. However our findings do not exclude the possibility that dendritic Ca^{2+} spikes might occur in pyramidal neurons not patched by us or at more distal parts of the apical dendrite. It proved difficult to reach more distal parts of the apical dendrite due to the fact that CA3 pyramidal neurons dendrites branch relatively early and decrease in diameter rapidly. Previous publications also managed a distance of 50 – 100 μm from the soma (Spruston *et al.*, 1995a; Urban & Barrionuevo, 1998). Moreover these results do not exclude the possibility that dendritic Ca^{2+} spikes might occur in CA3 pyramidal neurons *in vivo* (Bar-Yehuda *et al.*, 2008) or in neurons of older animals, as all publications regarding dendritic Ca^{2+} spikes have used older animals than we used (5 weeks and older; Perez-Garci *et al.*, 2006; Kamondi *et al.*, 1998; Gasparini *et al.*, 2006) and our inability to observe them might stem from the fact that the animals we used were too young (2 to 3 weeks). These results do suggest, however, that dendritic Ca^{2+} spikes are not necessary for generation of CA3 sharp waves *in vitro*.

5.5.3 Synaptic input on CA3 neurons

To investigate the excitatory currents and inhibitory currents CA3 neurons receive during sharp wave – ripple activity, neurons were clamped respectively at the reversal potential for inhibitory currents (-65 mV) and the reversal potential for excitatory currents (0 mV).

All CA3 neurons received an increase in excitatory and inhibitory charge transfer during a sharp wave. Whereas interneurons received a balanced increase of both excitation and inhibition, pyramidal neurons received significantly more inhibition. This again suggests that a large population of pyramidal neurons is actively silenced during sharp wave activity. Plotting the frequency of excitatory events (EPSCs) and inhibitory events (IPSCs) relative to

the peak of the field sharp wave revealed that all neurons receive an increase in synaptic input during a sharp wave. Again, inhibition was dominant, as the average frequency of inhibitory events was higher than that of excitatory events. Interestingly, the perisomatic-targeting interneurons received an increase in EPSC frequency before any of the other neurons, as well as a continued high frequency over a much broader time-period than most other neurons, suggesting they might be the first and the last neurons to be active during a sharp wave. Secondly, plotting the frequency of excitatory events (EPSCs) and inhibitory events (IPSCs) prior to sharp wave initiation revealed that all CA3 neurons receive a temporary reduction in synaptic activity ~200 ms prior to sharp wave initiation. This is corroborated by the observation that the membrane potential of most neurons shows a tendency towards hyperpolarisation prior to sharp wave initiation. We did not observe a plateau and build-up period of excitatory events prior to sharp wave initiation as seen prior to an epileptiform burst (Menendez de la Prida *et al.*, 2006).

5.6 Summary

The results from this chapter suggest that a sharp wave is the result of the synchronous firing of action potentials in a small population of pyramidal neurons and does not regularly involve the generation of dendritic Ca^{2+} spikes. Secondly, it suggests that the surrounding non-participating pyramidal neurons are silenced by inhibition. Finally, it suggests that most neurons receive a temporary reduction in synaptic activity ~200 ms prior to sharp wave initiation.

Inhibitory control of sharp wave initiation

6.1 Introduction

The observation that GABA_A receptors are necessary for sharp wave generation, combined with the fact that network activity seems to decrease prior to a sharp wave event, led to the hypothesis that GABA_A receptor-mediated inhibition might play a role in the initiation of sharp waves. To investigate this idea and find the source of GABA, whole-cell patch-clamp recordings in current-clamp mode were performed from anatomically-identified perisomatic-targeting interneurons, dendritic-targeting interneurons and interneuron-selective interneurons, as well as pyramidal neurons. The neurons were repeatedly depolarized to supra-threshold levels with 500 ms long current pulses at a frequency of 0.1 Hz. Concurrently, both the sharp wave incidence, as well as the excitation and inhibition in the network were monitored.

6.2 Stimulation of CA3 neurons

It has been reported that a single pyramidal neuron in CA3 can initiate a population event in disinhibited hippocampal slices (Miles & Wong, 1983; Menendez de la Prida *et al.*, 2006). In slices with intact inhibition, however, we did not observe any effect on population event incidence by activating a single pyramidal neuron (Figure 6.1, $n = 22$ pyramidal neurons). In contrast, the activation of a single interneuron could increase sharp wave incidence by $\sim 20\%$ immediately after and up to 1.5 seconds following the depolarizing step. This ability to influence sharp wave generation was interneuron subclass specific. We identified all those interneurons that increased the average sharp wave incidence by more than 1 SD above baseline

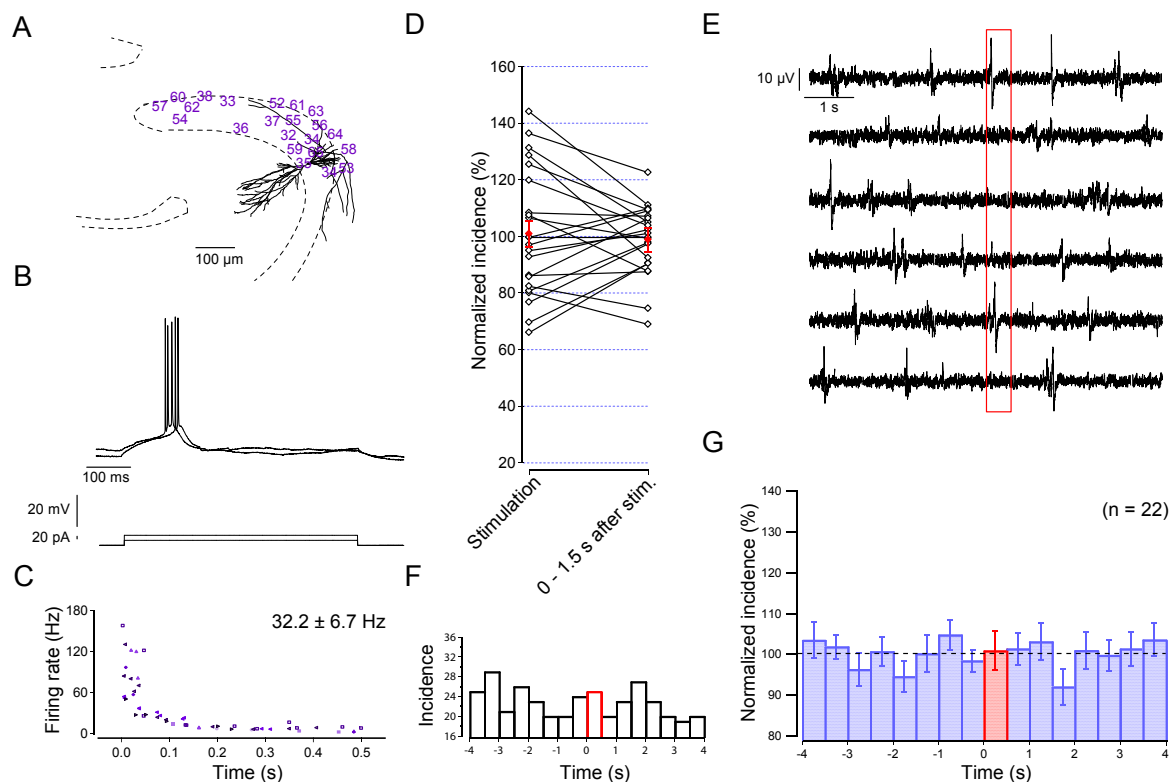


Figure 6.1 An individual pyramidal neuron does not affect sharp wave incidence

The effects of single cell firing on sharp wave generation was tested for anatomically-identified pyramidal neurons ($n = 22$). (A) an example of the anatomy of a CA3 pyramidal neuron, recovered via intracellular biocytin labelling, (B) an example trace of the response of an individual pyramidal neuron to suprathreshold 500 ms current injection which in this case leads to the generation of a burst, (C) a plot of spike rate during suprathreshold 500 ms current injection for individual neurons (indicated by different colours) as well as the overall average spike rate for pyramidal neurons, (D) a plot of sharp wave incidence during depolarisation, and 0-1.5 s after depolarisation of pyramidal neurons, (E) example traces of the response to individual suprathreshold 500 ms current injection (red bar) on sharp wave events, (F) plot of sharp wave incidence 4 seconds before stimulation, during stimulation (stimulation between $t = 0$ s and 0.5 s indicated by red bar) and 3.5 seconds after stimulation for a single neuron and (G) averaged for a population of neurons.

incidence (within 1.5 s following their activation) as PTI. Out of 24 anatomically-identified PTI recorded, 13 increased the average sharp wave incidence by more than 1 SD above baseline following stimulation (Figure 6.2; $p < 0.05$, ANOVA, $n = 13$ perisomatic-targeting interneurons; pairwise comparison: pre-stimulus baseline vs. $t = 0.5 - 1.0$ s, $t = 1.0 - 1.5$ s, and $t = 1.5 - 2.0$ s, $p < 0.01$, post hoc *LSD*). We will refer to these neurons as successful PTI. During stimulation of these PTI the number of detected sharp waves was reduced, although not significantly ($92.9 \pm 7.0\%$; pre-stimulus baseline vs $t = 0 - 0.5$ s; $p > 0.05$, post hoc *LSD*). Both the suppression and subsequent enhancement in sharp wave incidence were local to the area of axonal arborisation of the stimulated PTI, without significant influence on surrounding

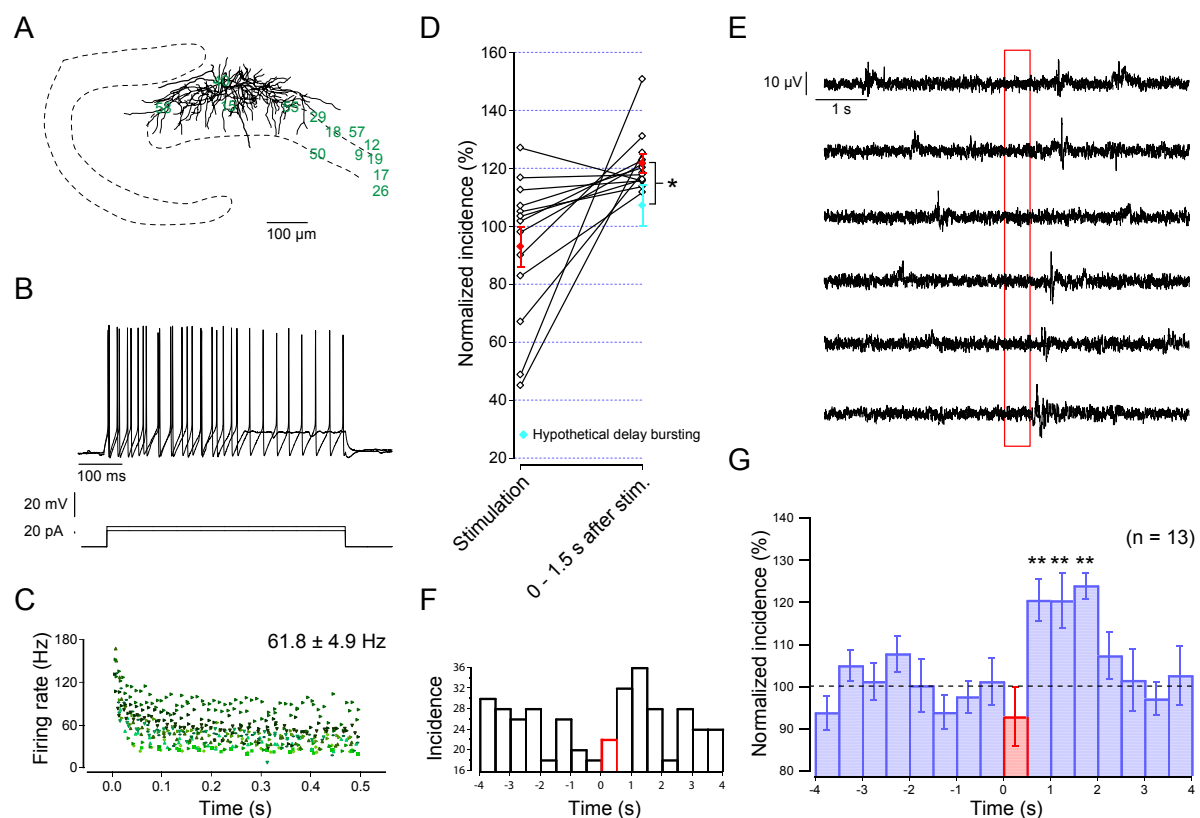


Figure 6.2 An individual perisomatic-targeting interneuron can suppress and subsequently enhance sharp wave incidence

The effects of single cell firing on sharp wave generation was tested for anatomically-identified perisomatic-targeting interneurons ($n = 13$). (A) an example of the anatomy of a CA3 perisomatic-targeting interneuron, recovered via intracellular biocytin labelling, (B) an example trace of the response of an individual perisomatic-targeting interneuron to suprathreshold 500 ms current injection, (C) a plot of spike rate during suprathreshold 500 ms current injection for individual neurons (indicated by different colours) as well as the overall average spike rate for perisomatic-targeting interneurons, (D) a plot of sharp wave incidence during depolarisation, and 0-1.5 s after depolarisation of perisomatic-targeting interneurons, (E) example traces of the response to individual suprathreshold 500 ms current injection (red bar) on sharp wave events, (F) plot of sharp wave incidence 4 seconds before stimulation, during stimulation (stimulation between $t = 0$ s and 0.5 s indicated by red bar) and 3.5 seconds after stimulation for a single neuron, and (G) averaged for a population of neurons. Only stimulation of perisomatic-targeting interneurons produced a significant effect on network activity, with a reduction in sharp wave incidence during stimulation (red bar), and a significant increase in sharp wave incidence immediately and up to 1.5 s after stimulation.

sharp wave incidence (Figure 6.3). The increase in incidence after stimulation of the PTI was not the result of a delayed generation of sharp waves which were unable to occur during stimulation. The actual average increase in the 1.5 seconds after stimulation ($120.6 \pm 3.0\%$) was significantly greater than the predicted net increase due to a simple delay ($107.1 \pm 7.0\%$; $p < 0.05$, paired-samples t test, $n = 13$; Figure 6.2). Furthermore, neither the average firing rate of the PTI, nor the baseline sharp wave incidence, was correlated to the changes in sharp wave incidence induced by stimulating these PTIs (Figure 6.4). Stimulation of the remaining PTI ($n = 11$) did not increase the average sharp wave incidence in the 1.5 seconds following activation, in fact, a small reduction in sharp wave incidence was observed immediately

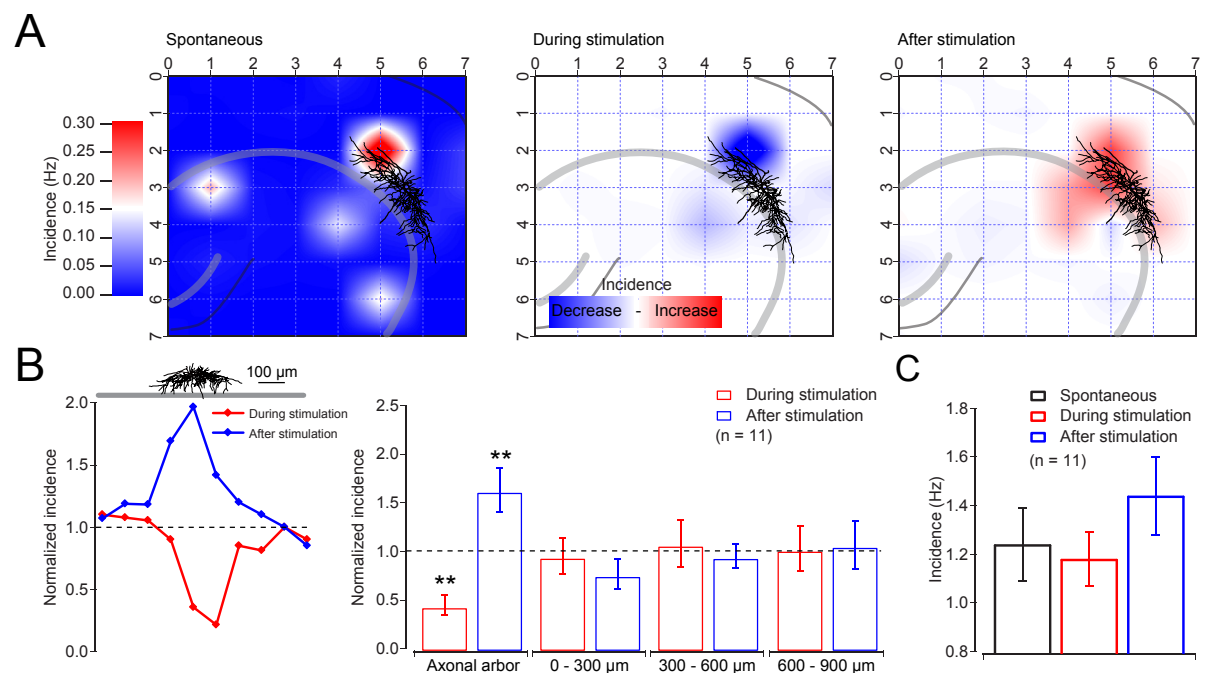


Figure 6.3 The suppression and post-inhibitory enhancement of sharp waves is local to the axonal arborization of the stimulated perisomatic-targeting interneuron

(A) Reconstruction of a single perisomatic-targeting interneuron and the spatial incidence of spontaneous sharp waves (left). During stimulation of the perisomatic-targeting interneuron a local decrease in sharp wave incidence was observed (middle). After stimulation there was a local post-inhibitory enhancement of sharp wave incidence (right). Plotted is the difference between the incidence during stimulation and after stimulation relative to baseline.

(B) Left, Spatial distribution of sharp wave incidence ratio during (red) and following stimulation (blue) relative to baseline. This ratio is plotted relative to the axonal arbor of a perisomatic-targeting interneuron. Right, Plot of differential incidence during and after stimulation within the extent of the axonal arbor, and at 0 – 300 μm , 300 – 600 μm and 600 – 900 μm distance from the axonal arbor. Over all cells, the change in sharp wave incidence during and after stimulation of perisomatic-targeting interneurons was significant only for the area of the axonal arborization (suppression, 0.45 ± 0.10 , $p < 0.01$; enhancement, 1.63 ± 0.23 , $p < 0.05$; one-sample t test, $n = 11$).

following stimulation (Figure 6.5; ANOVA; $p < 0.05$; $n = 11$ perisomatic-targeting interneurons; pairwise comparison: baseline vs. time period 0.5–1.0 s, $p < 0.05$, baseline vs. 1.0–1.5 s, $p > 0.05$ and baseline vs. 1.0–1.5 s, $p < 0.05$, post hoc *LSD*). We will refer to these neurons as unsuccessful PTI. No significant differences were observed between the two populations of PTI in either their average firing rate (successful PTI, 61.8 ± 4.9 Hz and unsuccessful PTI, 51.2 ± 5.7 Hz, $p > 0.05$, independent-samples *t* test, Figure 6.2 and 6.5), location (Figure 6.2A and 6.5A) or axon length (successful PTI, 715 ± 93 μm and unsuccessful PTI, 533 ± 67 μm , $p > 0.05$, independent-samples *t* test; $n = 10$ and $n = 9$). Detailed anatomical analysis of both successful and unsuccessful PTI revealed that of the successful PTI 5 out of 13 could

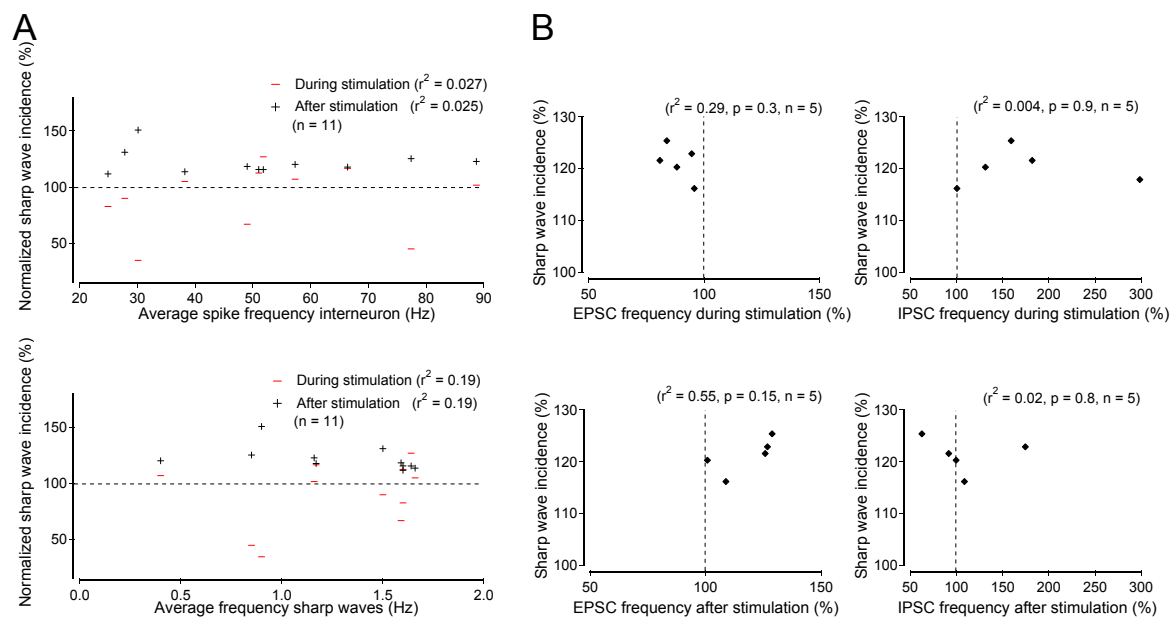


Figure 6.4 Further characterization of successful perisomatic-targeting interneurons

(A) For the successful PTI there was no correlation between their average spiking frequency with either the observed decrease or increase of sharp waves during and after stimulation. Furthermore, there was no correlation between the incidence of spontaneous sharp waves in the slice with the relative decrease or increase in sharp wave incidence during, and after, stimulation.

(B) No significant correlation was observed between changes in EPSC and IPSC frequency after stimulation of successful PTI and changes in sharp wave incidence.

be classified as putative axo-axonic neurons and 4 out of 13 could be classified as putative basket cells. Analysis of the unsuccessful PTI revealed that 1 out of 11 could be classified as putative axo-axonic neurons and 3 out of 11 could be classified as putative basket cells. PTI with predominant axonal arbourisation in stratum oriens and the neighbouring lower part of the stratum pyramidale and containing short axon collaterals sticking perpendicular into the pyramidal cell layer were classified as putative axo-axonic neurons, whilst those PTI with axonal arbourisation in both the stratum oriens and the stratum lucidum (and occasionally the stratum radiatum) as well as extensive arbourisation in the stratum pyramidale were classified as putative basket cells (Hajos *et al.*, 2004). Finally, activation of DTI (Figure 6.6, $n = 18$

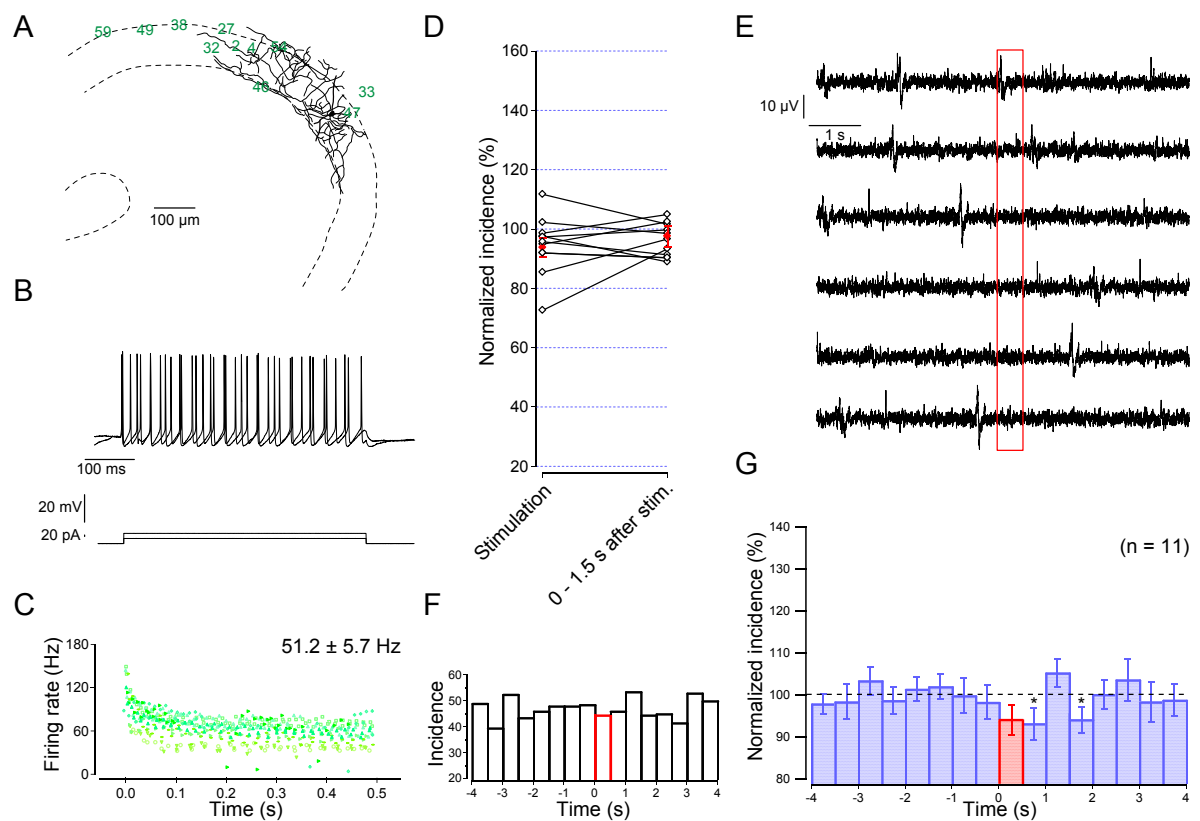


Figure 6.5 A subset of perisomatic-targeting interneurons does not affect sharp wave incidence

The effects of single cell firing on sharp wave generation was tested for a subset of anatomically-identified perisomatic-targeting interneurons ($n = 11$). (A) an example of the anatomy of an unsuccessful CA3 perisomatic-targeting interneuron, recovered via intracellular biocytin labelling, (B) an example trace of the response of an individual perisomatic-targeting interneuron to suprathreshold 500 ms current injection, (C) a plot of spike rate during suprathreshold 500 ms current injection for individual neurons (indicated by different colours) as well as the overall average spike rate for unsuccessful perisomatic-targeting interneurons, (D) a plot of sharp wave incidence during depolarisation, and 0-1.5 s after depolarisation of perisomatic-targeting interneurons, (E) example traces of the response to individual suprathreshold 500 ms current injection (red bar) on sharp wave events, (F) plot of sharp wave incidence 4 seconds before stimulation, during stimulation (stimulation between $t = 0$ s and 0.5 s indicated by red bar) and 3.5 seconds after stimulation for a single neuron and (G) averaged for a population of neurons.

dendritic-targeting interneurons) or ISI (Figure 6.7, $n = 2$ putative interneuron-selective interneurons) did not affect the network activity, even though their average firing rate (DTI; 53.4 ± 5.3 Hz and ISI; 68.8 ± 20 Hz) was not significantly different from the PTI ($p > 0.05$, independent-samples t test) and their location within CA3 similar (Figure 6.6A and 6.7A). A subset of interneurons ($n = 6$) could not be classified according to their anatomy mostly because of a lack of clear axonal arbourisation. Stimulation of these neurons did not lead to an increase in sharp wave incidence (Figure 6.8)

These results suggest that the ability of individual interneurons to increase the local incidence of sharp wave generation is restricted to a subpopulation of PTI.

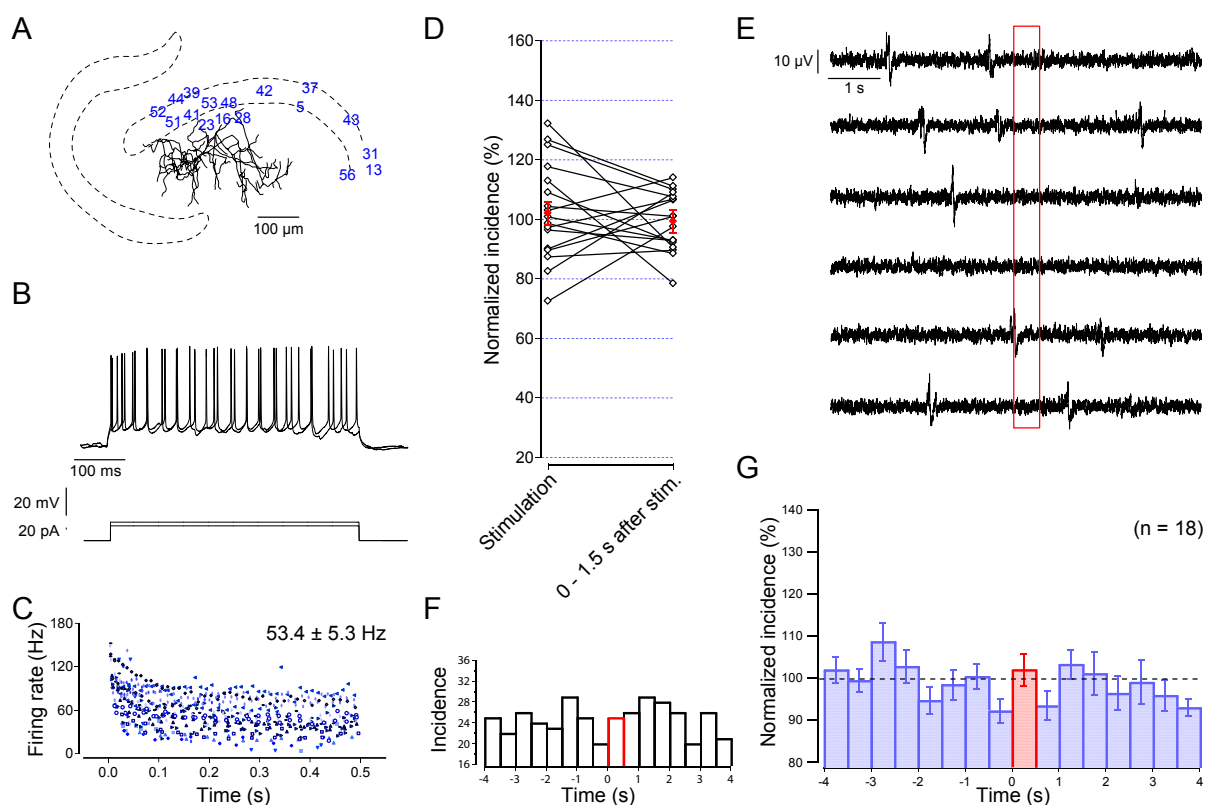


Figure 6.6 An individual dendritic-targeting interneuron does not affect sharp wave incidence

The effects of single cell firing on sharp wave generation was tested for anatomically-identified dendritic-targeting interneurons ($n = 18$). (A) an example of the anatomy of a CA3 dendritic-targeting interneuron, recovered via intracellular biocytin labelling, (B) an example trace of the response of an individual dendritic-targeting interneuron to suprathreshold 500 ms current injection, (C) a plot of spike rate during suprathreshold 500 ms current injection for individual neurons (indicated by different colours) as well as the overall average spike rate for dendritic-targeting interneurons, (D) a plot of sharp wave incidence during depolarisation, and 0-1.5 s after depolarisation of dendritic-targeting interneurons, (E) example traces of the response to individual suprathreshold 500 ms current injection (red bar) on sharp wave events, (F) plot of sharp wave incidence 4 seconds before stimulation, during stimulation (stimulation between $t = 0$ s and 0.5 s indicated by red bar) and 3.5 seconds after stimulation for a single neuron and (G) averaged for a population of neurons.

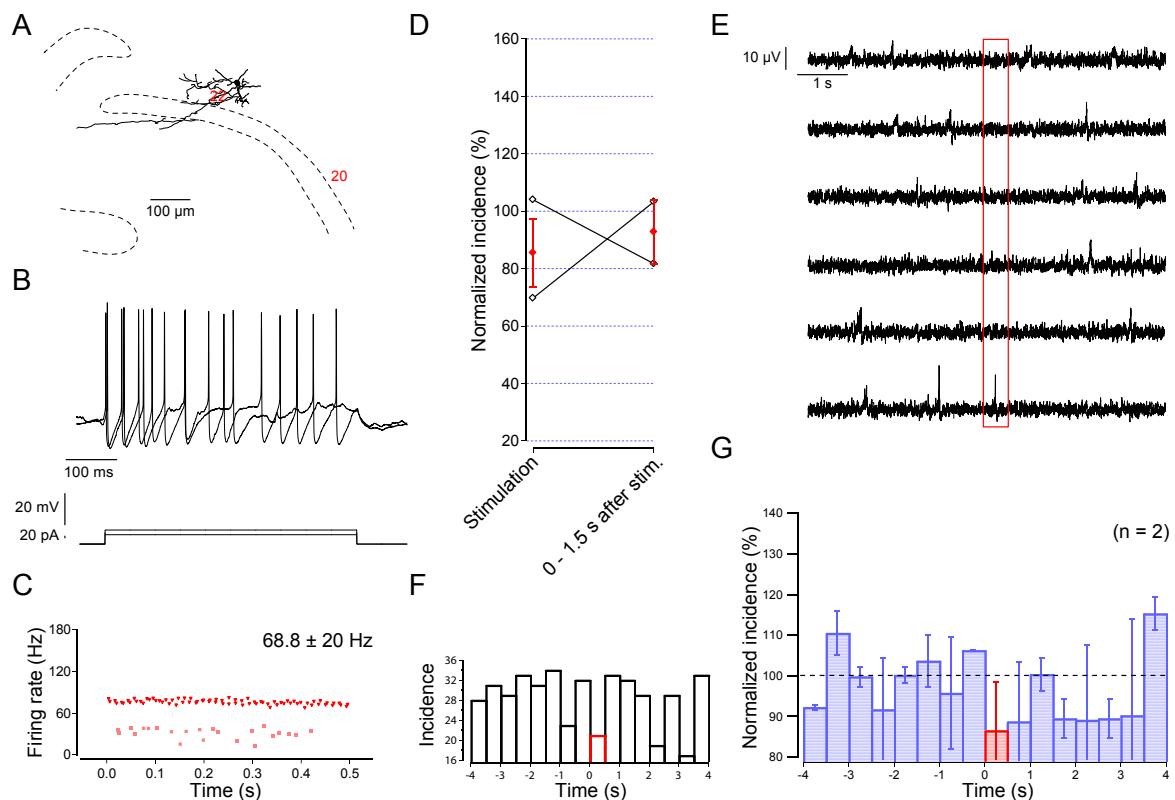


Figure 6.7 An individual putative interneuron-selective interneuron does not affect sharp wave incidence
 The effects of single cell firing on sharp wave generation was tested for anatomically-identified interneuron-selective interneurons ($n = 2$). (A) an example of the anatomy of a putative CA3 interneuron-selective interneuron, recovered via intracellular biocytin labelling, (B) an example trace of the response of an individual putative interneuron-selective interneuron to suprathreshold 500 ms current injection, (C) a plot of spike rate during suprathreshold 500 ms current injection for individual neurons (indicated by different colours) as well as the overall average spike rate for putative interneuron-selective interneurons, (D) a plot of sharp wave incidence during depolarisation, and 0-1.5 s after depolarisation of putative interneuron-selective interneurons, (E) example traces of the response to individual suprathreshold 500 ms current injection (red bar) on sharp wave events, (F) plot of sharp wave incidence 4 seconds before stimulation, during stimulation (stimulation between $t = 0$ s and 0.5 s indicated by red bar) and 3.5 seconds after stimulation for a single neuron and (G) averaged for a population of neurons.

6.3 Mechanism underlying the post-inhibitory increase

To investigate the possible mechanism underlying the ability of an individual PTI to influence the occurrence of sharp waves, whole-cell recording of interneurons in current-clamp mode was performed, combined with simultaneous recordings of either a pyramidal cell or an interneuron in voltage-clamp mode. Sharp wave activity was concomitantly recorded with planar multi-electrode arrays (Figure 6.9). A total of 28 pairs were patched, of which 6 included a PTI in current-clamp mode, whose firing led to a post-inhibitory increase in sharp wave incidence.

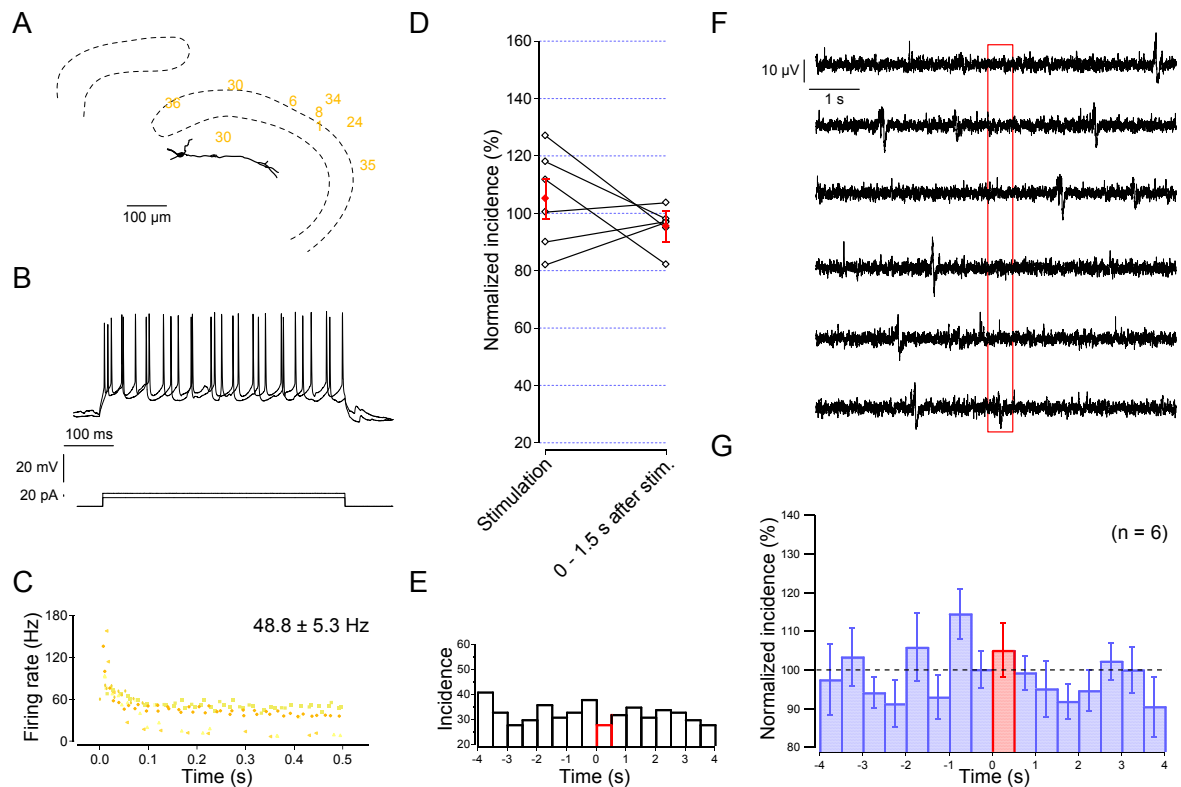


Figure 6.8 A subset of interneurons of unclassified anatomy does not affect sharp wave incidence

The effects of single cell firing on sharp wave generation was tested for a subset of interneurons whose anatomy could not be recovered ($n = 6$). (A) an example a neuron whose anatomy could not be recovered, (B) an example trace of the response of these neurons to suprathreshold 500 ms current injection, (C) a plot of spike rate during suprathreshold 500 ms current injection for individual neurons (indicated by different colours) as well as the overall average spike rate, (D) a plot of sharp wave incidence during depolarisation, and 0-1.5 s after depolarisation, (E) example traces of the response to individual suprathreshold 500 ms current injection (red bar) on sharp wave events, (F) plot of sharp wave incidence 4 seconds before stimulation, during stimulation (stimulation between $t = 0$ s and 0.5 s indicated by red bar) and 3.5 seconds after stimulation for a single neuron and (G) averaged for a population of neurons (G).

Interneurons in current-clamp mode were repeatedly depolarized above spike threshold for 500 ms, at a frequency of 0.1 Hz, and their effect on the overall synaptic activity in the network was measured in the neuron held in whole-cell voltage-clamp mode. The number of excitatory events were counted from recordings when the neuron was kept at the reversal potential for inhibitory currents (-65 mV). The number of inhibitory events were counted from recordings when the neuron was kept at the reversal potential for excitatory currents (0 mV). The 500 ms of spiking in individual successful PTI resulted in a significant decrease

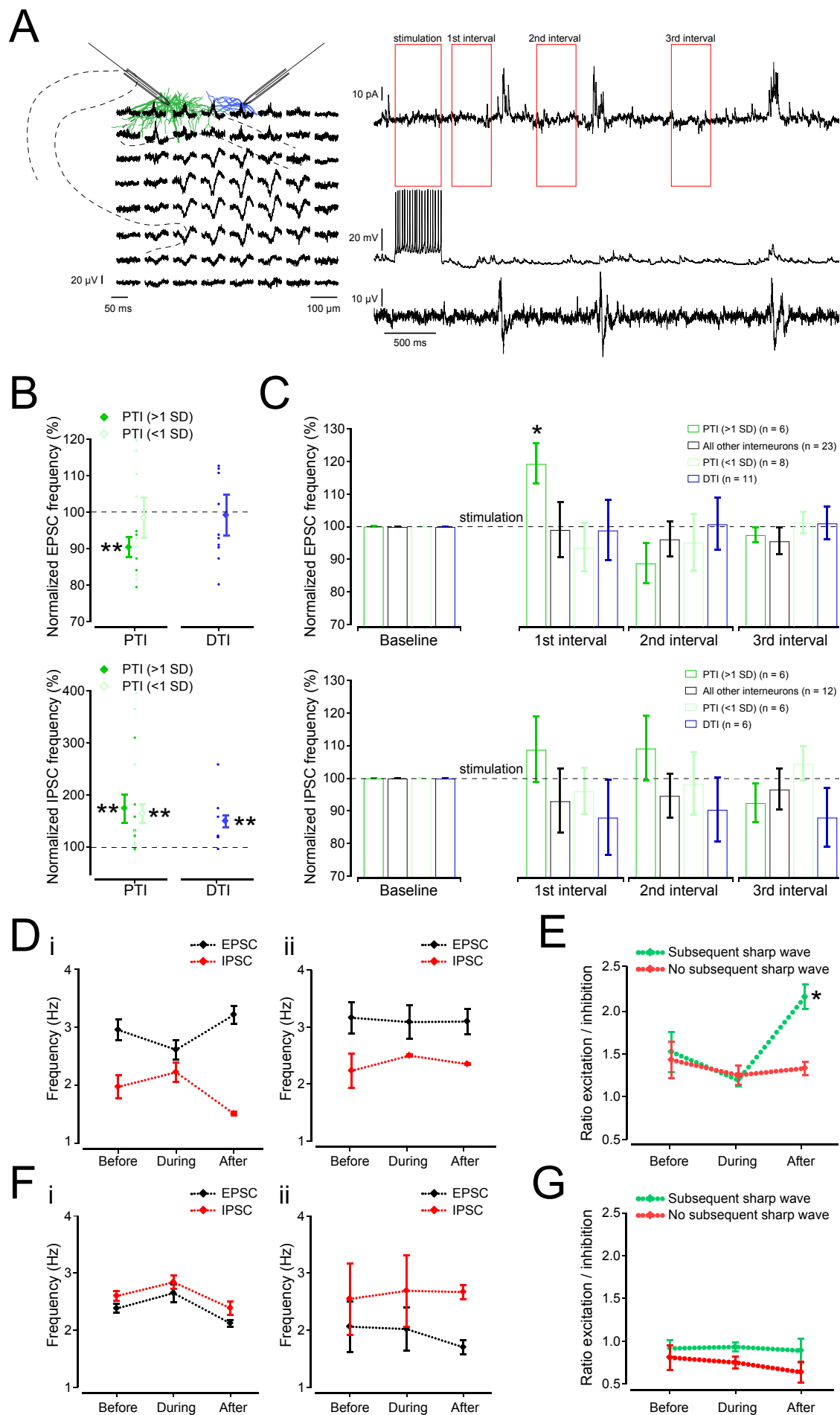


Figure 6.9 A single perisomatic-targeting interneuron can trigger a transient increase in excitation over inhibition

(A) Left, Diagram of 2 reconstructed interneurons and their location in hippocampal CA3. Simultaneous field recordings of ongoing sharp wave activity was combined with whole-cell current-clamp of an interneuron, in this case a perisomatic-targeting interneuron (green cell) and whole-cell voltage-clamp of another cell, in this case a dendritic-targeting interneuron (blue cell), held consecutively at -65 mV (for recording of EPSCs) and 0 mV (for recording of IPSCs). Right, Intracellular traces of dendritic-targeting interneuron held in whole-cell voltage-clamp at 0 mV and a perisomatic-targeting interneuron held in whole-cell current-clamp with concurrent field recording of sharp waves. Illustrated as red boxes are the four time periods used when determining PSC frequencies.

(B) Suprathreshold 500 ms current steps given to successful PTI ($n = 6$) led to a significant decrease in EPSC frequency during stimulation (top). Current injection in unsuccessful PTI ($n = 8$) and all DTI ($n = 11$) did not lead to a significant change in EPSC frequency during stimulation. Stimulation of all interneurons led to a significant increase in IPSC frequency during stimulation (bottom).

(C) Normalised EPSC (top) and IPSC frequency (bottom), as measured from a cell held in whole-cell voltage-clamp. Measurements were made before stimulation (baseline), after stimulation but before subsequent sharp wave (1st interval), after 1st subsequent sharp wave but before 2nd sharp wave (2nd interval), and after 2nd sharp wave but before 3rd sharp wave (3rd interval) (see Experimental Procedures). Only after stimulation of successful PTI was there a significant increase in subsequent EPSC frequency ($119.3 \pm 6.2\%$) whereas stimulation of other types of interneuron led to a decrease in EPSC frequency. Changes in IPSC frequency did not reach statistical significance.

(D, E) Frequency of EPSCs and IPSCs before, during and after individual stimulation events in successful PTI (see Experimental Procedures). Stimulation events were split in those that were followed by a sharp wave (within 1.5 s) (Di) and those that did not (Dii). Only after stimulation events followed by a sharp wave is there a concurrent increase in EPSC frequency and decrease IPSC frequency. This shift results in a significant increase in the ratio of excitation to inhibition (Ei) (baseline 1.5 ± 0.23 ; during stimulation 1.2 ± 0.07 ; after stimulation 2.1 ± 0.14).

(F, G) Same as in (D, E), but in unsuccessful PTI. No shift in the ratio of excitation to inhibition was seen after stimulation, whether the stimulation was followed by a sharp wave or not.

in EPSC frequency ($90.8 \pm 2.8\%$; $p < 0.01$; one-sample t test; $n = 5$; Figure 6.4B and 6.9B), similar to that seen in the network prior to sharp wave initiation (see Figure 5.4E), whereas stimulation of DTI as well as unsuccessful PTI had no significant effect on EPSC frequency (respectively $99.1 \pm 5.6\%$ and $98.4 \pm 5.5\%$; one-sample t test; $p > 0.05$; $n = 8$ and $n = 18$; Figure 6.9B). Stimulation of all interneuron types led to an increase in IPSC frequency as recorded in the other neuron (successful PTI, $174.8 \pm 27.3\%$, unsuccessful PTI 164.5 ± 18.1 , and DTI $150.3 \pm 11.3\%$; $p < 0.01$; one-sample t test; $n = 5$, $n = 6$ and $n = 6$).

Next, we measured both the EPSC and IPSC frequency after stimulation (normalised to baseline). We looked at three time segments: just after stimulation but before subsequent sharp wave (first interval), between the first and second sharp wave (second interval), and between the second and third sharp wave (third interval). We looked at three groups of interneurons; six successful PTI whose activity increased sharp wave incidence by more than 1 SD, eight unsuccessful PTI which failed to do so, and the remaining 14 interneurons of which 11 were

DTI. We observed that immediately after stimulation of those PTI that successfully enhanced sharp wave incidence, there was a significant increase in EPSC frequency ($19.3 \pm 6.2\%$, $p < 0.05$; paired-samples t test; $n = 5$), along with a smaller increase in IPSC frequency ($8.8 \pm 10.3\%$) (also see Figure 6.4B, page 103). Corroborating this result, stimulation of these PTI led to an increased EPSC charge transfer with a concurrent decrease in IPSC charge transfer (respectively to $118.0 \pm 4.7\%$ and $72.2 \pm 4.5\%$ of baseline; $p > 0.05$; one-sample t test; $n = 5$ and $n = 4$). In contrast, stimulation of the other PTI and DTI led to a decrease in EPSC frequency (to $93.6 \pm 7.4\%$ and $98.9 \pm 9.3\%$ of baseline; Figure 6.9C, top), along with a small decrease of IPSC frequency (to $96.7 \pm 8.0\%$ and $87.2 \pm 11.2\%$ of baseline, Figure 6.9C, bottom), neither of which was significant.

Finally, we looked in further detail at individual stimulation events within the population of successful (Figure 6.9D, E) and unsuccessful PTI (Figure 6.9F, G). We divided the stimulation events into those that led to a subsequent sharp wave (Figure 6.9Di, Fi) and those that did not (Figure 6.9Dii, Fii). A significant shift in the ratio of EPSC to IPSC frequency was only observed after stimulation of successful PTI, and only when stimulation was followed by a sharp wave (Figure 6.9Di, E; independent-samples t test; $p < 0.05$). In contrast, no such difference was seen in unsuccessful PTI, whether interneuron stimulation was followed by a sharp wave or not (Figure 6.9F, G).

These results show a specific ability of at least a subpopulation of PTI to elicit a post-inhibitory transient increase in excitation over inhibition in the network, facilitating subsequent sharp wave generation.

6.4 Discussion

I set out to investigate the role of inhibition in the initiation of sharp wave – ripple population bursts by stimulating several classes of anatomically-identified CA3 interneurons and monitoring the effect of their firing on the sharp wave – ripple incidence in the network. Next, this stimulation protocol was combined with simultaneous recording of the overall synaptic activity in the network as measured from another single neuron held in whole-cell voltage-clamp mode. These results show that activity of a single perisomatic-targeting interneuron can initiate sharp waves. Secondly, they show that activity of a single perisomatic-targeting interneuron can transiently increase the ratio of excitation to inhibition in the local network facilitating sharp wave initiation.

6.4.1 Stimulation of pyramidal neurons

It has been reported that a single pyramidal neuron in CA3 can initiate a population event in disinhibited hippocampal slices (Miles & Wong, 1983; Menendez de la Prida *et al.*, 2006). Furthermore, it has been suggested that CA3 pyramidal neurons could act as burst-initiator cells in the initiation of sharp wave – ripple population bursts (Buzsaki, 1989; Buzsaki *et al.*, 1990; Buzsaki & Chrobak, 1995). It has been proposed that this is mediated by changes in synaptic connectivity in hippocampal CA3 which occur during exploration (Buzsaki & Chrobak, 1995) and that these synaptic weights control population burst initiation and recruitment in subsequent rest and sleep stages (Behrens *et al.*, 2005). Sharp wave bursts are thought to be initiated by neurons with the strongest synaptic connectivity or recent place cell related activity (Csicsvari *et al.*, 2007), and then spread to other neurons with less synaptic strengths (Buzsaki & Chrobak, 1995).

However, we found that a single CA3 pyramidal neuron was not able to induce a sharp wave in a hippocampal slice with intact inhibition. It is necessary to be cautious when interpreting this result. Firstly, the sample size covers only 0.7% (22 out of 3000; see Chapter 5 Discussion, page 94) of the total number of CA3 pyramidal neurons present in a 400 μm

hippocampal slice. Secondly, the population of pyramidal neurons that were patched might have been a biased sample. The limited penetration into the tissue using IR-DIC microscopy would bias towards superficial pyramidal neurons that might have lost large parts of their axonal arbourisation due to the slicing procedure, resulting in a vastly reduced connectivity to downstream targets. Lastly, these findings do not exclude the possibility that a concerted activation of a larger population of pyramidal neurons could induce a sharp wave. Electrical activation of the stratum pyramidale using a monopolar stimulating electrode did not lead to convincing generation of sharp wave – ripples however (data not shown).

6.4.2 Stimulation of perisomatic-targeting interneurons

Activation of individual perisomatic-targeting interneurons resulted in a subsequent transient (up to 1.5 seconds after stimulation) increase in the incidence of sharp wave – ripple population bursts. As discussed in the main introduction it is possible to distinguish three types of perisomatic-targeting interneuron: the parvalbumin-positive basket cells, the cholecystokinin-positive basket cells and the parvalbumin-positive axo-axonic cells (Somogyi, 1977; Freund & Buzsaki, 1996; Klausberger *et al.*, 2003). Detailed anatomical investigation of the axonal arbourisation enabled a distinction between putative axo-axonic and putative basket cells. Electron microscopy will be necessary to confirm this, however, and immunocytochemistry would enable detection of potential expression of parvalbumin and cholecystokinin. The group of successful perisomatic-targeting interneurons contained a high proportion of axo-axonic interneurons (5 out of 13). These axo-axonic interneurons are especially interesting with regard to the initiation of sharp waves as they have been shown to fire preferentially prior to a sharp wave and are silent during a sharp wave, although this is only established for the CA1 hippocampal region *in vivo* under anaesthesia (Klausberger *et al.*, 2003). Some putative basket interneurons were also found in the group of successful perisomatic-targeting interneurons (4 out of 13). This suggests that at least two types of perisomatic-targeting interneuron have the capability to induce sharp waves *in vitro*. No difference was seen between the average spike rate, location or axonal arbourisation between the group of successful and unsuccessful perisomatic-targeting interneurons. Some other factor might

influence the ability of perisomatic-targeting interneurons to successfully influence sharp wave initiation. It might be that connectivity to other perisomatic-targeting interneurons through gap junctions distinguishes the two groups. These connections would enable the recruitment of a larger population of perisomatic-targeting interneurons which could synapse on the same subpopulation of pyramidal neurons.

Perisomatic-targeting interneurons controlling sharp wave events might be recruited by a small subpopulation of pyramidal neurons in CA3 (Buzsaki & Chrobak, 1995), but could also be excited by external input from the dentate gyrus or entorhinal cortex. Although I and others show that the recurrent network in CA3 is sufficient to produce sharp wave – ripples intrinsically, and independently of input from the dentate gyrus or entorhinal cortex (Bragin *et al.*, 1995; Maier *et al.*, 2003; and see Figure 3.3C, page 49), there is evidence that interneurons are modulated by upstream targets. For example, surgical removal of the entorhinal cortex leads to an increase in CA1 ripple patterns during slow-wave sleep (Bragin *et al.*, 1995), and, recently, a number of studies have shown the influence of the cortical slow oscillation on sharp wave – ripple generation in the hippocampus both in rodents (Sirota *et al.*, 2003; Battaglia *et al.*, 2004; Isomura *et al.*, 2006; Molle *et al.*, 2006; Ji & Wilson, 2007) and humans (Clemens *et al.*, 2007). Moreover, intracellular recordings of individual hippocampal interneurons revealed membrane potential fluctuations in synchrony with neocortical slow oscillations (Hahn *et al.*, 2006, 2007).

6.4.3 Stimulation of dendritic-targeting interneurons

Activation of dendritic-targeting interneurons did not affect the incidence of sharp waves. This family of interneurons is very diverse and I did not distinguish between different types of dendritic-targeting interneuron but grouped them together as one class. This might have occluded an effect of particular subtypes of dendritic-targeting interneurons on sharp wave incidence. The firing of dendritic-targeting interneurons during sharp wave – ripple activity has not been described for every member, but for those that have been described it shows vastly different activity patterns for different members. For example, it has been shown that bistratified cells in CA1 fire preferentially during the ripple event (Klausberger *et al.*, 2004),

whereas oriens-lacunosum moleculare interneurons in CA1 preferentially fire outside of, but not during, sharp wave – ripple events (Klausberger *et al.*, 2003). Again it has to be kept in mind that this has been shown for the CA1 hippocampal region *in vivo* under anaesthesia and it is presently unknown what activity patterns these types exhibit in CA3 in unanaesthetized animals.

Although activation of this class of interneuron did not affect sharp wave initiation they might well have a role in sharp wave – ripple related plasticity. They could regulate the excitatory input or action potential back propagation in pyramidal neurons (Kampa *et al.*, 2007), the initiation of dendritic Ca^{2+} spikes as has been shown for cortical layer 5 pyramidal neurons (Murayama *et al.*, 2009) or play a role in the inhibition of non-participating pyramidal neurons (Figure 5.2F, page 89).

6.4.4 Stimulation of putative interneuron-selective interneurons

Activation of interneuron-selective interneurons did not influence the incidence of sharp waves as recorded in the field. Only a very small proportion of the interneurons that were patched consisted of interneuron-selective interneurons (2 out of 72). They were identified according to the presence of varicose axon collaterals or braids, but further immunostaining, for calretinin (Gulyas *et al.*, 1996) or vasoactive intestinal peptide (Acsady *et al.*, 1996) would be able to confirm the classification. Only a small number of interneuron-selective interneurons were patched so it is difficult to draw conclusions as to their role in sharp wave – ripple generation. They might well have a role during sharp wave – ripples e.g. in shutting down the spiking activity of axo-axonic interneurons during the sharp wave – ripple.

6.4.5 Mechanism underlying the post-inhibitory increase

The mechanisms by which a single perisomatic-targeting interneuron could subsequently increase sharp wave incidence were investigated by combining the stimulation protocol with simultaneous recording of the synaptic activity in the network as measured from another neuron held in whole-cell voltage-clamp mode. Both excitatory events and inhibitory events were measured by clamping the neuron at respectively the reversal potential for inhibitory

currents (-65 mV) and the reversal potential for excitatory currents (0 mV). The frequency of these events were calculated for three time periods, firstly, just after stimulation of the perisomatic-targeting interneuron but before a subsequent sharp wave, secondly, after the first sharp wave but before the second sharp wave and lastly, after the second sharp wave but before the third sharp wave. To avoid contamination of these time periods by activity changes around the edges of the sharp waves (e.g. I show that synaptic activity is reduced prior to sharp wave initiation), I calculated the frequency of events in a 400 ms time window centred between the start and end of the time period. This analysis revealed that activity of a single perisomatic-targeting interneuron can lead to a reduction in the EPSC frequency (and increase in IPSC frequency) with a subsequent increase in EPSC frequency in the network. Activity in all other interneurons only produced a temporary increase in IPSC frequency during their spiking but had no significant effect on either the EPSC or IPSC frequency in the network afterwards. More detailed analysis of the EPSC and IPSC frequencies before, during and after stimulation in either successful or unsuccessful perisomatic-targeting interneurons revealed that a significant shift in the ratio of EPSC to IPSC frequency was only observed after stimulation of the successful perisomatic-targeting interneurons and only when stimulation was followed by a sharp wave. The increase in EPSC frequency could be the reflection of an increase in excitability, or an increase in the synchronization of a subset of pyramidal neurons. These possibilities are difficult to dissociate. However, our results suggest that spiking activity of perisomatic-targeting interneurons could facilitate the generation of sharp waves by providing a temporary inhibition or silencing of spike activity within a subpopulation of pyramidal neurons, after which rebound excitation can occur (Cobb *et al.*, 1995; Harris *et al.*, 2001). From our data, it is not possible to distinguish between the possibilities that the failure of some perisomatic-targeting interneurons to influence sharp wave initiation is due to the network state or different subclasses of perisomatic-targeting interneuron.

The observation that EPSC frequency decreases upon stimulation of all classes of interneurons, as well as a reduction in sharp wave incidence by stimulation of perisomatic-targeting interneurons, suggests that GABA is inhibitory under our conditions. It has been shown that GABA could have a directly depolarising, excitatory effect in young animals,

as well as in certain neuronal types and cortical states in the adult brain (Cohen *et al.*, 2002; Gullledge & Stuart, 2003; Wozny *et al.*, 2003; Banke & McBain, 2006; Szabadics *et al.*, 2006). However, we used mature rats in which neurons most likely exhibit a GABA_A receptor reversal potential which is negative to resting membrane potential (Luhmann & Prince, 1991; Owens *et al.*, 1996; but see Tyzio *et al.* 2008). Nevertheless, none of these observations exclude the possibility that GABA might be excitatory in a subset of neurons that were not recorded by us, or that GABA could be depolarising in specific subregions of the pyramidal neuron (e.g. axon initial segment; Szabadics *et al.*, 2006). Very recently, it has been shown in slices that GABAergic input to all regions of pyramidal neurons (dendrite, soma and axon initial segment) is inhibitory (Glickfeld *et al.*, 2009), arguing against the possibility that interneurons directly excite pyramidal cells.

6.5 Summary

These results suggest that a specific subclass of GABAergic interneuron, i.e. perisomatic-targeting interneurons, control sharp wave initiation by providing a temporary inhibition or silencing of spike activity within a subpopulation of pyramidal neurons, after which rebound excitation can occur.

Discussion and Conclusion

7.1 Main findings

In this thesis the cellular mechanisms involved in the initiation of hippocampal population burst activity, i.e. sharp wave – ripples, were investigated in an *in vitro* hippocampal slice preparation. The main findings of this thesis work are:

1. Transverse hippocampal slices superfused with an altered ACSF at a high flow rate (3 – 6 ml/min) can maintain stable sharp wave – ripple population bursts in submerged conditions with similar characteristics to those observed *in vivo*.
2. Sharp wave – ripples *in vitro* are generated by changing subpopulations of pyramidal neurons in CA3 with non-participating pyramidal neurons actively silenced by GABA_A receptor-mediated inhibition.
3. GABA_A receptor-mediated inhibition is necessary for the initiation of sharp waves.
4. An individual perisomatic-targeting interneuron can suppress, and subsequently enhance, local sharp wave generation.
5. This post-inhibitory enhancement is associated with a transient increase in excitation over inhibition in the local network.

7.1.1 Slice conditions for sharp wave – ripples *in vitro*

I investigated the conditions during which sharp wave – ripples could be obtained in submerged hippocampal slices *in vitro* as this would enable a more detailed investigation of this network activity. I found that stable sharp wave – ripples, with similar characteristics to those found *in vivo*, could be obtained by superfusing slices with an altered ACSF at a high flowrate (3 – 6 ml/min) which provided a sufficient oxygen concentration to the tissue (Figure 3.7, page 54). Although an altered ACSF was necessary, there was no need for NMDA or metabotropic glutamate receptor-mediated changes in excitability or plasticity, neither for translation or transcription (Figure 3.1, page 45).

7.1.2 Characteristics of sharp wave – ripples *in vitro*

I found the temporal characteristics of sharp wave – ripples to be similar to those described *in vivo* (Table 1, page 61). Using planar multi-electrode arrays in submerged conditions enabled the investigation of their spatial characteristics. This revealed them to be locally generated events, which could arise in all subfields of CA3 independently and covered approximately one third of the CA3 stratum pyramidale (Figure 3.8, page 57). I found them to be generated by firing of action potentials in a small population of pyramidal neurons with non-participating pyramidal neurons actively silenced by inhibition (Figure 5.2, page 89).

7.1.3 Role of inhibition in sharp wave – ripple generation

Pharmacological investigation revealed that both AMPA and GABA_A receptor-mediated events are obligatory for sharp wave generation. The need for AMPA receptor-mediated events was expected as they contribute to the fast synaptic transmission between CA3 pyramidal neurons (Figure 4.1, page 70). The need for GABA_A receptors-mediated events was less expected. As inhibition was necessary for silencing the population of non-participating pyramidal neurons it was expected that block of inhibition would lead to an enhancement of sharp wave – ripple population burst activity. However, block of only approximately 50% of the phasic GABA_A receptor-mediated inhibition did not lead to an enhancement of sharp wave – ripples, but blocked their generation completely (Figure 4.1, page 70).

7.1.4 Initiation of sharp wave – ripples *in vitro*

To investigate the source of the phasic GABA_A receptor-mediated inhibition, I stimulated anatomically-identified perisomatic-targeting interneurons, dendritic-targeting interneurons and interneuron-selective interneurons whilst simultaneously recording sharp wave – ripple activity with planar multi-electrode arrays. I found that a single perisomatic-targeting interneuron could suppress and subsequently enhance the local generation of sharp waves (Figure 6.2, page 101). None of the other classes of interneuron were able to influence sharp wave – ripple generation.

7.1.5 Mechanism of initiation

To investigate the mechanism by which individual perisomatic-targeting interneurons could facilitate the generation of sharp wave – ripple population bursts, I stimulated anatomically-identified perisomatic-targeting interneurons while recording the overall excitation and inhibition in the network from another neuron held in whole-cell voltage-clamp mode. I found that activity of a single perisomatic-targeting interneuron can lead to a transient increase in the EPSC to IPSC frequency ratio in the network preceding sharp wave initiation (Figure 6.9, page 108).

7.2 Validity of approach

When evaluating the results of this thesis it is important to consider whether or not the experimental approach used is appropriate for addressing the aims.

7.2.1 Studies in slice preparations

Slice conditions

The extracellular conditions which neurons experience in a slice differ vastly from what they experience in the intact brain. Any of the following factors might influence the intrinsic properties of neurons and as a result the properties of the network as a whole:

Firstly, it is still unknown what the precise ionic conditions are of the extracellular environment of hippocampal neurons, although estimates have been made from perfusion of the hippocampus (McNay & Sherwin, 2004) and from CSF measurements (Jones & Keep, 1987, 1988).

Secondly, most if not all neuromodulatory connections from subcortical nuclei are severed in the preparation of hippocampal slices. Moreover, any compounds which normally find their way to the brain through the bloodstream also are absent. It has been shown that neuromodulators such as serotonin, acetylcholine and noradrenaline can act as regulators of cortical activity (Kruglikov & Rudy, 2008), cortical plasticity (Seol *et al.*, 2007) and hippocampal plasticity (Scheiderer *et al.*, 2008). It was shown that GABA release from fast spiking interneurons in the cortex can be inhibited by activation of muscarinic, serotonin and adenosine receptors (Kruglikov & Rudy, 2008). Furthermore it has been shown that the polarity of SDTP is influenced by cholinergic and adrenergic activation in layer II/III pyramidal neurons of the visual cortex (Seol *et al.*, 2007).

Thirdly, slice preparations are normally kept at a lower than *in vivo* temperature, ranging from room temperature (20 °C) to close to physiological temperatures (35 °C). Although initially it had been shown that there is no difference in the reliability of transmitter release between slices kept at room temperature and at 32 °C – 37 °C (Allen & Stevens, 1994), a recent study found that local excitatory transmission between local pairs of pyramidal neurons in layer II/III of the visual cortex slices is more reliable at 36 °C compared to room temperature (Hardingham & Larkman, 1998). Hippocampal slices used in this thesis were kept at 32 °C and it is at present unknown how the change from 37 °C in the intact brain to 32 °C in a slice affects the neurons.

Reduced connectivity

It must be kept in mind that a 400 µm hippocampal slice does not retain as much connectivity as can be found in the intact hippocampus. The commissural connections between the CA3 pyramidal neurons from the two hemispheres are not present and connections from the dentate gyrus and entorhinal cortex to CA3 might have been reduced also. Neurons in the

intact brain receive high amounts of input resulting in, for example noisy fluctuations in membrane potential, whereas neurons often do not receive any input in slice conditions due to a reduction in connectivity or silencing of the network through ionic or pharmacological conditions. Recently, it has been shown that this difference can affect the intrinsic properties of pyramidal neurons governing spike initiation and it has been proposed that they can switch from being resonators *in vivo* to being integrators *in vitro* (Prescott *et al.*, 2008).

Increased connectivity

Although a hippocampal slice retains a lot of the connections between the neurons it is not known to what extent the connections of the neurons are affected by the slicing procedure. It has been shown that warming of hippocampal slices after a period of cooling down (as occurs during the slicing procedure) can lead to an extensive proliferation and an increase in the number of spines on CA1 pyramidal neurons (Kirov *et al.*, 2004). It is not known if these spines are functional, but if they are this would change the connectivity of the CA3 circuitry within the slice relative to that in the intact animal, which would have implications for the interpretation of these results. I show that a single perisomatic-targeting interneuron is able to recruit a population of pyramidal neurons into generating a sharp wave population burst. However, it is possible that the connectivity between pyramidal neurons in the slice is very different from that found in CA3 *in vivo* and that activity in a single perisomatic-targeting interneuron might not show this effect in the intact hippocampus.

7.2.2 Neuron sampling bias

The use of IR-DIC visualization of hippocampal neurons biases the sampling to the first 100 – 150 μm of the slice. Firstly, there might be a difference between neurons located at the surface of the hippocampal slice and those found deeper in the tissue. For example, it might be that the entire population of neurons are more excitable at the surface of the slice. This would make them more prone to fire and exhibit rebound excitation. Therefore a subset of perisomatic-targeting interneurons found deeper in a hippocampal slice and targeting a less excited network might not have had the effect described in this thesis. Furthermore, the

distinction between successful and unsuccessful perisomatic-targeting interneurons might be less about anatomical subtypes, and more the result of the depth of the perisomatic-targeting interneuron patched and stimulated, as there might be differences in the preservation of their axonal arbour or the population of pyramidal neurons they target.

7.2.3 Data analysis

Data analysis can also affect the results reported in this thesis. It is important to keep in mind that separating the two populations of perisomatic-targeting interneurons, in those that were successful and unsuccessful in initiating sharp wave – ripples using the criterion of > 1 SD above baseline sharp wave incidence, might artificially separate two populations. This separation would skew both populations to two extremes. Reassuringly, after all perisomatic-targeting interneurons are grouped together I still observe a significant increase after stimulation. Further immunocytochemical and electron microscope classification schemes might be able to separate the perisomatic-targeting interneurons into more meaningful groups.

7.3 Implications

7.3.1 Sharp wave – ripple population bursts

The main results of this thesis suggest that, in addition to the pyramidal neurons with the strongest synaptic connectivity (Buzsaki & Chrobak, 1995) or recent place-cell related activity (Csicsvari *et al.*, 2007), inhibitory neurons can assist in selecting those pyramidal neurons that initiate a sharp wave – ripple population burst. It could be the release from inhibition, resulting in rebound excitation, which initiates a sharp wave replay event. If axo-axonic interneurons in CA3 also fire preferentially prior to sharp wave – ripples, as their CA1 counterparts do (Klausberger *et al.*, 2003), they may have a role in selecting the subpopulation of CA3 pyramidal neurons that initiate the sharp wave replay event (Freund, 2003). Their preference to make synaptic connections on the axon initial segment of pyramidal neurons would make them well suited to influence axonal output (Somogyi, 1977; Miles *et al.*, 1996).

If the selective activation of a subset of pyramidal neurons involves some form of plasticity between the perisomatic-targeting interneuron and pyramidal neurons is unknown. It has been shown that the formation of boutons between interneurons and pyramidal neurons in hippocampal cultures is very different from that seen between excitatory neurons (Wierenga *et al.*, 2008). Newly formed GABAergic boutons are very transient and only appear at pre-existing axon-dendrite crossings without dendritic or axonal protrusions, whereas excitatory connections involve the growth of dendritic spines towards axons which are thought to be actively involved in the formation of excitatory synapses. Although it is not known if these findings hold for *in vivo* perisomatic-targeting interneurons it would suggest substantial structural constraints on the generation and plasticity of GABAergic synapses between perisomatic-targeting interneurons and pyramidal neurons (Wierenga *et al.*, 2008). It is also not known if plasticity occurs between the afferents and the perisomatic-targeting interneuron and if so by what rules (Lamsa *et al.*, 2005; Lamsa *et al.*, 2007; Lu *et al.*, 2007; Galvan *et al.*, 2008).

The results from this thesis raise the question whether the perisomatic-targeting interneurons are activated by inputs inside or outside of hippocampal CA3. As discussed in detail at the end of Chapter 6, perisomatic-targeting interneurons controlling sharp wave events might be recruited by a small subpopulation of pyramidal neurons in CA3 (Buzsaki & Chrobak, 1995), but could also be excited by external input from the dentate gyrus (Mori *et al.*, 2004; Mori *et al.*, 2007) or entorhinal cortex, and there is strong evidence that the cortical slow oscillation (seen during slow-wave sleep) influences sharp wave – ripple generation in the hippocampus both in rodents (Sirota *et al.*, 2003; Battaglia *et al.*, 2004; Isomura *et al.*, 2006; Molle *et al.*, 2006; Ji & Wilson, 2007) and humans (Clemens *et al.*, 2007). Furthermore, intracellular recordings of individual hippocampal interneurons revealed membrane potential fluctuations in synchrony with neocortical slow oscillations, suggesting that cortical activity might influence CA3 interneurons involved in sharp wave generation (Hahn *et al.*, 2006, 2007). Interestingly, a study in humans showed that when subjects were learning a hippocampal-dependent declarative memory task with a particular odour present, presenting the same odour during subsequent slow-wave sleep significantly improved the retention, as

measured the following day (Rasch *et al.*, 2007). Hippocampal independent procedural tasks and presenting the odour during REM sleep did not have an effect. Furthermore it was shown by fMRI measurements that the hippocampus was activated during odour presentation. This study suggests that cortical input can reach the hippocampus and affect its activity. These combined findings suggest that the cortex might well influence assembly reactivation and might do this, at least in part, through activation of perisomatic-targeting interneurons.

If the findings described in this thesis hold for the intact brain it suggests that perisomatic-targeting interneurons can initiate population bursting by recruiting specific populations of pyramidal neurons. This would be a new function for perisomatic-targeting interneurons and suggest that sharp wave – ripple population bursts could be regulated by inhibition rather than excitation.

7.3.2 Epileptiform bursting

The results presented in this thesis suggest that inhibition not only keeps population bursts in check, but is actively involved in the production of the population bursts as well. Specifically, GABA_A receptor-mediated inhibition was involved in both the restriction and initiation of burst activity, with pentobarbital, which enhances GABA_A receptor function and is used as an anti-epileptic, leading to an increase in the incidence of bursting. In contrast, the generation of pathological epileptiform bursts seems to depend on a reduction of GABA_A receptor-mediated inhibition which facilitates mutual synaptic excitation (Traub & Wong, 1982) and leads to the synchronous firing of pyramidal neurons (Menendez de la Prida *et al.*, 2006). The common idea that epileptiform activity is the result of a decrease in inhibition leading to run-away excitation might have to be expanded to include the possibility that pathological perisomatic inhibition might lead to runaway excitation as well. It has been shown in human epileptic tissue that perisomatic inhibition is retained in the CA1 region of the hippocampus (Wittner *et al.*, 2005) and the dentate gyrus (Wittner *et al.*, 2001) and might even be increased in the dentate gyrus (Isokawa-Akesson *et al.*, 1989). It was shown for the dentate gyrus that the number of parvalbumin-positive perisomatic-targeting neurons seems reduced, although it could not be excluded that they were still present and had lost their parvalbumin expression,

but that overall synaptic inhibition was left intact. There seemed to be an increase in axo-axonic cells, as well as an increase in inhibitory contacts at the axon initial segments of granule cells. It was suggested that hyper-innervation of axon initial segments may lead to a more effective synchronization of granule cell firing and could in fact contribute to the generation or amplification of epileptic seizures (Wittner *et al.*, 2001).

More recently, it has been shown that nicotinic enhancement of GABA release can aggravate seizure generation in several models of autosomal dominant nocturnal frontal lobe epilepsy (Klaassen *et al.*, 2006; Mann & Mody, 2008), further emphasizing the possibility that inhibition could facilitate pathological burst generation.

7.4 Conclusion

In conclusion, I suggest that a subclass of perisomatic-targeting interneuron can select the subset of pyramidal neurons that initiate a sharp wave – ripple replay event. This is a new function for this class of interneuron and it remains to be investigated if this holds for initiation of replay events *in vivo* and in other brain regions, as well as for pathological burst activity. Finally, it implies that interneurons, rather than merely modulating pyramidal cell activity, can play an integral part in the local information processing that takes place in the hippocampal network.

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ADDENDUM 1

INTRODUCTION

In this thesis the population of perisomatic-targeting interneurons was separated in those that are successful in controlling sharp waves and those that were unsuccessful. The criterion to distinguish the two was the ability of a single perisomatic-targeting interneuron to increase sharp wave incidence more than 1 SD above baseline sharp wave incidence.

A more common method used in *in vivo* analysis is so called ‘shuffling of spikes’. This method was used to see if individual perisomatic-targeting interneurons can increase sharp wave incidence, in the 1.5 seconds following stimulation, above what could be expected by chance (with $p < 0.05$).

METHODS

Sharp wave times were taken from recordings. From this the inter-sharp wave interval (ISI) was calculated. The ISI's were shuffled with a Gaussian random number generator (‘gnoise’ function in Igor 5.0), which returns a random value from a Gaussian distribution. The random number generator is initialized using the system clock when Igor starts. This almost guarantees that you will never get the same sequence twice. The Gaussian distribution is achieved using a Box-Muller transformation of uniform random numbers and based on a Linear Congruential generator by L'Ecuyer with added Bayes-Durham shuffle.

The shuffled ISI's were converted back in sharp wave times. The shuffled sharp wave times were plotted relative to the stimulation times and the average number of sharp waves before stimulation and the average number of sharp waves after stimulation (within 1.5 seconds following stimulation) were counted and the percentage increase of sharp waves was calculated. This was repeated a 1000 times.

If the observed percentage increase in sharp wave incidence following stimulation was within the top 50 percentages (of a total of a 1000 percentages) generated by the shuffling of sharp waves, the perisomatic-targeting interneurons was classified as successful ($p < 0.05$).

RESULTS

The addendum shows all perisomatic-targeting interneurons grouped together ($n = 24$). The average sharp wave incidence was significantly increased at $t = 1.0 - 1.5$ and $t = 1.5 - 2$ ($p < 0.05$, ANOVA, $n = 24$; pairwise comparison: pre-stimulus baseline vs. $t = 1.0 - 1.5$, $p < 0.01$ and vs. $t = 1.5 - 2.0$, $p < 0.05$).

Plotting the average sharp wave incidence during and after stimulation (in B) reveals a large variability in the ability of single perisomatic-targeting interneurons to increase sharp wave incidence. Some of the perisomatic-targeting interneurons are not able to increase sharp wave incidence or are followed by a reduction in sharp wave incidence.

Separating the population of perisomatic-targeting interneurons according to a significant increase over a chance increase using the shuffling method is shown in C. Two populations were created. One population which was unsuccessful in significantly altering sharp waves incidence ($p > 0.05$, ANOVA, $n = 18$). Another population which was successful in significantly altering sharp wave incidence ($p < 0.01$, ANOVA, $n = 8$; pairwise comparison: pre-stimulus baseline vs. $t = 0.5 - 1.0$ and $t = 1.0 - 1.5$, $p < 0.01$ and vs. $t = 1.5 - 2.0$, $p < 0.05$).

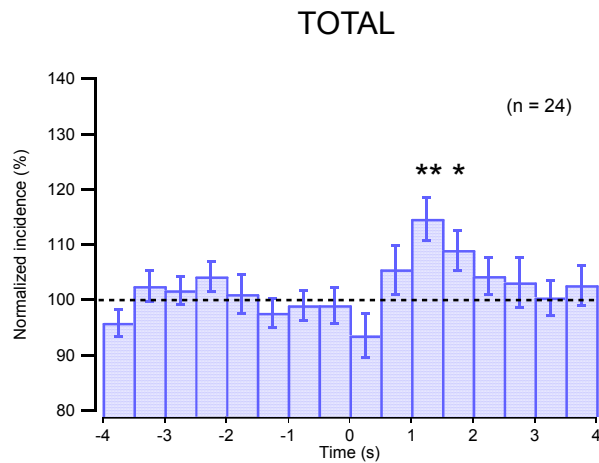
The number of successful perisomatic-targeting interneurons is lower than that found by the >1 SD method (> 1 SD = 13, shuffle = 6). The following are the p values of the perisomatic-targeting interneurons using the shuffling method which were all significant using the >1 SD method (**$p = 0.01$** , $p = 0.15$, $p = 0.12$, $p = 0.07$, **$p = 0.00$** , $p = 0.06$, $p = 0.07$, **$p = 0.01$** , **$p = 0.04$** , $p = 0.14$, $p = 0.17$, **$p = 0.04$** , **$p = 0.00$**). In bold are indicated the significant ones. Some of the remaining p values are very close to the significant criterion of $p < 0.05$.

Of the 6 significant perisomatic-targeting interneurons, three were putative axo-axonic cells, one a putative basket cell and two could not be classified conclusively.

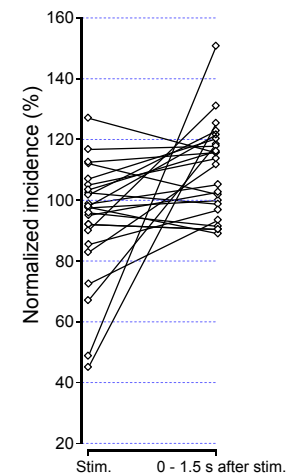
CONCLUSION

This method is more stringent than the >1 SD method used initially. It does not alter further findings of the thesis significantly, as the majority of perisomatic-targeting interneurons used for further analysis were significant in both the >1 SD and shuffling method.

A



B



C

