DISSERTATION

SINGLE AND REPETITIVE PAIRED-PULSE SUPPRESSION: DOES IT PROVIDE AN ANALYSIS OF SYNAPTIC INHIBITION IN EPILEPSY RESEARCH?

Submitted by
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In partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Fall 2007

COLORADO STATE UNIVERSITY

July 13, 2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SIMON WALDBAUM ENTITLED ""SINGLE AND REPETITIVE PAIRED-PULSE SUPPRESSION: DOES IT PROVIDE AN ANALYSIS OF SYNAPTIC INHIBITION IN EPILEPSY RESEARCH?" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

SINGLE AND REPETITIVE PAIRED-PULSE: DOES IT PROVIDE AN ANALYSIS OF SYNAPTIC INHIBITION IN EPILEPSY RESEARCH?

The paired-pulse technique has a long history of use in neurophysiological studies in the hippocampus, particularly in epilepsy research where it has been a convenient but indirect measure of synaptic inhibition. Most investigators have used a single paired-pulse protocol, while others have utilized repetitive paired pulses. Nearly all of the electrophysiological evidence that inhibition is increased after synaptic reorganization in the hippocampus derives from this technique. This study investigated the dependence of single and repetitive paired-pulse suppression on stimulus parameters and levels of membrane excitability in the perforant path-granule cell network. The hypothesis was tested that slightly reduced $GABA_A$-receptor mediated inhibition results in an increase in paired-pulse suppression. Single paired-pulse suppression was analyzed as a function of stimulus intensity and interpulse interval with field-potential recordings from the granule cell layer and stimulation of the perforant path. Six different interpulse intervals (20-200 ms) were used at stimulus intensities that evoked responses ranging from 25% to 100% (i.e., the maximum). Paired-pulse suppression during a repetitive train of 10 paired pulses was analyzed as a function of stimulation intensity, interpulse interval and frequencies of 0.1-4.0 Hz. Stimulation intensities of 75% to 100% of maximum resulted in a higher degree of paired-pulse

suppression, as did interpulse intervals from 20-60 ms. At lower frequencies, single and repetitive paired-pulse protocols yielded similar levels of paired-pulse suppression. The degree of paired-pulse suppression, however, depended heavily on the interplay of stimulation intensity, interpulse interval and stimulus frequency, particularly when repetitive paired pulses were given at higher frequencies. The amplitude of the population spike produced by the conditioning pulse progressively increased during stimulation at higher frequencies (1.0-4.0 Hz). Alterations in ionic conditions from 3.0 to 6.0 mM [K^+] and from 1.0 to 2.5 mM [Ca^{++}] showed that paired-pulse suppression was maximal when membrane excitability was highest. Under physiological ionic conditions, increased levels of paired-pulse suppression were measured during bath application of the $GABA_A$-receptor antagonist SR-95531, which reduced $GABA_A$-receptor mediated inhibition. Therefore, the paired-pulse technique erroneously measured increased rather than decreased levels of "inhibition" under conditions of slightly increased excitability. These results indicate that the paired-pulse technique is not a reliable or accurate measure of synaptic inhibition, and strongly challenges the electrophysiological evidence supporting the "hyperinhibition" hypothesis after epilepsy-associated synaptic reorganization in the hippocampus.

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ACKNOWLEDGEMENTS

I would like to thank Drs. F.E. Dudek, P.A. Williams, W.A. Pouliot, J.L. Hellier and Mr. D.J. Ferraro for their valuable input and support. I would also like to thank my parents, Drs. B.S. and N.Z. Waldbaum, and my wife, Michelle L. Waldbaum, for their support, kindness and generosity.

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CHAPTER 1

INTRODUCTION

1.1 The hippocampal formation

The hippocampal formation appears as a curved bi-lateral cortical structure in the floor and medial wall of the temporal horn of the lateral ventricle. It is traditionally defined as including the tightly packed principal layers of the Cornu Ammonis 1 (CA1), CA2, and CA3 regions, the dentate gyrus, the subicular complex, and the entorhinal cortex. The two principal cell types, pyramidal cells and granule cells, make up 96-98% of the neuropil (Cassell & Brown 1977; Seress & Pokorny 1981). The hippocampal sub-regions are connected by the "tri-synaptic circuit" which begins with afferent axons of the perforant path originating in the entorhinal cortex (Lothman et al. 1992). These axons synapse on dendrites of granule cells within the molecular layer of the dentate gyrus. Next, the axons of the granule cells travel through the hilus and synapse on dendrites of CA3 pyramidal neurons. The axons of CA3 neurons divide into two fiber projections, one forms the commissural pathway that projects to the contralateral hippocampus via the corpus callosum, and the other forms the Schaffer collateral pathway which synapses on the dendrites of CA1 pyramidal neurons. Lastly, the axons of CA1 neurons project to the subiculum and the entorhinal cortex. The tri-synaptic circuit provides a schematic of the flow of synaptic information through

the hippocampus, however, it is a simplified view of the true connectivity that exists.

The dentate gyrus is primarily composed of granule cells, excitatory mossy cells, and a variety of inhibitory interneurons sub-types. The dentate is thought to act as a "gate" to incoming electrical activity from the entorhinal cortex (Lothman et al. 1992; Strigner & Lothman 1992). The "gate" property of the dentate gyrus is based on granule cells having a high level of tonic inhibition, a relatively negative resting potential, a high threshold for action potential generation, and a high degree of spike accommodation to maintained depolarization compared to pyramidal cells in the hippocampus (Staley et al. 1992). The inhibitory GABA-activated chloride channels of the granule cells are high-conductance and can shunt excitatory synaptic events (Staley & Mody 1992) making it more difficult to depolarize these neurons in response to incoming synaptic activity, and blocking electrical activity from spreading further into the hippocampus. During "maximal dentate activation", the high threshold of the dentate gyrus can break down allowing the spread of electrical activity to other areas of the hippocampus (Lothman et al. 1992; Heinemann et al. 1992; Stringer & Lothman 1992). While this "gating" property may normally restrict electrical activity, a parallel pathway from the entorhinal cortex projecting directly to the CA3 and CA1 areas may still transmit electrical activity into the hippocampus regardless of the properties of the dentate gyrus.

The hilus is a relatively unorganized sub-region of the dentate gyrus containing granule cell axons and heterogenous groups of neurons including

excitatory mossy cells and inhibitory interneuron sub-types (Amaral 1978). The granule cell axons, or mossy fibers, synapse on the dendrites of CA3 neurons as well as inhibitory interneurons via axon collaterals. The soma and dendrites of mossy cells are mainly contained within the hilus. Their axons innervate the inner one-third of the molecular layer ipsilaterally, and contralaterally their primary targets are granule cell dendrites. Mossy cell axons are also known to emit collaterals that may contact inhibitory interneurons (Amaral 1978; Ribak et al. 1985; Buckmaster et al. 1996). The complex synaptic connections of the hilus have made the investigation of normal and pathological hippocampal circuitry a difficult task.

1.2 Inhibitory interneurons of the hippocampus

Hippocampal inhibitory interneuron sub-types have distinct anatomical and functional properties which have allowed them to be classified by their chemical markers, location of their post-synaptic targets, and electrophysiological characteristics. Somatostatin-containing interneurons are found in all hippocampal subfields, synapse preferentially on distal dendrites of granule cells and are implicated primarily in feed-back inhibitory mechanisms (Leranth et al. 1990; Blasco-Ibanez & Freund 1995; Katona et al. 1999; Buckmaster et al. 2002). Parvalbumin-containing interneurons synapse preferentially at or near the soma of granule cells and their axon terminal fields overlap with that of basket and chandelier cells, suggesting that these cell types contain parvalbumin (Kosaka et al. 1987; Katsumaru et al. 1988; Soriano et al. 1990). Calretenin-

containing interneurons are found in all layers of the hippocampus and dentate gyrus (Jacobowitz & Winsky 1991; Gulyas et al. 1992; Miettinen et al. 1992; Resibois & Rogers 1992). The majority of neuro-peptide Y-positive interneurons have their soma's in the hilus and along the hilar granule cell border (Miettinen & Freund 1992). Dendrites of the hilar residing group remain there and do not penetrate the molecular layer, while those at the hilar granule cell border send dendrites through stratum moleculare. Their axon terminals are seen in the outer one third of the molecular layer and in the granule cell layer and hilus. Cholecystokinin-positive interneurons are found in all layers of the hippocampus and dentate gyrus and represent about 10% of all GABAergic cells (Greenwood et al. 1981; Sloviter & Nilaver 1987; Harris et al. 1985; Kosaka et al. 1985). The distribution of their dendritic trees allows these cells to receive input from the perforant path as well as from local mossy fiber collaterals, implicating them in both feed-forward and feedback inhibitory mechanisms. Vasoactive intestinal peptide -positive interneurons are present in all layers of the hippocampus and dentate gyrus, but not with uniform distribution (Loren et al. 1979; Acsady et al. 1996; Sims et al. 1980). In the dentate gyrus these cells are found within or close to the granule cell layer, and in lesser numbers in the outer molecular layer and hilus. Substance-P containing interneurons are found in the hippocampus and dentate gyrus and are thought to have local and extrinsic origins (Ljungdahl et al. 1978; Roberts et al. 1984; Davies & Kohler 1985). A population of GABAergic interneurons has also been identified that projects from the ipsilateral to contralateral hippocampus that affects intrahippocampal inhibitory networks and

is involved in both feed-forward and feedback inhibitory responses (Buzsaki & Eidelberg 1981; Buzsaki 1984). Although many interneuron sub-types have been described and classified, their exact functional roles are still being defined.

Basket cells are the most prevalent class of inhibitory interneurons and play a large role in a variety of hippocampal inhibitory mechanisms.Their soma's are found in the granule cell layer or within 50 μm of it, and within the molecular layer of the dentate gyrus. Five types of basket cells have been described in the dentate gyrus including pyramidal, horizontal, fusiform, inverted fusiform, and multipolar neurons (Ribak & Seress 1983). Basket cells are known to have heterogenous afferent connections and predominantly innervate the perisomatic regions of principal cells. Pyramidal shaped basket cells have their somas mainly located at the granule cell hilar border with dendrites reaching through the molecular layer. Axons of all types of basket cells enter the granule cell layer and inner molecular layer where they form a large plexus extending up to 1 mm in the longitudinal axis forming divergent synaptic connections on the somata and proximal dendrites of granule cells (Struble et al. 1978). Basket cells have been identified as the interneurons primarily involved in inhibitory feedback mechanisms, however many of their dendritic trees are in a position to theoretically be activated in both feed-forward and feedback manners.

Inhibitory interneurons of the hippocampus have unique electrophysiological properties distinguishing them from principal cells. Inhibitory interneurons are characterized as possessing a spike duration of less than 1.2 ms, a relatively high discharge rate of 5-80 Hz, and repetitive and high frequency

firing in response to afferent activation (Finch & Babb 1977; Schwartzkroin & Mathers 1978; Buzsaki & Eidelberg 1981; Knowles & Schwartzkroin 1981; Buzsaki & Eidelberg 1982). It has also been reported that inhibitory interneurons have a lower threshold for action potential generation then principal cells (Buzsaki & Eidelberg 1982; Ashwood et al. 1984; Lacaille 1991). Post-synaptic potentials (PSP's) recorded from interneurons in CA1 to afferent stimulation reveal a multi-component response. First, a fast AMPA-receptor mediated excitatory postsynaptic potential (EPSP), then an early $GABA_A$-receptor mediated inhibitory postsynaptic potential (IPSP) lasting approximately 80 ms, and a late $GABA_B$-receptor mediated IPSP which peaks at 100-130 ms. In a subset of interneurons a delayed slow EPSP has been observed (Buhl et al. 1994; Miles 1991; Miles et al. 1996; Lacialle 1991). Thus, interneurons can be reliably distinguished from principal cells and from each other based on their distinct anatomical and chemical differences, post-synaptic targets, and electrophysiological properties. This overview of hippocampal inhibitory interneurons illustrates the complexity involved in isolating and identifying specific cell types involved in the study of synaptic inhibition in the hippocampus.

1.3 Feedback and feed-forward inhibitory circuits

Hippocampal inhibitory interneurons may be activated by feed-forward or feedback mechanisms, each of which affects excitability of principal cell populations (Fig. 1.1). Feed-forward inhibition acts via extra and intra-

hippocampal inputs directly activating interneurons that synapse on principal cells, reducing the probability of principal cell firing. Certain interneuron subtypes

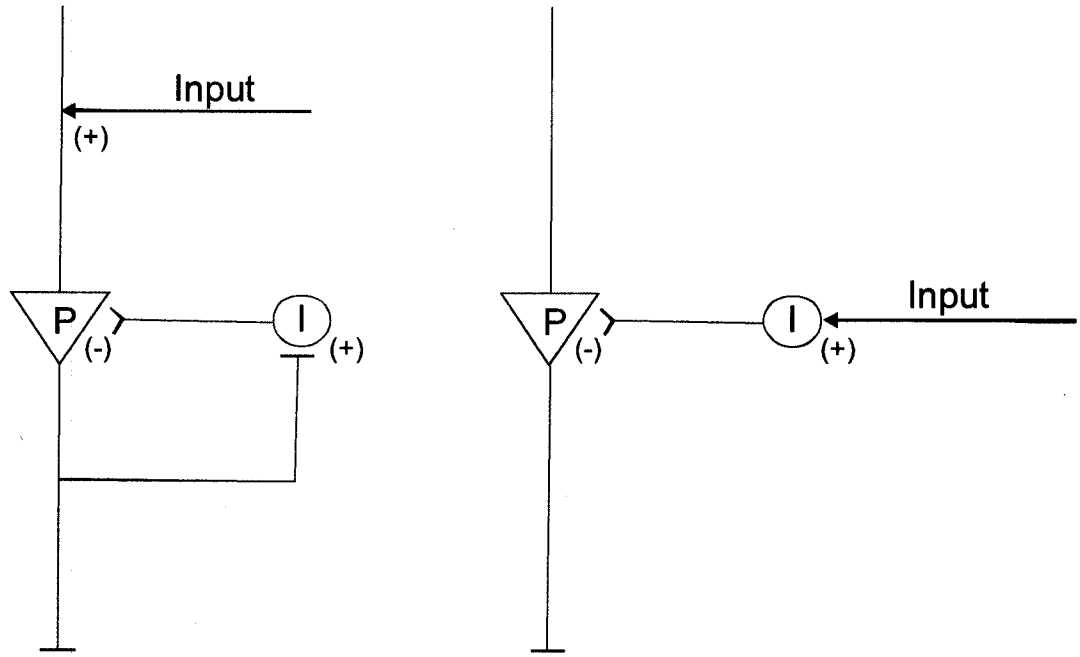


Fig. 1.1 Schematic of feedback and feed-forward inhibitory circuits. (P), principal neuron, (I), inhibitory interneuron.

located in stratum lacunosum moleculare and the molecular layer of the dentate gyrus are innervated exclusively by extrahippocampal afferents and involved in feed-forward circuits only. Other interneuron subtypes located in the pyramidal and granule cell layers and the hilus may be shared between feed-forward and feedback systems (Buzsaki 1984). It has been reported that many of the interneurons involved in feed-forward circuits respond earlier, and have lower activation thresholds than principal cells for population spike generation when stimulated by the perforant path. This phenomenon is partly based on the presence of tonic recurrent inhibition that elevates the activation threshold of the

principal cells (Freund & Buzsaki 1996; Buzsaki & Eidelberg 1982). Feed-forward inhibitory circuits play a crucial role in hippocampal inhibition and may be activated alone, or in conjunction with feedback circuits to influence principal cell excitability.

Feedback inhibition occurs via axon collaterals of principal cells forming excitatory synapses on inhibitory interneurons. These interneurons are then activated to inhibit a group of principal cells, including those that initially activated them. The feedback inhibitory mechanism is mediated by the basket cells of the subgranular zone and accounts for the tonic and post-activation suppression of the granule cells (Andersen et al. 1966). A single principal cell can innervate hundreds of interneurons, which then in turn innervates 1,000-3,000 principal cells (Buhl et al. 1994; Sik et al. 1995). Hence, a single principal neuronal discharge may result in inhibition of tens of thousands of principal cells. The effectiveness of feedback inhibition is dependent upon the output strength from the principal cells that subsequently drive the inhibitory interneurons. Afferent input to the hippocampus has the potential to activate either feedback, feed-forward, or both inhibitory circuits simultaneously. However, it still remains to be determined what type of stimulation patterns preferentially activates each circuit individually.

1.4 The study of synaptic inhibition in the hippocampus

Early investigations into the electrophysiology of hippocampal neurons helped to build the foundation for modern day research in epilepsy. Important

findings include the graded nature of postsynaptic potentials and the direct relationship between neuronal depolarization to excitation, and hyperpolarization to inhibition. Kandel et al. (1961) observed that virtually all hippocampal pyramidal cells underwent a large hyperpolarization following initial activation. These IPSP's were explained by a recurrent inhibitory system terminating on the cells' soma. The characteristics of the IPSP's were similar in a variety of cells, produced by all afferent inputs, and had a long duration and sizable amplitude. Although IPSP's were confirmed in virtually all hippocampal principal neurons, it remained unclear which cells were the source of the recurrent inhibition. Andersen et al. (1963,1964) demonstrated that the basket cell was responsible for the recurrent inhibition reported by Kandel et al. Andersen et al. (1963) reported that following pyramidal cell discharge, axon collaterals would synapse onto and excite the basket cells, which went onto discharge impulses along divergent axons producing IPSP's back on to the pyramidal cells. Lomo (1971) demonstrated that when stimulating the perforant path and recording extracellularly from the granule cell layer, a negative going population spike was observed superimposed on the positive going EPSP. The population spike represented the synchronous discharge of granule cells, whose amplitude was graded in correlation to the number of synchronously firing cells.

The lability of hippocampal inhibition in response to previous stimulation has been observed by many investigators. The decline of membrane potentials in pyramidal cells following tetanic stimulation has been reported to be the result of a breakdown in recurrent inhibition (Andersen & Lomo 1969). Finch & Babb

(1977) recording from a basket cell in vivo and reported a response decrement to repeated stimulation that began after the first stimulus and led to a corresponding collapse in the recurrent inhibitory response. Ben-Ari et al. (1979), recording in CA3 pyramidal cells reported that as stimulus frequency was raised from 1-3 Hz to 3-10 Hz the IPSP's became smaller, disappeared completely at $\geq$10 Hz, and that the effectiveness of GABA diminished during repetitive stimulation. Alger & Teyler (1976) in an in vitro slice preparation noted a consistent response decrement of granule cells following low-frequency repetitive stimulation. Lee et al. (1980) recorded extracellularly from interneurons in CA1 in vitro and reported that in response to repetitive stimulation, interneurons produced a potentiated response which was dependent on stimulus intensity. In vivo, certain interneuron sub-types in the dentate gyrus and CA1 have shown an increased responsiveness to perforant path afferent repetitive stimulation, suggesting that with low current intensity IPSP's will be potentiated in principal cells and alter local network excitability (Buzsaki & Eidelberg 1982). It is clear from these studies that synaptic inhibition in the hippocampus is variable, and exhibits a great deal of plasticity in response to previous patterns of stimulation.

Andersen et al. (1966) were one of the first to use the "double-shock" technique, later known as the paired-pulse technique, in an attempt to measure levels of hippocampal inhibition. They demonstrated the inhibitory effect of the granule cell IPSP's resulting from perforant path stimulation, whereby a conditioning stimulation depressed all signs of spike discharge resulting from a test stimulation at a time-course of 50 ms. The test pulse produced population

spike was not inhibited, however, until the intensity of the conditioning pulse reached a threshold of population spike production. The efficiency of the perforant path excitatory synapses increased as the frequency of stimulation was increased, and an increase in stimulus intensity produced a longer lasting inhibitory effect. Without the availability of more direct means to measure levels of synaptic inhibition in vivo, the paired-pulse technique was and is used as a marker for inhibitory function.

1.5 Altered states of inhibition in experimental epilepsy

Animal models that mimic the hippocampal pathology of human temporal lobe epilepsy (TLE) have been used to investigate the mechanisms of chronic epileptogenesis. Two commonly used models are status epilepticus (SE) and kindling, which have provided an opportunity to study the reduction of GABAergic inhibition and/or the increase of glutamatergic excitation that are known to occur in the human pathology. Consistently affected regions in both human and experimental epilepsy are the hippocampus and dentate gyrus, where primarily CA3 and CA1 pyramidal neurons and interneurons are lost and granule cell reorganization takes place (Margerison & Corsellis 1966; Babb et al. 1984,1991). Hilar neuron loss is a key neuropathological feature of temporal lobe epilepsy, whereby the loss of inhibitory interneurons could reduce inhibitory control and contribute to seizure production (Falconer et al. 1964; Mouritzen Dam 1979; de Lanerolle et al. 1989). Impaired afferent drive, due to principal cell loss, has also been proposed to be responsible for altered states of inhibition (Sloviter 1991b;

Bekenstein & Lothman 1993). Some investigators have reported that GABAergic interneurons in the dentate gyrus survive in human and experimental models of temporal lobe epilepsy (Sloviter 1989; Babb et al. 1989; Davenport et al. 1990) while others have reported significant losses of GAD-positive neurons (Houser & Esclapez 1996; Obenaus et al. 1993). Nissl staining in kainate-treated rats has shown a 52% reduction of hilar neurons with somatostatin-positive and calretinin-positive interneurons being particularly vulnerable (Sperk et al. 1992; Magloczky & Freund 1993, Buckmaster & Dudek 1997; Babb et al. 1989; Sloviter 1989). Some studies report that loss of parvalbumin and CCK-positive interneurons in kainate-treated rats remains in the control range and appear to be largely spared (Best et al. 1993; Magloczky & Freund 1993; Buckmaster & Dudek 1997). However, some treated animals with marked hyperexcitable responses in the dentate gyrus have a significant loss of parvalbumin and CCK-positive interneurons. Other studies report a significant loss of parvalbumin-positive cells in similar numbers to somatostatin-positive celll loss (Kobayashi & Buckmaster 2003). Excitatory mossy cells are the most numerous cells of the hilus, and are also highly vulnerable to seizure induced loss. Along with somatostatin-positive inhibitory interneurons, mossy cells account for 97% of the hilar neuron loss seen in epileptic rats (Buckmaster & Jongen-Relo 1999). Differences in the number and type of neuron loss reported may be partly due to the use of different models and inconsistencies in protocols between labs, and further underscores the complexity of epileptogenesis.

The hypothesis that mossy fiber sprouting causes enhanced recurrent excitation in the dentate gyrus contributing to epileptogenesis was first advanced experimentally by Tauck and Nadler (1985). They showed that mossy fiber reorganization in hippocampal slices from kainate-treated rats was associated with paired-pulse potentiation of field potential responses and multiple population spikes to hilar stimulation. The hilus was stimulated in order to activate granule cell axons selectively and excite feedback circuits, because perforant path stimulation would activate a major extrinsic excitatory synaptic input and be more difficult to interpret. Thus, Tauck and Nadler (1985) observed mildly "hyperexcitable" responses 2-3 weeks after kainate-induced SE.

Sloviter (1992) used perforant path stimulation and field-potential recording from the granule cells in anesthetized rats to argue that the dentate gyrus was "hyperexcitable" to repetitive paired-pulse stimulation shortly after SE. The hyperexcitability was reported to decrease progressively over 2 months while mossy fiber sprouting occurred. He concluded that the initial hyperexcitability was due to decreased inhibition resulting from the SE, and the recovery of inhibition resulted from mossy fiber sprouting and formation of new synapses from granule cells to inhibitory interneurons. Parallel experiments with simultaneous intra- and extracellular recording from hippocampal slices of kainate-treated rats several months after SE showed that GABA-mediated inhibition was relatively intact, and responses to perforant path and hilar stimulation were similar to controls (Cronin et al. 1992). In the presence of $GABA_A$-receptor antagonists, dentate granule cells in hippocampal slices from

normal rats showed an antidromic action potential to hilar stimulation and only one or a few action potentials to perforant-path stimulation (Fricke & Prince 1984; Wuarin & Dudek 1996). In kainate-treated rats with mossy fiber sprouting, however, hilar stimulation evoked robust bursts and afterdischarges in bicuculline (Wuarin & Dudek 1996). These responses had electrical properties resembling a recurrent excitatory circuit (Traub & Wong 1982; Miles & Wong 1987). Similarly, slight elevations in $[K^+]_o$, which both depolarizes neurons and depresses $GABA_A$-receptor mediated inhibition, caused bursting in slices from kainate-treated rats with mossy fiber sprouting, but not in rats studied before sprouting could occur or in saline-treated rats (Patrylo & Dudek 1998; Hardison et al. 2000). These experiments, and comparable studies from several other laboratories (Lynch & Sutula 2000; Molnar & Nadler 1999), provide evidence that mossy fiber sprouting is not associated with hyperexcitability in normal medium. However, under conditions of elevated $[K^+]_o$ and/or $GABA_A$-receptor antagonists the local excitatory circuit generates all-or-none bursts and afterdischarges that do not occur in the dentate gyrus of control rats.

Epilepsy-associated changes in the level of hippocampal inhibition are a common finding, but whether inhibition is decreased or increased is highly controversial. Evidence for a decrease in inhibition is supported mainly by the seizure-induced loss of GABAergic inhibitory interneurons (Sloviter 1987; Obenaus et al. 1993; Buckmaster & Dudek 1997; Gorter et al. 2001; Houser & Esclapez 1996; Kobayashi & Buckmaster 2003). The "dormant basket cell" hypothesis states that temporal lobe epilepsy (TLE) is associated with loss of

excitatory mossy cells that normally project to GABAergic basket cell interneurons leading to hyperexcitability (Sloviter 1991b). These "dormant" basket cells are hypothesized to become re-innervated by sprouted mossy fibers. Despite the known loss of inhibitory interneurons, "hyperinhibition" in the epileptic hippocampus has been reported (Tuff et al. 1983; King et al. 1985; de Jonge & Racine 1987; Stringer & Lothman 1989; Milgram et al. 1991; Haas et al. 1996; Buckmaster & Dudek, 1997; Wilson et al. 1998; Harvey and Sloviter 2005; Sloviter et al. 2006) based mainly on surviving GABAergic interneurons or mossy fibers sprouting axon collaterals forming new inhibitory synapses with granule cells and other inhibitory interneurons (Sloviter 1992; Kotti et al. 1997; Bausch 2005; Sloviter et al. 2006). Spontaneous IPSC's in granule cells have been reported to increase in brain slices from kindled rats, coupled with a reduction of presynaptic inhibition of GABA release (Buhl et al. 1996). It has been hypothesized that inhibition may fail due to a decrease in GABA release, a lack of sensitivity to GABA by principal cells (Thompson & Gahwiler 1989; During et al. 1995), or the depolarizing action of GABA (Staley et al. 1995). Despite the potentially significant role of impaired inhibition in epilepsy models, direct recordings from inhibitory interneurons during epileptiform activity are rare and the possibility also exists that excitation overrides existing inhibition (Sutula et al. 1988).

1.6 Research aims and hypotheses

Synaptic inhibition is critically important for normal brain function and largely depends on a relatively small number of heterogeneous interneurons that use gamma-aminobutyric acid (GABA) as their neurotransmitter (Meldrum 1975). GABA acts on two pharmacologically distinct types of receptors (i.e., $GABA_A$ and $GABA_B$), which are responsible for fast and slow IPSC's, respectively (Ogata 1990). For in vivo studies, few methods are available to quantitate changes in GABAergic inhibition across time and animal preparations. Intracellular recordings provide direct measurements of IPSP's of individual neurons but are limited by difficulty in obtaining stable, high-quality recordings for long periods, sensitivity to membrane potential, and only possible in vivo in anesthetized animals. Thus, assessments of the strength of GABA-mediated inhibition with intracellular recordings are potentially the most direct but also the most difficult. The paired-pulse technique has been used extensively in normal and epileptic animals to study synaptic inhibition because of its' ability to repeatedly measure activity of large neuronal populations in a relatively stable manner (Andersen et al. 1966; Tuff et al. 1983; Stringer & Lothman 1989; Hellier et al. 1999). In awake animals, it is the only possible electrophysiological measurement of "inhibition." The rationale supporting the technique is that suppression of the response to a test pulse (i.e. second stimulus) after a conditioning pulse (i.e. first stimulus) reflects the amount of synaptic inhibition (Andersen et al. 1966). Paired-pulse stimulation has typically been performed with a single pair of pulses, but it has also been argued that a repetitive paired-pulse protocol must be utilized to

accurately measure inhibition (Sloviter 1991a; Swanson et al. 1998; Wilson et al. 1998; Harvey & Sloviter, 2005; Sloviter et al. 2006). Paired-pulse suppression in the dentate gyrus is interpreted to be caused by GABAergic synaptic inhibition resulting from the recurrent activation of basket-shaped interneurons (Andersen 1964), as well as interneurons activated directly by afferent stimulation. Therefore, paired-pulse suppression is equated to GABAergic synaptic inhibition. The lack of quantifiable evidence for a direct relationship between paired-pulse suppression and GABAergic inhibition, however, places the technique into question as a reliable, or even accurate means of assessing inhibition.

The fact that the paired-pulse technique has been used historically to assess levels of inhibition does not qualify it as an adequate and accurate means of measurement, and raises the question of how valid is the paired-pulse technique at determining changes in inhibition? Although it has been argued that repetitive stimulation is needed to measure the full strength of inhibition, GABAergic inhibition is known to fade during repetitive activation of inhibitory synapses (Ben-Ari et al. 1979; McCarren & Alger 1985; Thompson and Gahwiler 1989). It is unclear how dependent the results of paired-pulse suppression are on the parameters used to study it. Can changes in paired-pulse suppression be used to accurately measure changes in synaptic inhibition? The use of different paired-pulse protocols begs the question of their relative strengths and weaknesses. Changes in $[K^+]_o$ and $[Ca^{++}]_o$ occur in response to neuronal activation and in epileptic tissue (Moody et al. 1974; Fisher et al. 1976; Fritz & Gardner-Medwin 1976; Heinemann et al. 1977; Benninger et al. 1980; Krnjevic et

al. 1980; Somjen & Giacchino 1985), thus affecting levels of membrane excitability. It is unclear how these changes may influence measurements of paired-pulse suppression. The strong dependence of the "hyperinhibition" and "dormant basket cell" hypothesis on this technique as supportive evidence (Tuff et al. 1983; King et al. 1985; Haas et al. 1996; Wilson et al. 1998; Sloviter 1987; Sloviter 1991b; Sloviter et al. 2003) justifies a detailed analysis of it's validity.

The goal of the study conducted and presented here was to assess how the parameters used in the paired-pulse technique (i.e. single and repetitive paired pulses) affect measured levels of suppression in the dentate gyrus of normal rats in vivo and in a hippocampal slice preparation. The hypothesis was that variable levels of suppression can be obtained using the paired-pulse technique depending on the interplay of the stimulus parameters leading to erroneous interpretations about the state of GABA-mediated "inhibition." The first aim was to assess how the parameters of stimulation intensity, frequency, interpulse interval, and the repetitive nature of stimulation affects levels of suppression in the dentate gyrus of anesthetized rats. Acutely implanted stimulation and recording electrodes in the perforant path and granule cell layer, respectively, were used to obtain field-potential recordings and determine paired-pulse measurements. The second aim was to determine how paired-pulse parameters affect levels of suppression in hippocampal slices prepared from normal rats. Paired-pulse measurements were determined by obtaining field-potential recordings from the granule cell layer while stimulating the perforant path. The third aim was to examine how paired-pulse suppression was affected

by changes in levels of membrane excitability. Alterations in $[K^+]_o$ and $[Ca^{++}]_o$ were made during paired-pulse tests to determine changes in suppression. The fourth aim was to investigate how paired-pulse measurements may change when $GABA_A$-receptor mediated inhibition was reduced. Application of a $GABA_A$-receptor antagonist was applied to hippocampal slices during paired-pulse tests to determine changes in suppression. This is the first study to parametrically investigate the paired-pulse technique as a valid and reliable measure of hippocampal inhibition.

CHAPTER 2

METHODS

2.1 Surgical procedure and electrode placement

Adult male Sprague-Dawley rats (180-250 gm; Harlan, Indianapolis, IN) were used for all experiments. All animals were pre-treated with dexamethasone (4mg/kg) and atropine (2.0 mg/kg) to prevent cardiorespiratory complications before being anesthetized with 2% isoflurane and placed in a stereotaxic unit (Kopf Instruments; Tujunga, CA). Temperatures were maintained using a heating pad. The skull was exposed by making a mid-sagittal incision on the scalp, and the skin reflected with hemostats. The periosteum was removed and haemostasis achieved with application of cotton tips or a cautery pen. Using bregma as a reference, five small holes were drilled into the skull using a Dremel and #105 bit according to the coordinates in Figure 2.1. A ground electrode made of exposed Teflon-coated steel wire (127.0 μm bare) (A-M Systems; Everett, WA) crimped with a female connector (220-P02-200, Wirepro Inc.; Salem, NJ) that spanned both hemispheres was secured just caudal to the frontal lobe support screw with dental cement. A Teflon-coated platinum-iridium recording electrode (76.2 μm) and bipolar stimulating electrode (114.3 μm) were then placed using stereotaxic coordinates.

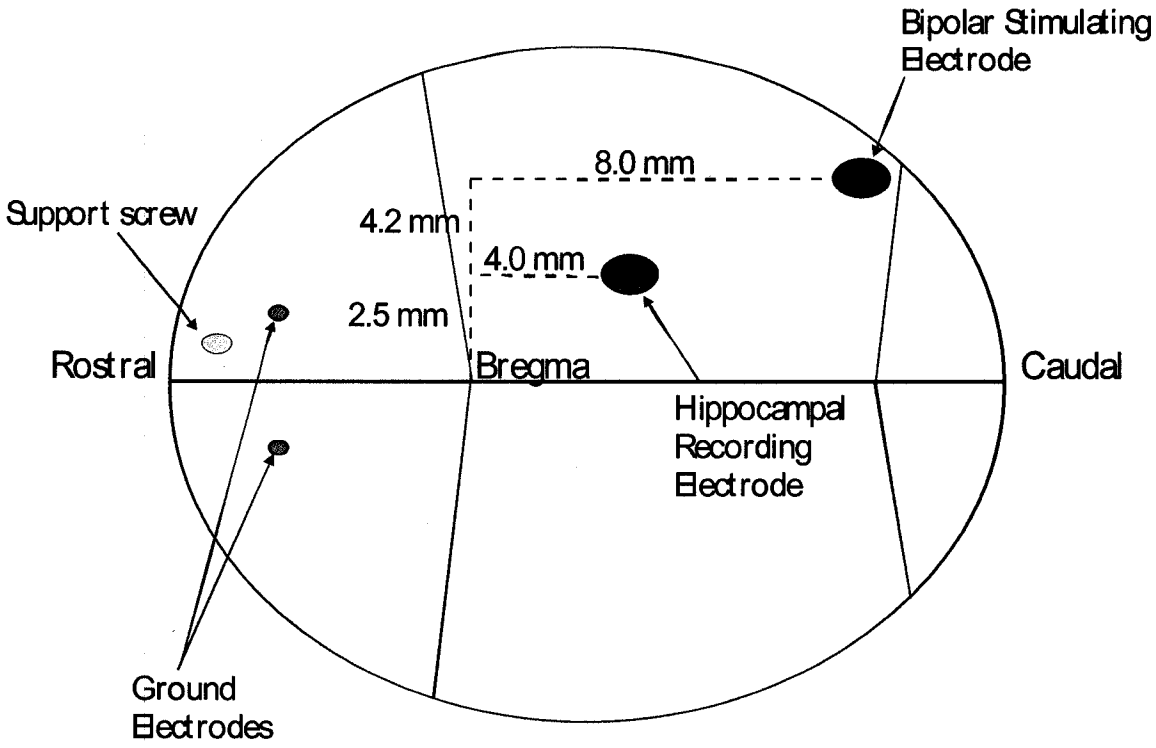


Fig. 2.1 Electrode placement. A hippocampal recording electrode was positioned in the dorsal dentate gyrus at the coordinates shown (3.0 mm below the dura). A bipolar stimulating electrode was positioned in the angular bundle at the coordinates shown (2.5-3.5 mm below the dura).

2.2 In vivo electrophysiology

In seven animals, the recording electrode was lowered into the hippocampus using stereotaxic coordinates and audio monitoring of spontaneous spikes until the dorsal blade of the granule cell layer was confirmed. At this point, the stimulating electrode was lowered into the ipsilateral angular bundle and low-frequency stimulation (duration 0.01 ms) was applied until the response showed a maximal amplitude population spike. The responses were filtered (0.5 Hz-10 kHz), amplified (100X), displayed on an oscilloscope, digitized (Neuro-corder, Neuro Data Instruments; NYC, NY) and stored on video tape. Once a response

consisting of a stable, short-latency EPSP and population spike was confirmed, the minimum intensity needed to produce the largest population spike to the conditioning stimulus was determined. Paired-pulse stimulation consisted of an initial conditioning pulse and a subsequent test pulse separated by the interpulse interval. Single paired pulses were delivered at an intensity of 25%, 50%, 75%, and 100% of maximum population spike amplitude and interpulse intervals of 20, 40, 60, 80, 100, and 200 ms. A 3-min period was allowed between stimulations. Repetitive paired pulses consisted of 10 consecutive stimulus pairs delivered at frequencies of 0.1 Hz, 1.0 Hz, 2.0 Hz, 3.0 Hz, and 4.0 Hz with the same interpulse intervals and stimulation intensities used to assess single paired-pulse responses. All data analyses were performed using pClamp 9.0 software (Axon Instruments, Foster City, CA). Population spike amplitudes were calculated as described by Alger and Teyler (1976) by measuring the mean of the amplitude from the peak of the initial positivity to the trough of the initial negativity, and from the trough of the initial negativity to the peak of the second positivity. Only those population spikes with distinct fast components were used for data analysis. The paired-pulse ratio (PPR) was calculated by dividing the amplitude of the test-pulse population spike by that of the conditioning-pulse population spike. Values <1.0 indicated suppression, and values >1.0 indicated facilitation. The two-tailed Student's t-test and one-way ANOVA were used to assess statistical significance between experimental groups.

2.3 Slice preparation and in vitro electrophysiology

i Field potential recordings. Brain slices were prepared from age-matched rats anesthetized with halothane and euthanized by decapitation. Brains were rapidly dissected out and placed in partially frozen oxygenated (95% O_2-5%CO_2) artificial cerebrospinal fluid (ACSF) composed of (in mM) 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.4 NaH_2PO_4, 1.3 $CaCl_2$, 1.3 $MgSO_4$, and 11 glucose. The cerebellum and anterior forebrain was then dissected out with coronal cuts and the brain glued to the chuck of a vibroslicer (Lancer vibrotome). Hippocampal slices (500 μm) were cut parallel to the base of the brain and incubated at 32-34° C for 1.5 h. Slices were then placed in an interface chamber that allowed the placement of both recording and stimulating electrodes, thermoregulated, and perfused with oxygenated ACSF. In experiments requiring a partial blockade of $GABA_A$-receptors, the antagonist SR-95531 (GABAzine) (Sigma S-106) (0.3 μM) was added to the ACSF and a minimum of a 15-min. wash in allowed. In experiments requiring a decrease in membrane excitability, $[Ca^{++}]$ was increased to 2.5 mM in the bath solution. In experiments requiring an increase in membrane excitability, $[K^+]$ was increased to 6.0 mM and $[Ca^{++}]$ was decreased to 1.0 mM in the bath solution. Field potential recordings were obtained from the granule cell layer with patch pipettes pulled from borosilicate glass capillaries (1.65 mm o.d. and 0.45 mm wall thickness) to a tip diameter of 1-2 μm and filled with1 M NaCl. Bipolar stimulating electrodes were made of two tightly wound Teflon-insulated platinum-iridium wires (75 μm diameter) and positioned in the medial perforant path to stimulate granule cells orthodromically.

ii Whole-cell patch clamp recordings. Brain slices were prepared as described above. Recordings were conducted at room temperature using glass pipettes that were pulled from borosilicate glass capillaries (o.d. 1.65 mm, i.d. 1.2 mm, Garner Glass, Claremont, CA) with a P-87 Flaming-Brown puller (Sutter Instruments, Novato, CA). The intracellular solution was composed of (in mM) 130 CsCl, 1 NaCl, 5 EGTA, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 5 QX314, and 2 ATP. The pH was adjusted to 7.2 with CsOH. When filled with this solution, the patch pipettes had resistances of 2-3 MΩ. The estimated series resistance was 6-10 MΩ. Only data without changes in series resistance during the experiment were included. Neurons were voltage-clamped at -75 mV and only one neuron per slice was recorded. Spontaneous excitatory post-synaptic currents (sEPSC's) were excluded from recordings by adding glutamate antagonist 6,7-dinitroquinoxaline-2-3-dione (DNQX, 50 μM) and DL-2-amino-5-phosphonopentanoic acid (AP-5, 50 μM). Miniature inhibitory post-synaptic currents (mIPSC's) were obtained by adding 2 μM tetrodotoxin (TTX). All signals were amplified with an Axopatch 1D amplifier (Axon Intstruments), low-pass filtered at 2 kHz and sampled at 10 kHz, and recorded with pClamp 9.0 software through a Digidata-1320A digitizer (Axon Instruments). A 0.1% solution of methylene blue delivered along with the SSP-saporin injections allowed visualization of the area of injection, and an estimate of the center of the injection site during the slice experiment. Recordings were performed within the injection zone and at locations outside of the injection zone.

2.4 Neurotoxin injection

Normal male Sprague-Dawley rats (180-250 gm) were initially prepared for surgery as described above. All instruments were autoclaved and maintained sterile with a glass-bead sterilizer. All equipment was sprayed with 95% alcohol. Rats were given prophylactic antibiotic penicillin (30,000 I.U.) subcutaneously 15 min. prior to surgery. The incision site was prepped with betadine scrub and lotion and isolated with sterile surgical towels. A ground electrode made of exposed Teflon-coated steel wire (127.0 μm bare) crimped with a female connector that spanned both hemispheres was secured just caudal to a frontal lobe support screw with dental cement. Using bregma as a reference, a small hole was made through the skull of the left hemisphere over the hippocampus. A glass microelectrode pulled on a Sutter electrode puller was glued to a Teflon-coated platinum-iridium recording electrode (76.2 μm) so the tips were adjacent to each other for audio monitoring of spontaneous spikes. The electrode was filled with 0.1 M PBS and SSP-saporin (Advanced Targeting Systems, IT-11-25) (0.4 ng/10 nl) and connected to plastic tubing connected to a 1 μl Hamilton syringe which was secured to the sterotaxic arm. In some experiments, 0.1% methylene blue was added to mark the injection site. The glass electrode was then lowered into the dorsal blade of the dentate gyrus using stereotaxic coordinates and audio monitoring of spontaneous spikes. Approximately 1 μl of SSP-saporin was injected into the dentate gyrus by manually lowering the plunger of the Hamilton syringe and waiting 20 min. After the injection was complete, the scalp incision was closed with 3-0 Dermalon (non-absorbable

monofilament nylon suture) and bupivicaine (0.5 ml, 0.5%) injected at the incision site for local anesthesia. Animals were monitored for a minimum of 7 days before being euthanized and used for immunocytochemistry or slice electrophysiology.

2.5 Immunocytochemistry

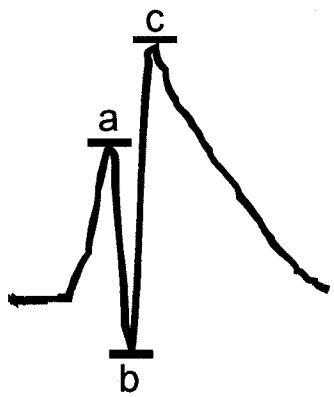
Immunocytochemistry was performed on naïve and experimental rats receiving injections of SSP-saporin. Rats were anesthetized with halothane, their brains removed and placed into ice cold ACSF for 1 min, the hippocampus was dissected out and placed into 4% paraformaldehyde for 24 h at 4° C. The hippocampus was then washed three times in 0.01 M PBS and placed in a 30% sucrose solution for 24 h at 4° C. Next, the hippocampus was sectioned using a freezing microtome into 35 µm sections and sequentially placed into PBS-filled wells. The tissue was put into a peroxidase block solution for 1 h, and then an antibody block solution for 1 h containing 10% normal goat serum. The tissue was subjected to primary antibody for neurokinin-1 receptors (Chemicon AB5060, 1:5000 dilution) or NeuN (Chemicon MAB377, 1:1000 dilution) overnight on a shaker at room temperature. After being washed three times with PBS, the tissue was placed into biotinylated secondary antibody (Vector Labs PK-6101) for 4 h at room temperature. Following three washes with PBS, the tissue was placed in ABC solution (Vector Labs PK-6101) for 4 h at room temperature, washed three times in PBS, and exposed to a DAB solution (Sigma D-0426). The tissue was then washed three more times in PBS and mounted on gelatin coated slides, dehydrated in alcohols, and coverslipped in permount.

CHAPTER 3

EFFECTS OF SINGLE AND REPETITIVE PAIRED-PULSE PARAMETERS ON LEVELS OF SUPPRESSION IN VIVO

3.1 Population spike amplitude measurement

Measurements of population spike amplitudes are difficult because they are never observed in isolation and always occur superimposed on EPSP's, which themselves vary in amplitude and latency. The population spike itself undergoes changes in latency which effect its relative appearance to the EPSP. Different approaches have been used to determine population spike amplitude because there has not been a universally accepted protocol, and this study followed the strategy used by Alger and Teyler (1976). The mean of the amplitudes of the descending and ascending portions of the spike were calculated. Measurements were made from the peak of the initial positivity to the trough of the initial negativity, and from the trough of the initial negativity to the peak of the second positivity (Fig. 3.1). This strategy maintains the information provided by other investigators, while remaining reasonably insensitive to potential shifts in EPSP parameters.



$$\text{Population Spike Amplitude} = \frac{(a-b) + (c-b)}{2}$$

Fig. 3.1 Measurement of the population spike amplitude. Population spike amplitude was determined by measuring the amplitude of the descending (a-b) and ascending portion (c-b) of the spike and calculating their mean value.

3.2 Stimulus-response relationship

A stimulus-response curve was plotted to determine how changes in relative intensity of the stimulus voltage affected the amplitude of the population spike produced by the conditioning pulse. The lowest voltage response that produced the maximum amplitude population spike was defined as 100%, and changes in stimulus intensity were calculated as percent changes from this value. Figure 3.2 illustrates that conditioning spike amplitude was not a linear function of stimulus voltage. Rather, stimulus intensities of 75%-100% produced responses smaller in amplitude than expected from a linear function of spike amplitude versus stimulus voltage. Hence, attempts to standardize paired-pulse experiments based on stimulus intensity may be difficult and inaccurate.

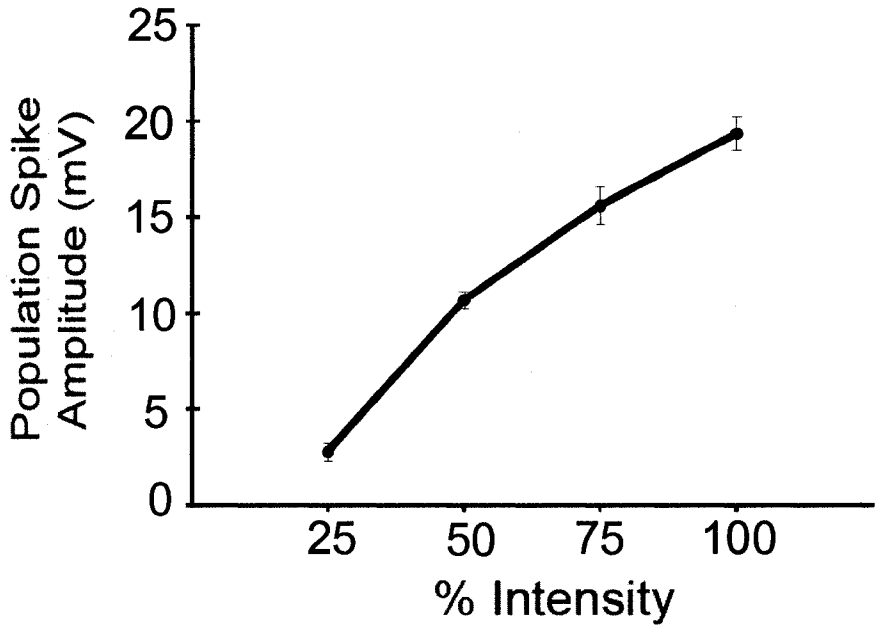


Fig. 3.2 The amplitude of the population spike was not a linear function of stimulus intensity. Stimulus-response curve of the population spike for the conditioning stimulus indicated that amplitude increased as stimulus intensity was raised, but intensities of 75%-100% of maximum produced lower amplitudes then predicted by a linear function (n=5).

3.3 Intensity dependent changes in single paired-pulse suppression

The amplitude of the population spike produced when granule cells fire synchronously is an indication of the number of cells that have been sufficiently activated to reach threshold (Andersen 1971). Theoretically, the more intense the stimulation to the perforant path, the greater the number of afferent fibers that are activated and the larger the amplitude of the population spike produced by the granule cells. Figure 3.3 illustrates that the amplitude of the population spike produced by the conditioning pulse became progressively larger as stimulus intensity was increased. This data indicates that stimulus intensity plays a direct role in determining the amplitude of the population spike to the conditioning

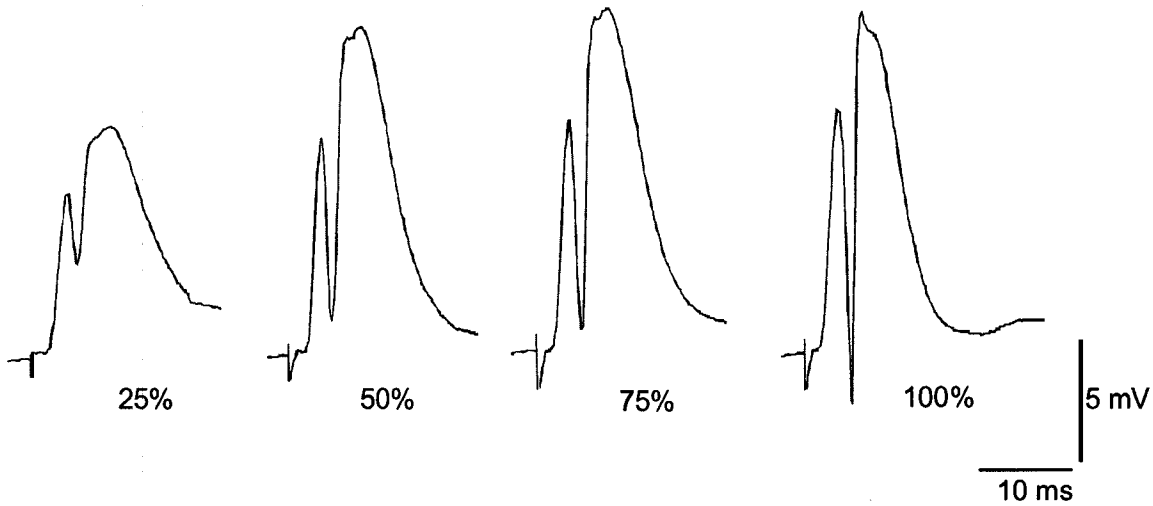


Fig. 3.3 The amplitude of the population spike to the conditioning pulse is dependent on stimulus intensity. Individual population spikes to the conditioning pulse from single paired-pulse responses at stimulus intensities between 25-100% of maximum. Note the increase in population spike amplitude as stimulus intensity was increased.

pulse, although a clearly linear relationship does not exist at higher intensities.

Single paired stimuli were used to assess the effect of stimulus intensity on levels of paired-pulse suppression. Figure 3.4 shows responses from the granule cell layer to single pairs of pulses separated by a 20 ms interpulse interval delivered to the perforant path at the range of stimulus intensities tested.

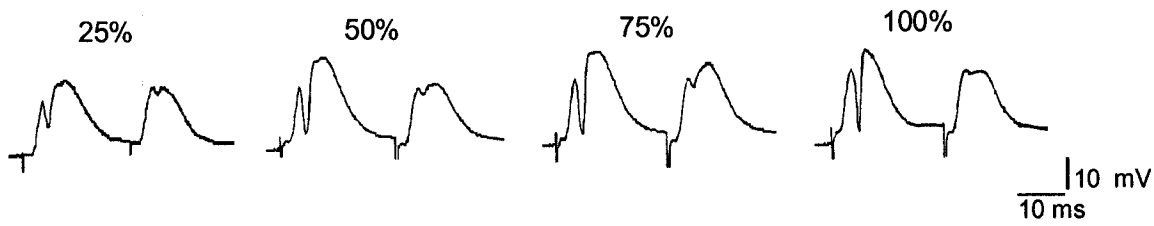


Fig. 3.4 Electrophysiological responses showing that paired-pulse suppression depends heavily on stimulus intensity. Single paired-pulse responses to stimulus intensities of 25%-100% of maximum with an interpulse interval of 20 ms. Traces are an average of 10 individual responses.

As the stimulus intensity increased, there was a corresponding increase in paired-pulse suppression as smaller amplitude population spikes to the test pulse were observed relative to the amplitude of the population spikes to the conditioning pulse. Stimulating at a high intensity (75%-100% of the intensity required to evoke the maximal population spike amplitude) produced the greatest levels of paired-pulse suppression when compared to stimuli of low intensity (50%-25% of the intensity required to evoke maximal population spike amplitude). These data indicate that stimulus intensity plays a direct role in determining the level of paired-pulse suppression.

3.4 Effect of interpulse interval on levels of single paired-pulse suppression

Single paired stimuli were used to assess the effects of interpulse interval on levels of paired-pulse suppression. Interpulse intervals of 20, 40, and 80 ms were analyzed most intensely due to findings suggesting GABAergic recurrent inhibition is strongest at $\leq$ 100 ms (Andersen et al. 1966a) and that alterations using $GABA_A$-receptor agents were restricted to intervals $\leq$ 100 ms (Tuff et al. 1983). A short interpulse interval of 20 ms produced the highest level of paired-pulse suppression, indicated by a small amplitude population spike produced by the test pulse (Fig. 3.5). As the interpulse interval increased, the population spike produced by the test pulse increased in amplitude, and the level of paired-pulse suppression decreased. Differences in the averaged paired-pulse ratios between 20, 40, and 80 ms were statistically significant at stimulus intensities of

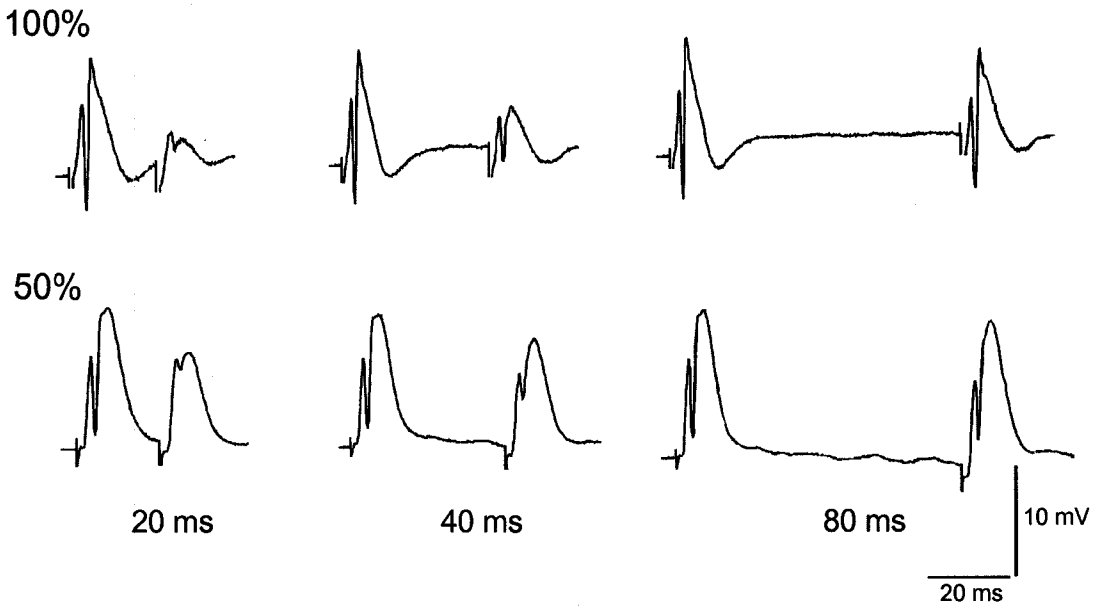


Fig. 3.5 Electrophysiological responses showing that paired-pulse suppression depends heavily on interpulse interval. Single paired-pulse responses with interpulse intervals of 20-80 ms and stimulus intensities of 100% and 50% of maximum. Traces are an average of 10 individual responses.

50%-100% with the exception of 20 to 40 ms with an intensity of 50% (one-way ANOVA $p < 0.05$). When stimulations were delivered with an interpulse interval of 100 ms, the paired-pulse response turned from suppression to facilitation, and then became suppressed again at an interpulse interval of 200 ms (Fig. 3.6). The latter response is presumably during the late phase of the GABAergic response (Tuff et al. 1983). Summary data for the effects of stimulus intensity and interpulse interval on paired-pulse suppression are shown in Figure 3.7, indicating that suppression decreased as stimulus intensity decreased and interpulse interval increased. These data demonstrate that when utilizing a single paired-pulse protocol, stimuli of higher intensities and shorter interpulse intervals produced the greatest levels of paired-pulse suppression.

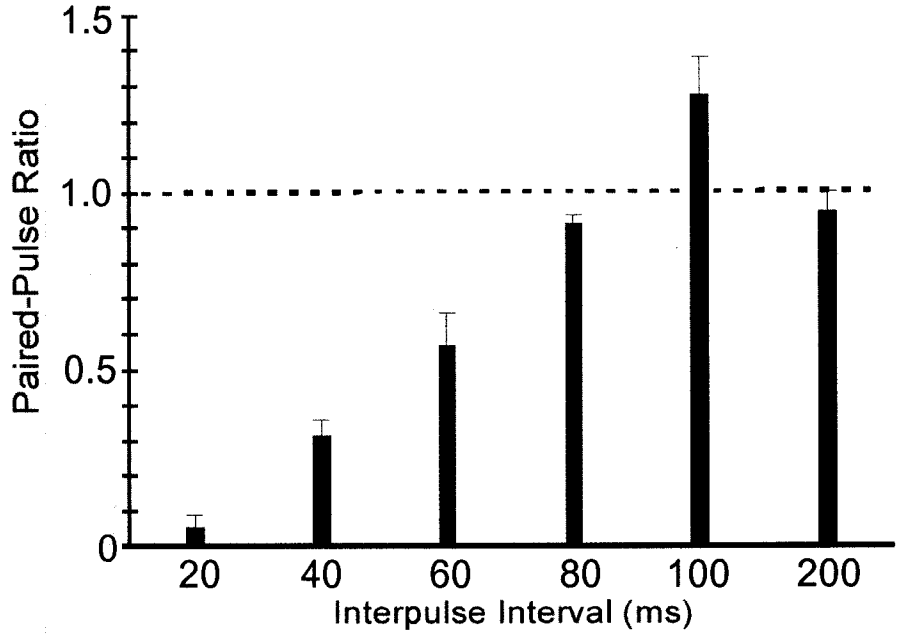


Fig. 3.6 Summary data showing that paired-pulse suppression levels change with interpulse interval. Histogram of the paired-pulse ratio from averaged single paired-pulse responses to interpulse intervals of 20-200 ms with a stimulus intensity that evoked initial responses of 75% of maximum population spike amplitude (n=7).

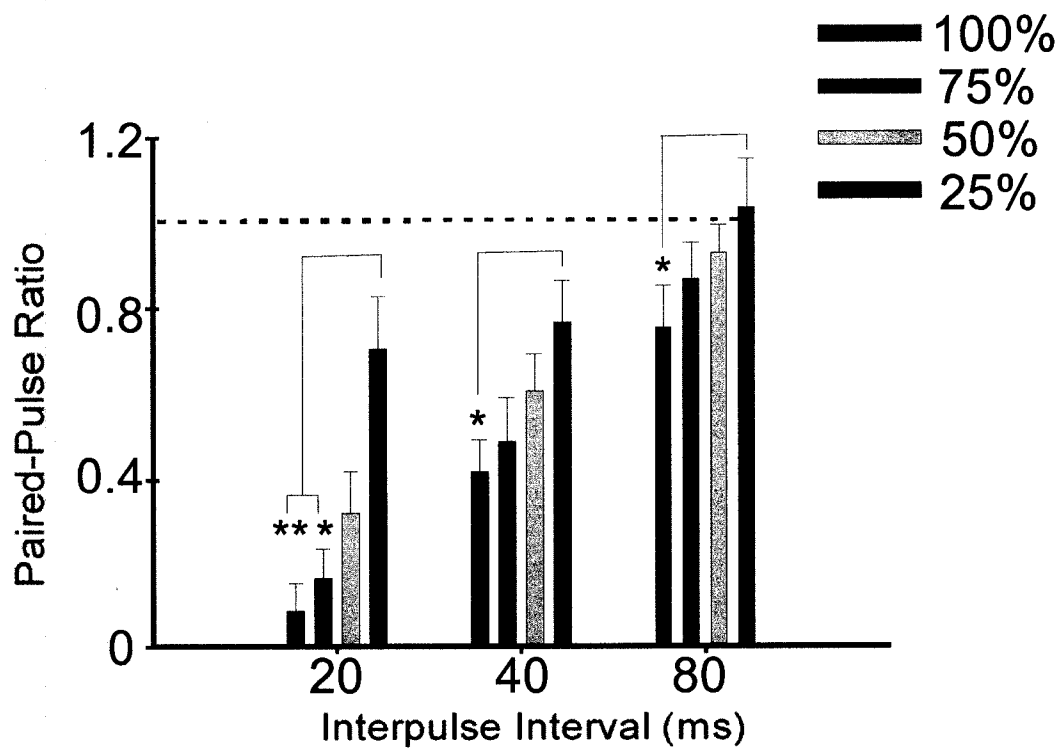


Fig. 3.7 Summary data showing that paired-pulse suppression depends on stimulus intensity and interpulse interval. Histogram of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of 25%-100% of the maximum population spike amplitude and interpulse intervals of 20-80 ms. *p< 0.05, **p< 0.01 (n=7).

3.5 Changes in paired-pulse suppression during a repetitive stimulus train

Consecutive stimulation of ten paired pulses was used to determine how repetitive stimulation effects levels of measured suppression. Interpulse intervals of 20, 40, and 80 ms were used with stimuli of 100%, 75%, 50%, and 25% of the intensity required to evoke the maximal population spike amplitude and frequencies of 0.1, 1.0, 2.0, 3.0, and 4.0 Hz. To establish if levels of suppression change during repetitive paired pulses, suppression was assessed at the first, fifth, and tenth pair in a paired-pulse train of ten consecutive pairs. Figure 3.8 shows that paired-pulse suppression progressively increased during a repetitive train, as indicated by population spikes of progressively smaller amplitude in response to the test pulses relative to the responses to the conditioning pulses. This effect was evident at all stimulation intensities except 25% of the maximum intensity where, presumably, the stimulation was too weak to produce an adequate inhibitory response (Fig. 3.9). This pattern was evident at all stimulus frequencies tested as well, with the exception of 0.1 Hz where little to no change was observed during the train, regardless of the stimulus intensity. Additionally, increasing the stimulus intensity of a repetitive paired-pulse train produced a corresponding increase in paired-pulse suppression (Fig. 3.9). These data

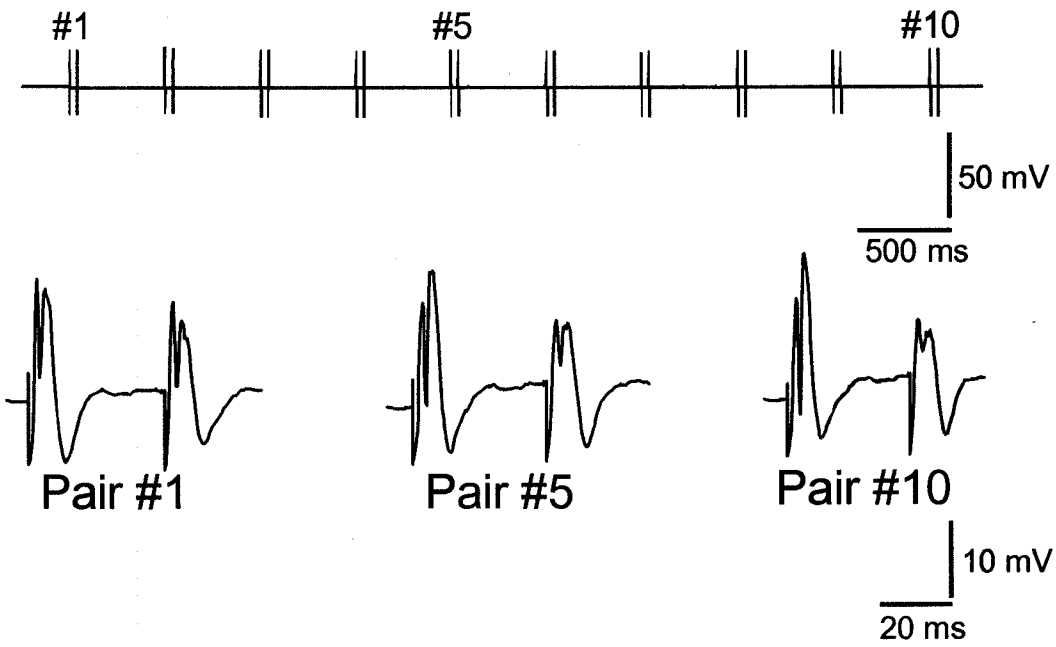


Fig. 3.8 The level of paired-pulse suppression increased progressively during a repetitive train of 10 consecutive paired pulses. Extracellular responses from the granule cell layer at the first, fifth and tenth pulses of a repetitive paired-pulse train delivered to the perforant path (stimulus intensity set at 75% of maximal population-spike amplitude, 2.0 Hz, 40 ms interpulse interval). Top trace shows a diagram of the complete 10 pulse train; bottom trace shows individual responses from the first, fifth, and tenth pairs from that train.

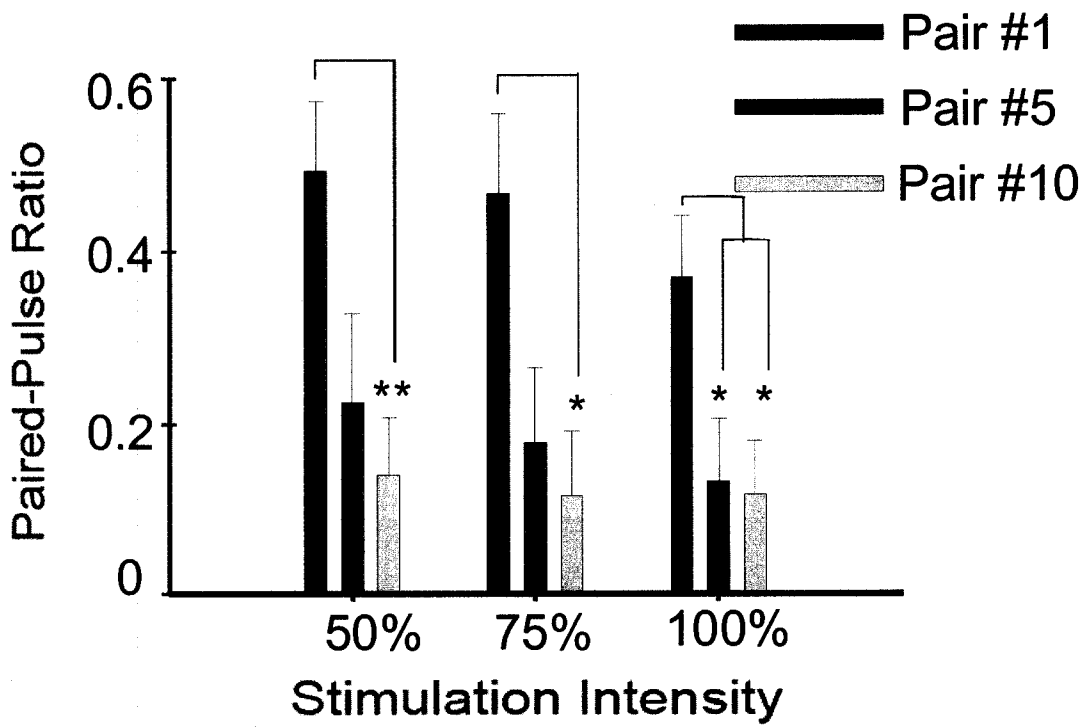


Fig. 3.9 Summary data showing the level of paired-pulse suppression increased progressively during a repetitive train of 10 consecutive paired pulses. Histogram of the paired-pulse ratio for averaged responses at the first, fifth, and tenth pairs in a repetitive train from 100%, 75%, and 50% of maximal population spike amplitude, 2.0 Hz, and 40 ms interpulse interval. *p< 0.05, **p< 0.01 (n=7).

indicate that the level of paired-pulse suppression increased during a repetitive train of 10 paired pulses at all but the lowest stimulus intensity and frequency tested.

3.6 Frequency-dependent changes in paired-pulse suppression

Results of repetitive paired-pulse tests are often presented using different frequencies of stimulation. These differences in protocol, and their subsequent results, raises the question of how frequency alone affects levels of paired-pulse suppression? Figure 3.10 shows that the level of paired-pulse suppression using single pairs of stimuli showed little difference from that produced by using low-frequency repetitive pairs of stimuli of 0.1-1.0 Hz. No statistically significant differences in suppression were found between single and low frequency stimulation. For a particular stimulus intensity and interpulse interval, the amount of paired-pulse suppression was relatively independent of stimulation when <1.0 Hz. As the stimulus frequency was increased to 4.0 Hz, a progressively larger degree of suppression was observed when compared to single pairs of stimuli, with a considerable increase at $\geq$1.0 Hz stimulation (Fig. 3.11). This pattern was preserved at all stimulus parameters with the exception of 25% intensity at 2.0 Hz and interpulse intervals of 40 and 80 ms when a decrease in paired-pulse suppression was observed compared to single paired-pulse. An increase in

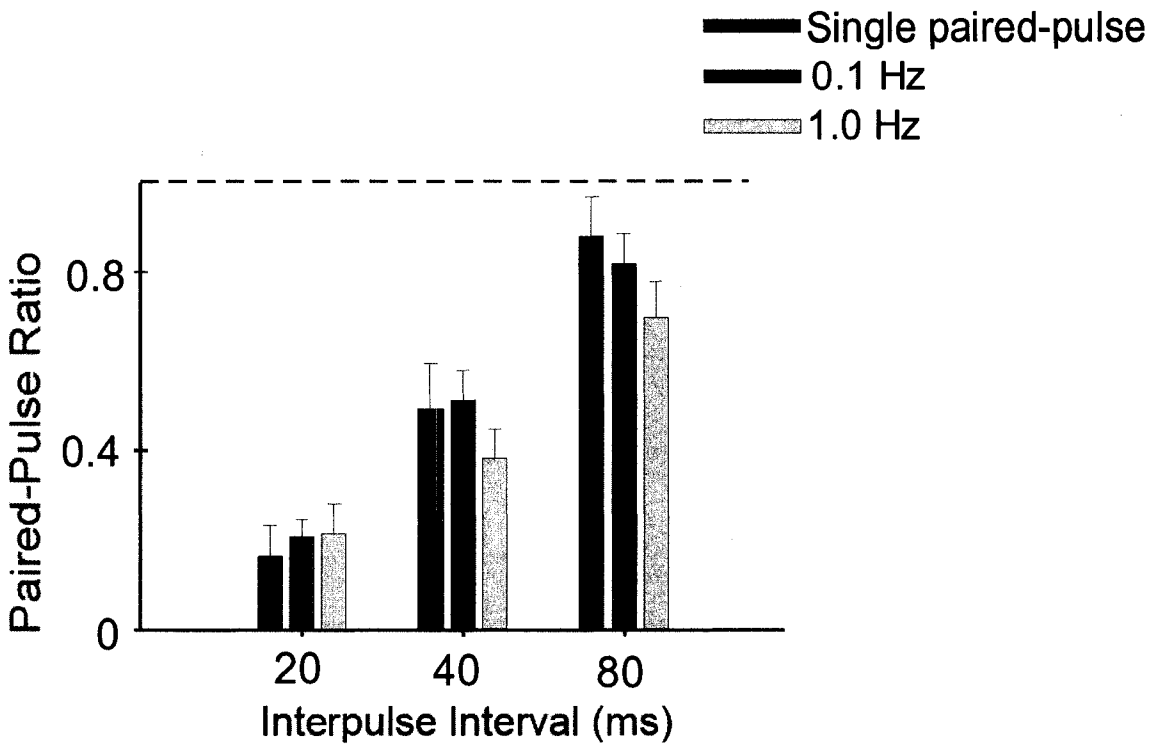


Fig. 3.10 Responses to single and repetitive paired pulses showed similar levels of paired-pulse suppression at low frequencies. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 0.1 Hz and 1.0 Hz and interpulse intervals of 20-80 ms (n=7). Intensity was 75% of maximum population-spike amplitude.

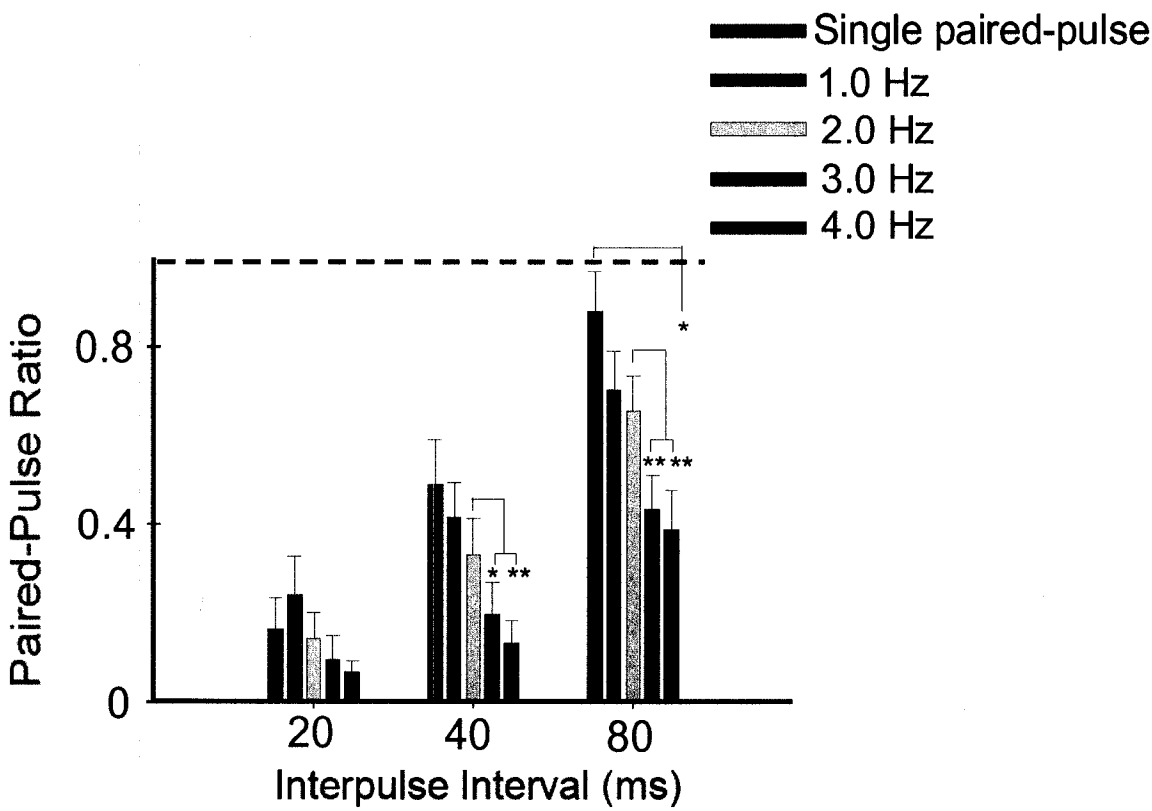


Fig. 3.11 Paired-pulse suppression increased progressively at higher frequencies of stimulation. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 1.0-4.0 Hz and interpulse intervals of 20-80 ms. Intensity was 75% of maximum population-spike amplitude. *p< 0.05, **p< 0.01, n=7.

paired-pulse suppression was observed at all but the lowest intensity as frequency was increased (Fig. 3.12). These data indicate that stimulating repetitively with pairs of pulses at low-frequency showed little difference in the level of paired-pulse suppression than stimulating with single pairs of pulses alone. A considerable difference in the levels of paired-pulse suppression only became evident when stimulating repetitively with frequencies > 1.0 Hz, and then an increase in frequency led to a progressive increase in paired-pulse suppression.

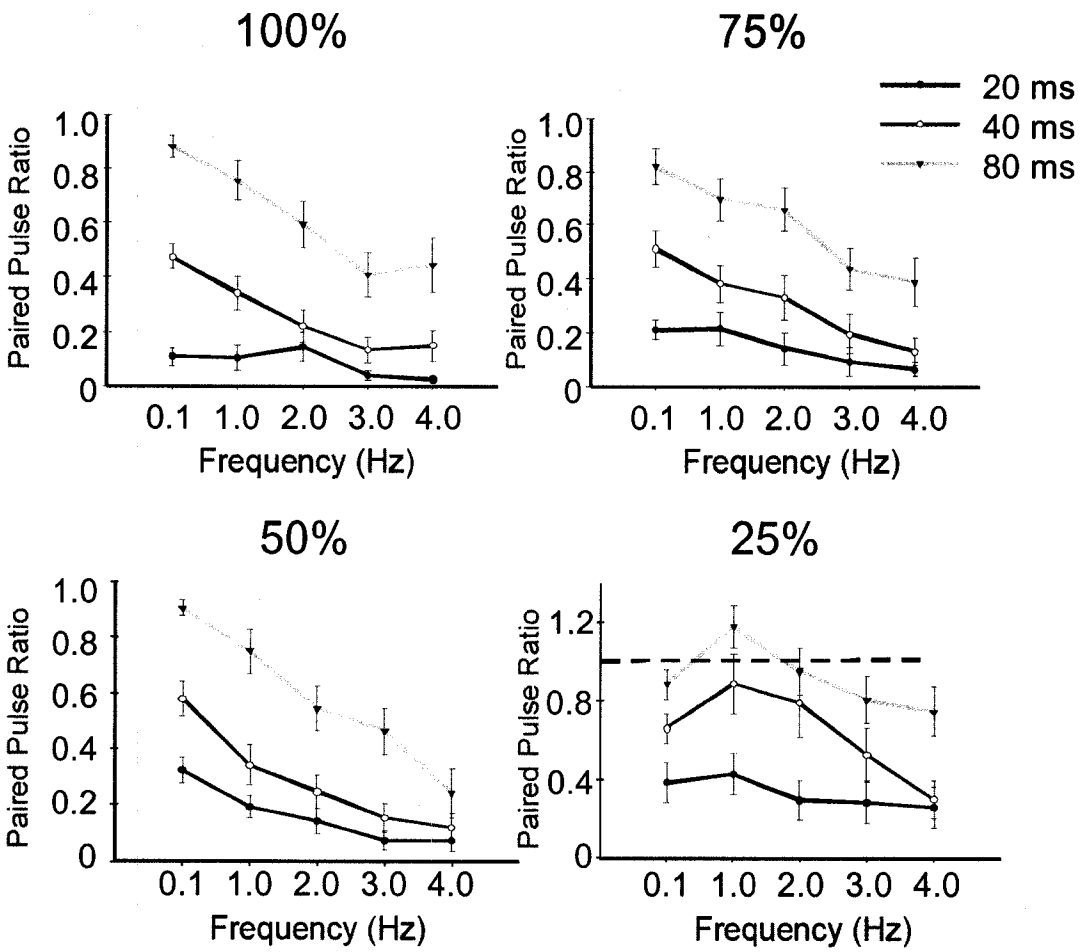


Fig. 3.12 Paired-pulse suppression increased as stimulus frequency increased at all but the lowest stimulation intensity. Plots of the paired-pulse ratio from averaged responses to repetitive paired-pulse stimulation at frequencies of 0.1-4.0 Hz and all stimulus intensities tested. For stimulus intensities that evoked responses from 50% to 100% of the maximal amplitude, the paired-pulse ratio decreased (i.e. paired-pulse suppression increased). Paired-pulse suppression did not occur consistently with higher interpulse intervals when stimulation intensity was 25% of maximum population-spike amplitude (n=7).

3.7 Conditioning pulse population spike amplitude during repetitive paired-pulse stimulation

In order for measurements of paired-pulse suppression to be reproducible and accurate, the amplitude of the population spike produced by the conditioning pulse must remain consistent. In order to determine if this was the case during

repetitive stimulation, the amplitudes of the population spikes in response to the conditioning pulse were compared during a repetitive train at all stimulus intensities, interpulse intervals and frequencies tested. Figure 3.13A shows a repetitive paired-pulse train resulting from stimulation of 75% intensity at 3.0 Hz.

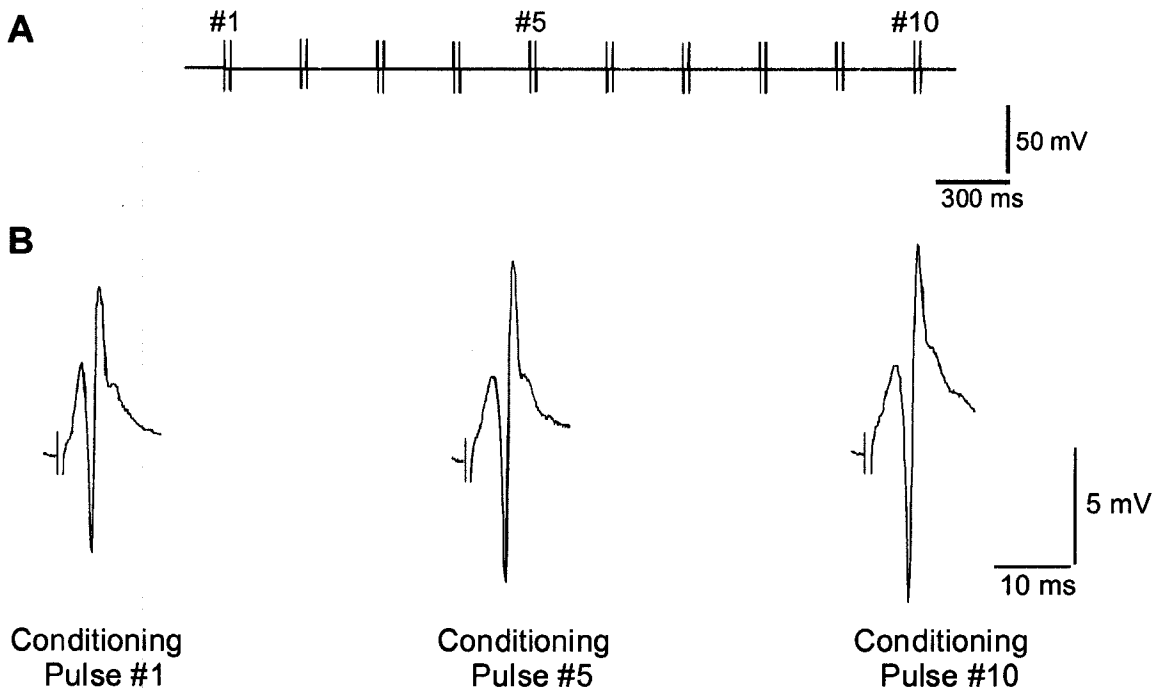


Fig. 3.13 The population spike responses to the conditioning pulse typically increased in amplitude during repetitive paired-pulse stimulation. A: Schematic diagram showing a train of 10 paired pulses delivered at 3.0 Hz and an intensity of 75% of maximum population-spike amplitude. B: Responses to the conditioning stimulus from the first, fifth, and tenth pairs in the repetitive paired-pulse train. Note that the responses to the conditioning stimulus became progressively larger during the stimulus train.

The population spike to the conditioning pulse from the first, fifth and tenth pairs are shown at a larger scale in Figure 3.13B, where their amplitudes increased progressively during the train. To further investigate changes in population spike amplitudes in response to the conditioning pulse, amplitude ratios were

calculated between the first and all subsequent conditioning pulse population spikes during a repetitive train. The amplitude ratio was determined by dividing the conditioning population spike amplitude of the second through tenth pairs, respectively, by the amplitude of the conditioning population spike of the first pair. Low-frequency stimulation of 0.1 Hz did not produce any considerable differences in conditioning pulse population spike amplitudes during a train, as indicated by an amplitude ratio consistently close to 1 (Fig. 3.14).

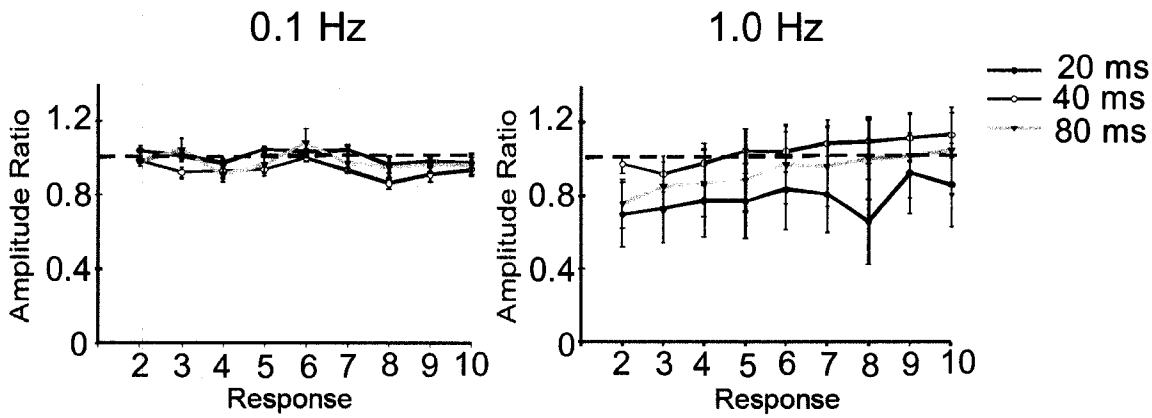


Fig 3.14 Population-spike amplitude in response to the conditioning stimulus at low frequency stimulation. Little change occurred in population spike amplitudes between the first and tenth pairs in a repetitive train at 0.1 Hz. At 1.0 Hz stimulation, population spike amplitudes initially decreased and then increased again. Intensity of stimulation was 75% of maximum population spike amplitude (n=7).

At 1.0 Hz, the amplitude ratio initially dropped below 1 and then progressively increased when using a stimulus intensity of 75% (Fig. 3.14). As stimulus frequencies were increased from 2.0-4.0 Hz the amplitude ratios progressively increased, with the largest values and degree of variability present at 4.0 Hz (Fig. 3.15). Differences were statistically significant at 2.0 Hz using interpulse intervals

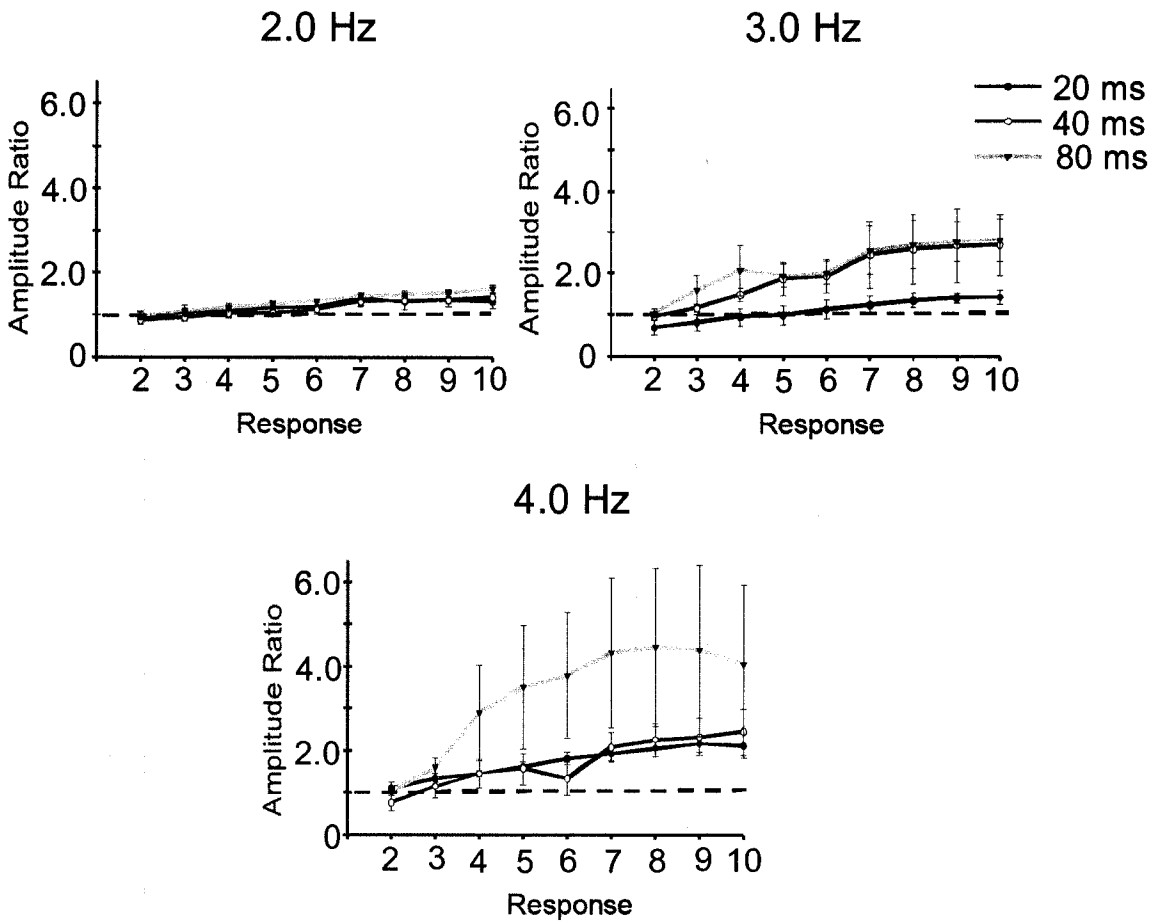


Fig 3.15 Population-spike amplitude in response to the conditioning stimulus increased at higher stimulation frequency. Conditioning pulse population spike amplitudes increased during a 10-pulse repetitive train at 2.0-4.0 Hz stimulation. Intensity of stimulation was 75% of maximum population spike amplitude.

of 40 and 80 ms, at 3.0 Hz using 80 ms, and at 4.0 Hz using 20 and 40 ms (one-way ANOVA, $p < 0.05$). These data indicate that the amplitude of the population spike to the conditioning pulse during repetitive paired-pulse activation increased. Further, stimulation at low frequencies (0.1-1.0 Hz) produced little variability in conditioning pulse population spike amplitude. However, as the frequency increased to 4.0 Hz the amplitudes of the population spikes in response to the

conditioning pulse progressively increased in amplitude and variability, especially at higher intensities.

CHAPTER 4

EFFECTS OF SINGLE AND REPETITIVE PAIRED-PULSE PARAMETERS ON LEVELS OF SUPPRESSION IN A HIPPOCAMPAL SLICE

4.1 Intensity dependent changes to single paired-pulse suppression

Single paired stimuli delivered to the perforant path were used to assess the effects of stimulus intensity on the amplitude of the population spike to the conditioning pulse in the granule cell layer of the dentate gyrus in a hippocampal slice preparation. Figure 4.1 illustrates that the amplitude of the population spike produced by the conditioning pulse became progressively larger as stimulus intensity was increased. This data indicates that stimulus intensity plays a

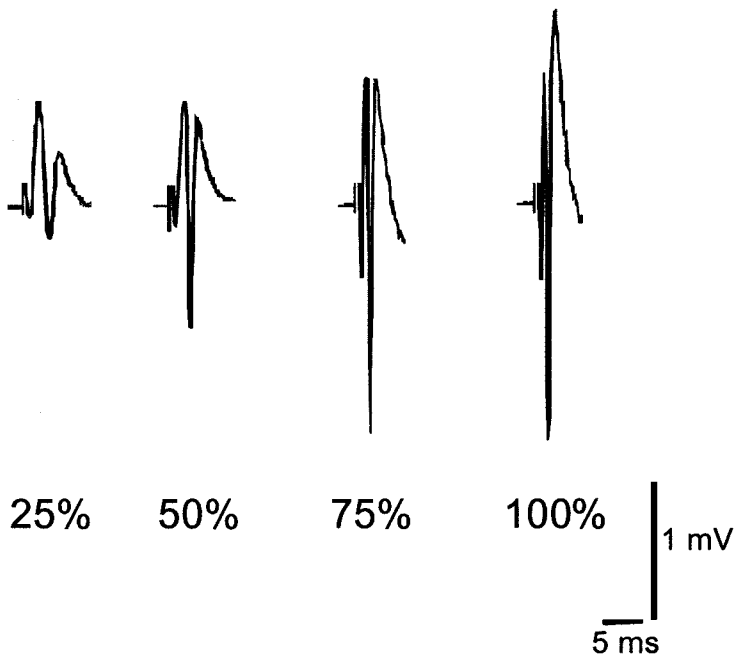


Fig. 4.1 The amplitude of the population spike to the conditioning pulse was dependent on stimulus intensity in a hippocampal slice. Individual conditioning population spikes from single paired-pulse responses at stimulus intensities between 25-100% of maximum.

direct role in determining the amplitude of the population spike to the conditioning pulse in a hippocampal slice, as observed in parallel experiments in vivo.

Single paired stimuli were used to assess the effects of stimulus intensity on levels of paired-pulse suppression in a hippocampal slice preparation. Figure 4.2 shows responses from the granule cell layer to single pairs of pulses delivered to the perforant path at the range of stimulus intensities tested.

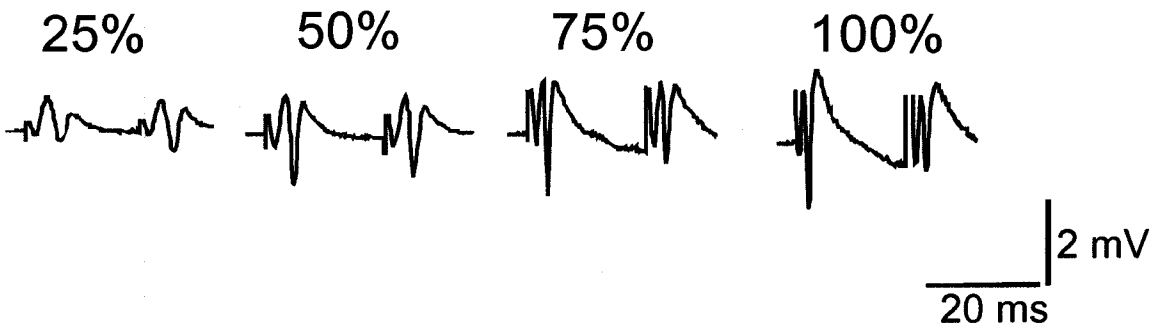


Fig. 4.2 Electrophysiological responses showing that paired-pulse suppression depends on stimulus intensity. Single paired-pulse responses to stimulus intensities of 25%-100% of maximum with an interpulse interval of 20 ms. Traces are an average of 10 individual responses.

As the stimulus intensity was increased, an increase in paired-pulse suppression was observed as population spike amplitudes to the test pulse decreased relative to the amplitude of the population spikes to the conditioning pulse. High stimulus intensities of 75%-100% of maximum amplitude produced the greatest levels of paired-pulse suppression when compared to lower intensity stimulation.

Stimulating at 25% of maximum amplitude produced paired-pulse facilitation, presumably, because the strength of the stimulation was not enough to recruit inhibitory circuits that survived the slice preparation. These data indicate that stimulus intensity plays a direct role in determining the level of paired-pulse suppression in a hippocampal slice as it does in vivo.

4.2 Effect of interpulse interval on levels of single paired-pulse suppression

Single paired stimuli were used to assess the effects of interpulse interval on levels of paired-pulse suppression. A short interpulse interval of 20 ms produced the highest level of paired-pulse suppression indicated by a low-amplitude population spike produced by the test pulse (Fig. 4.3). As the

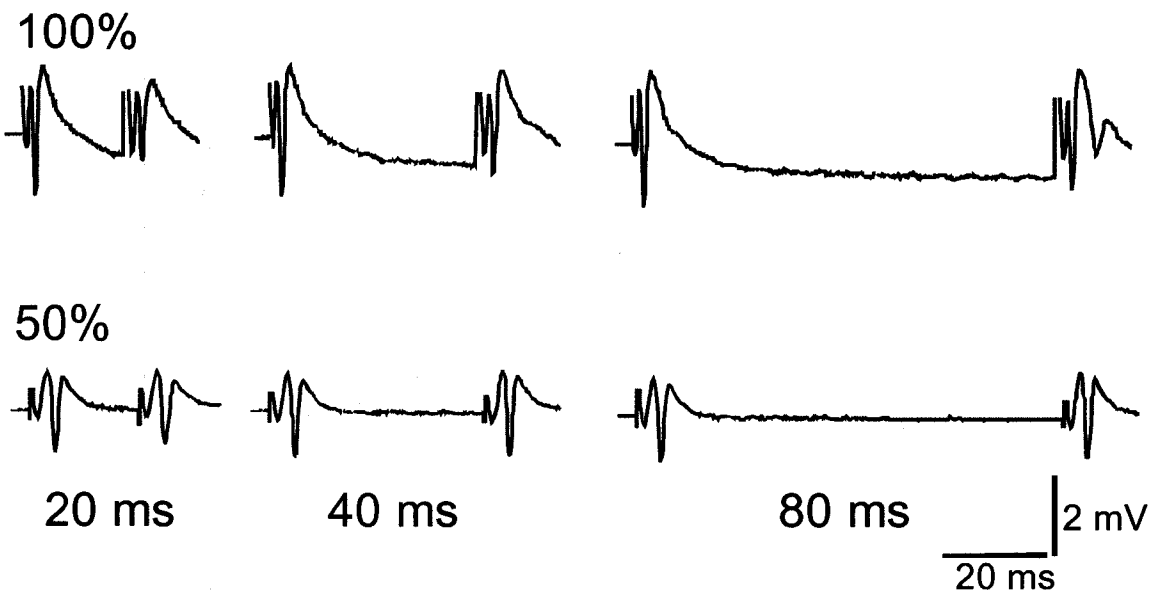


Fig. 4.3 Electrophysiological responses showing that paired-pulse suppression depends on interpulse interval. Single paired-pulse responses with interpulse intervals of 20-80 ms and stimulus intensities of 100% and 50% of maximum. Traces are an average of 10 individual responses.

interpulse interval increased from 20 to 80 ms, the population spike produced by the test pulse increased in amplitude and the level of paired-pulse suppression decreased. Figure 4.4 illustrates that stimuli of higher intensities and shorter interpulse intervals produced the greatest levels of paired-pulse suppression. Differences in the averaged paired-pulse ratios between interpulse intervals of 20, 40 and 80 ms were statistically significant at 100% intensity using interpulse intervals of 20 and 80 ms (one-way ANOVA, $p < 0.01$). This pattern was also observed in vivo, but levels of suppression were more pronounced than in a hippocampal slice.

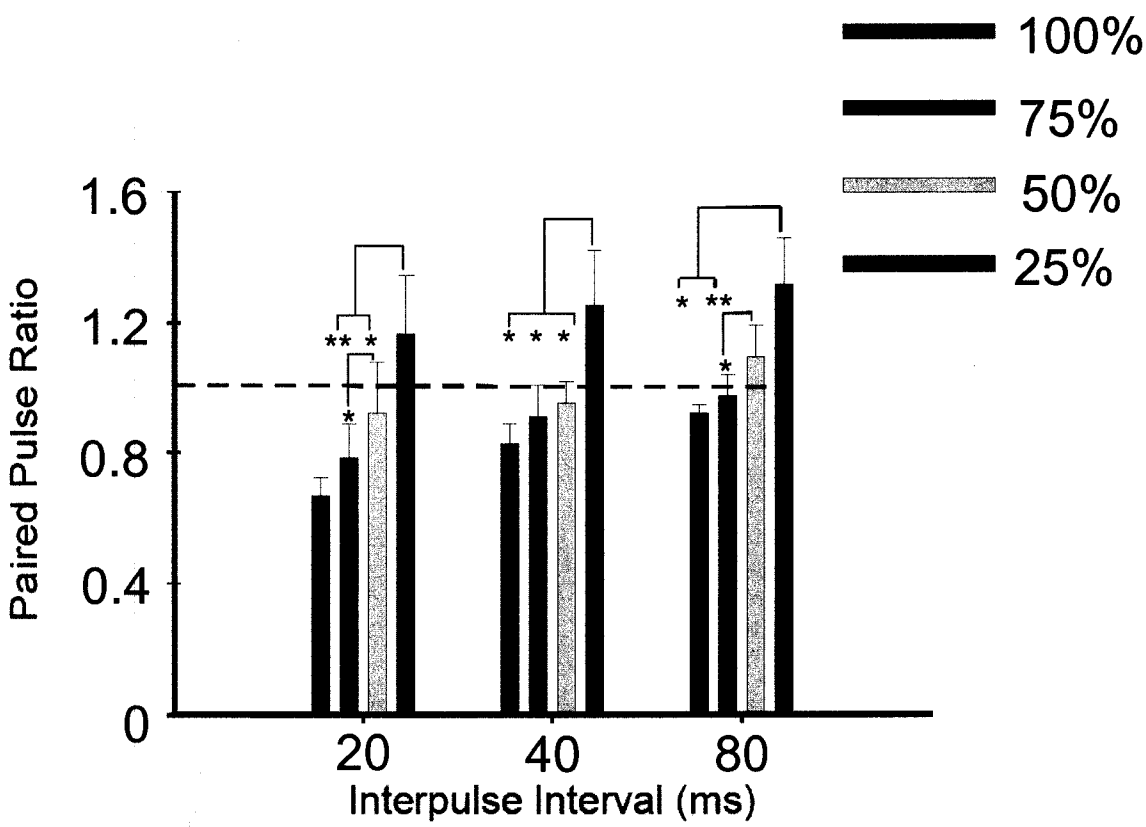


Fig. 4.4 Summary data showing that paired-pulse suppression depends on stimulus intensity and interpulse interval. Histogram of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of 25%-100% of the maximum population spike amplitude and interpulse intervals of 20-80 ms.

Note the decrease in paired-pulse suppression as stimulus intensity was decreased and interpulse interval was increased, and the lack of paired-pulse suppression with an intensity of 25%. *p< 0.05, **p< 0.01 (n=7).

4.3 Changes in paired-pulse suppression during a repetitive train

Paired-pulse ratios were calculated at the first, fifth, and tenth pairs in a train of ten consecutive pairs to determine changes in the level of suppression during repetitive stimulation. Paired-pulse suppression typically decreased during a repetitive train in a hippocampal slice, as indicated by progressively larger population spikes in response to the test pulses relative to the responses to the conditioning pulses (Fig. 4.5). Stimulating at 2.0 Hz at all intensities tested,

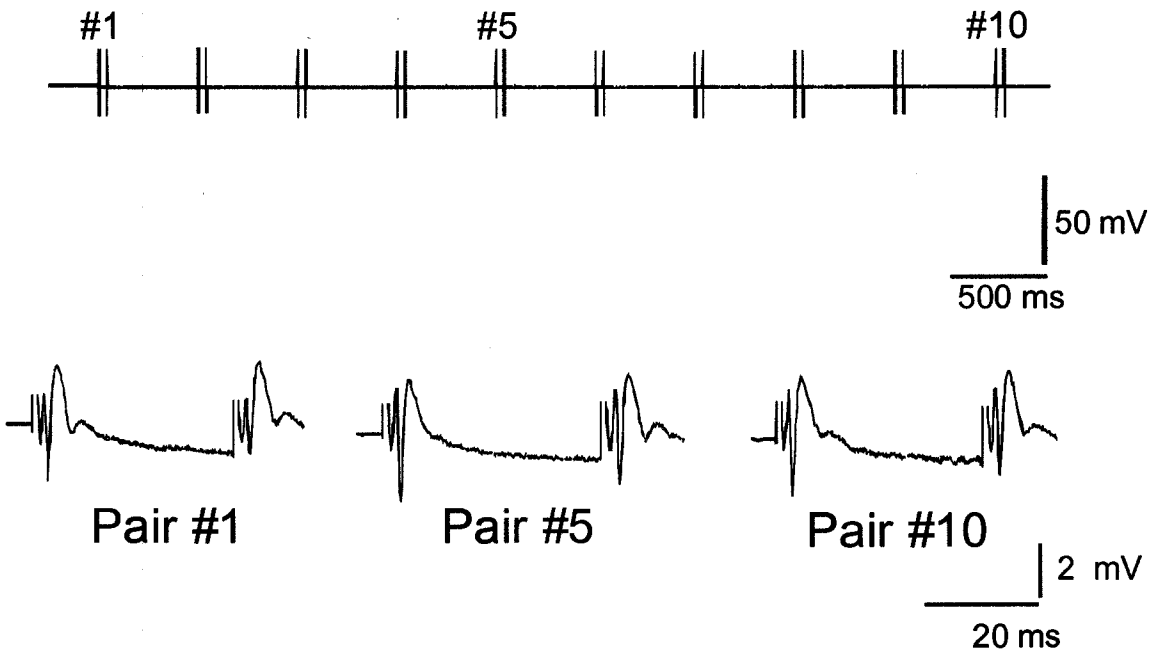


Fig. 4.5 The level of paired-pulse suppression decreased during a repetitive train of 10 consecutive paired pulses. Extracellular responses from the granule cell layer at the first, fifth and tenth pulses of a repetitive paired-pulse train delivered to the perforant path (stimulus intensity set at 75% of maximal population-spike amplitude, 2.0 Hz, 40 ms interpulse interval). Top trace shows a diagram of the complete 10 pulse train; bottom trace shows individual responses from the first, fifth, and tenth pairs from that train.

suppression decreased by the fifth pair and then increased by the tenth pair, however no statistically significant differences were found (Fig. 4.6).

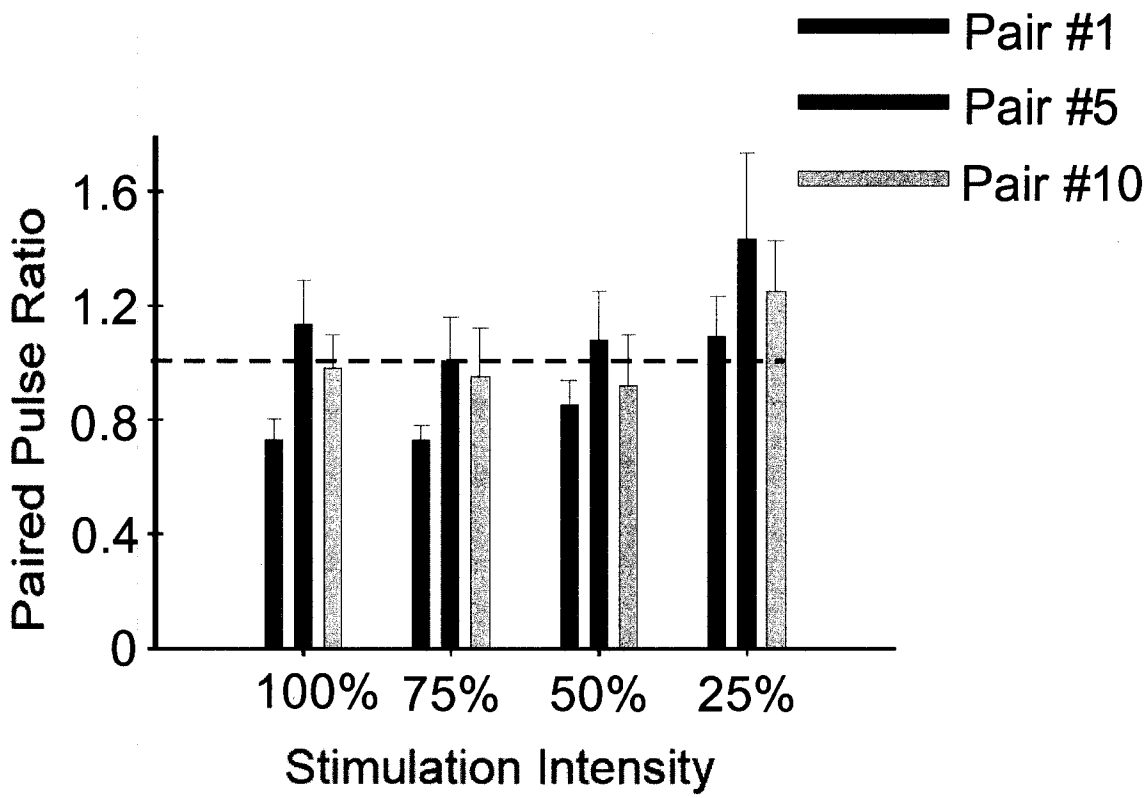


Fig. 4.6 Summary data showing the level of paired-pulse suppression decreased during a repetitive train of 10 consecutive paired pulses. Histogram of the paired-pulse ratio for averaged responses at the first, fifth, and tenth pairs in a repetitive train from 25%-100% of maximal population-spike amplitude, 2.0 Hz, and 40 ms interpulse interval (n=8).

When the rate of stimulation was increased to 4.0 Hz, levels of suppression either showed little change or increased by the end of a repetitive train, although not statistically significant fashion (Fig. 4.7). These data indicate that the level of paired-pulse suppression was sensitive to increases in stimulus intensity, and the pattern of suppression during a repetitive train was frequency dependent.

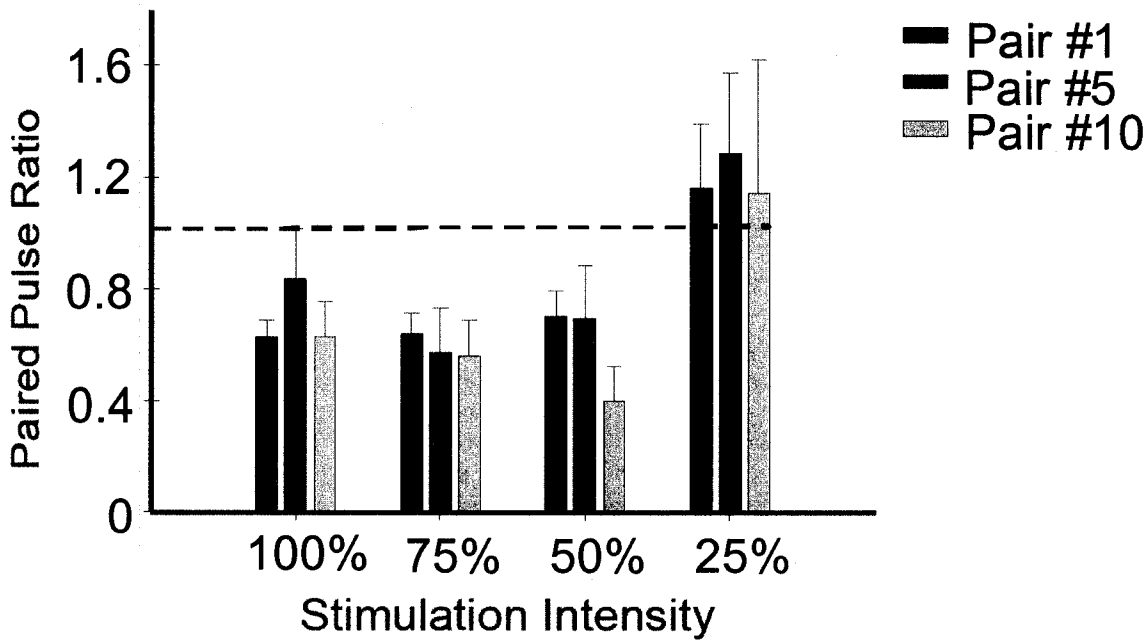


Fig. 4.7 Summary data showing changes in the level of paired-pulse suppression during a repetitive train of 10 consecutive paired pulses at high frequency stimulation. Histogram of the paired-pulse ratio for averaged responses at the first, fifth, and tenth pairs in a repetitive train from 25%-100% of maximal population-spike amplitude, 4.0 Hz, and 20 ms interpulse interval (n=8).

4.4 Frequency dependent changes in paired-pulse suppression

The level of paired-pulse suppression using single pairs of stimuli showed little difference from that produced by using low-frequency repetitive pairs of stimuli of 0.1-1.0 Hz (Fig. 4.8). As the stimulus frequency was increased to 4.0 Hz, a progressively larger degree of suppression was seen when compared to single pairs of stimuli (Fig. 4.9). Paired-pulse suppression increased considerably at $\geq$ 2.0 Hz stimulation. At all stimulus intensities when frequency was increased from 0.1-4.0 Hz, paired-pulse suppression either showed little change or increased (Fig. 4.10). At a stimulus intensity of 100% of maximal amplitude,

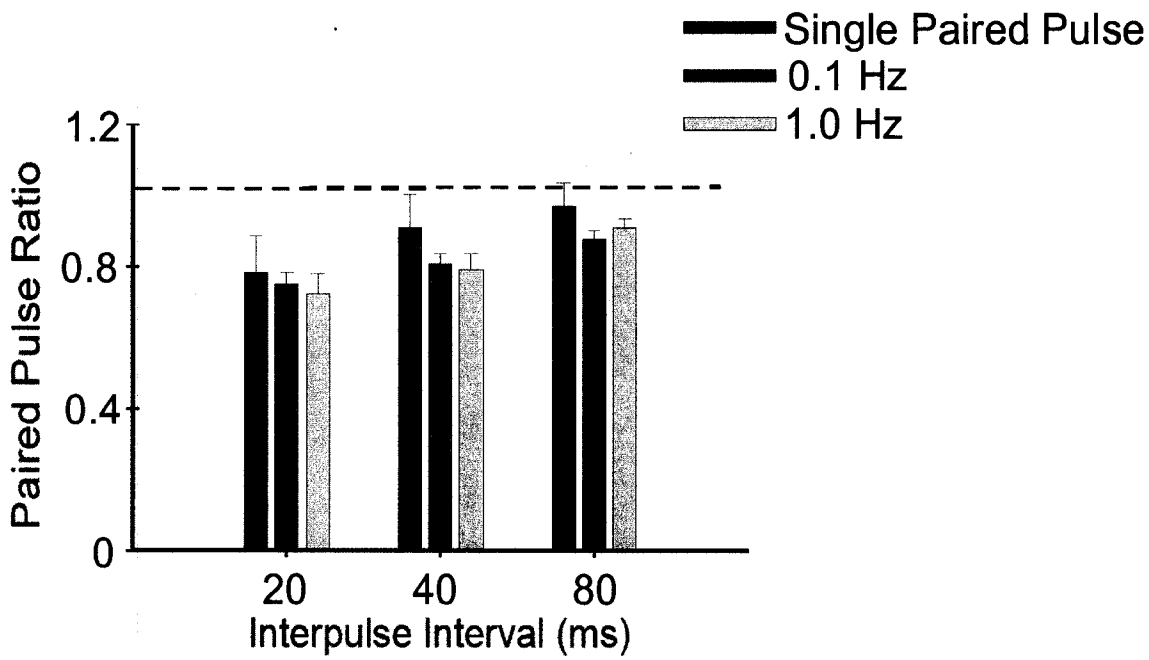


Fig. 4.8 Responses to single and repetitive paired pulses showed similar levels of paired-pulse suppression at low frequencies. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 0.1 Hz and 1.0 Hz and interpulse intervals of 20-80 ms. Intensity was 75% of maximum population-spike amplitude (n=8).

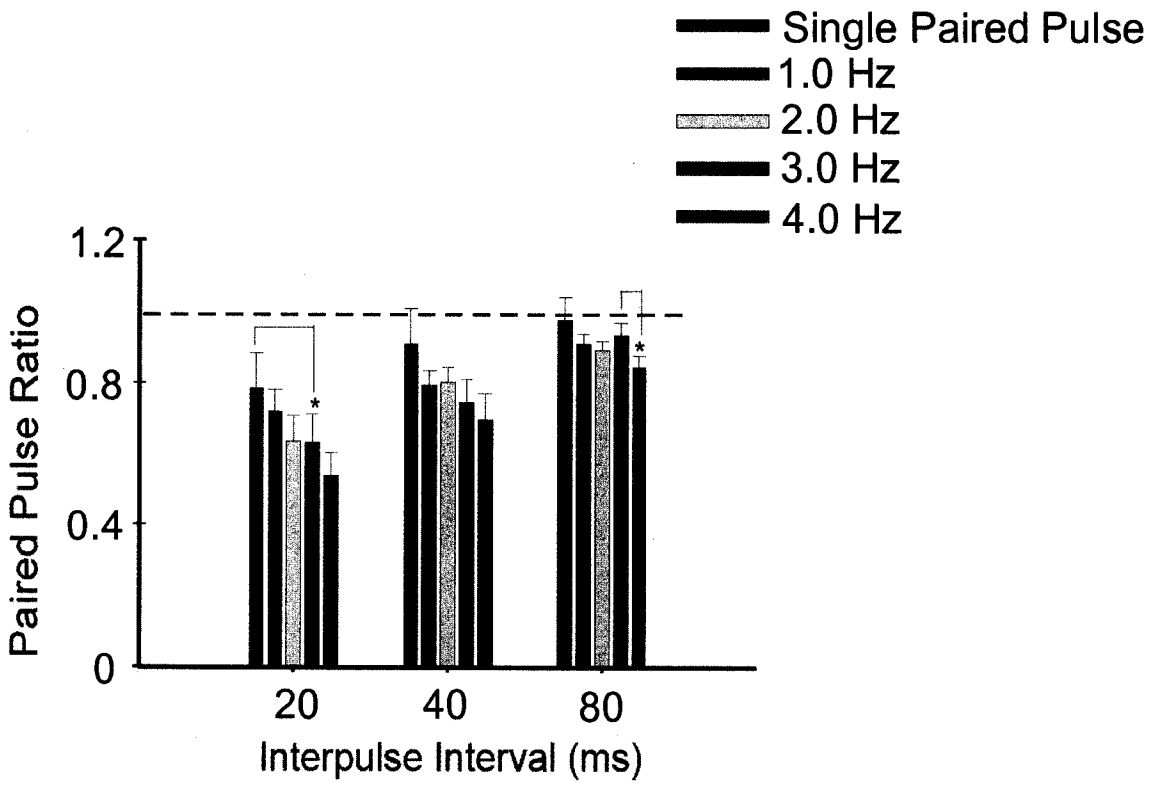


Fig. 4.9 Paired-pulse suppression increased at higher frequencies of stimulation. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 1.0-4.0 Hz and interpulse intervals of 20-80 ms. Intensity was 75% of maximum population-spike amplitude. *p< 0.05 (n=6).

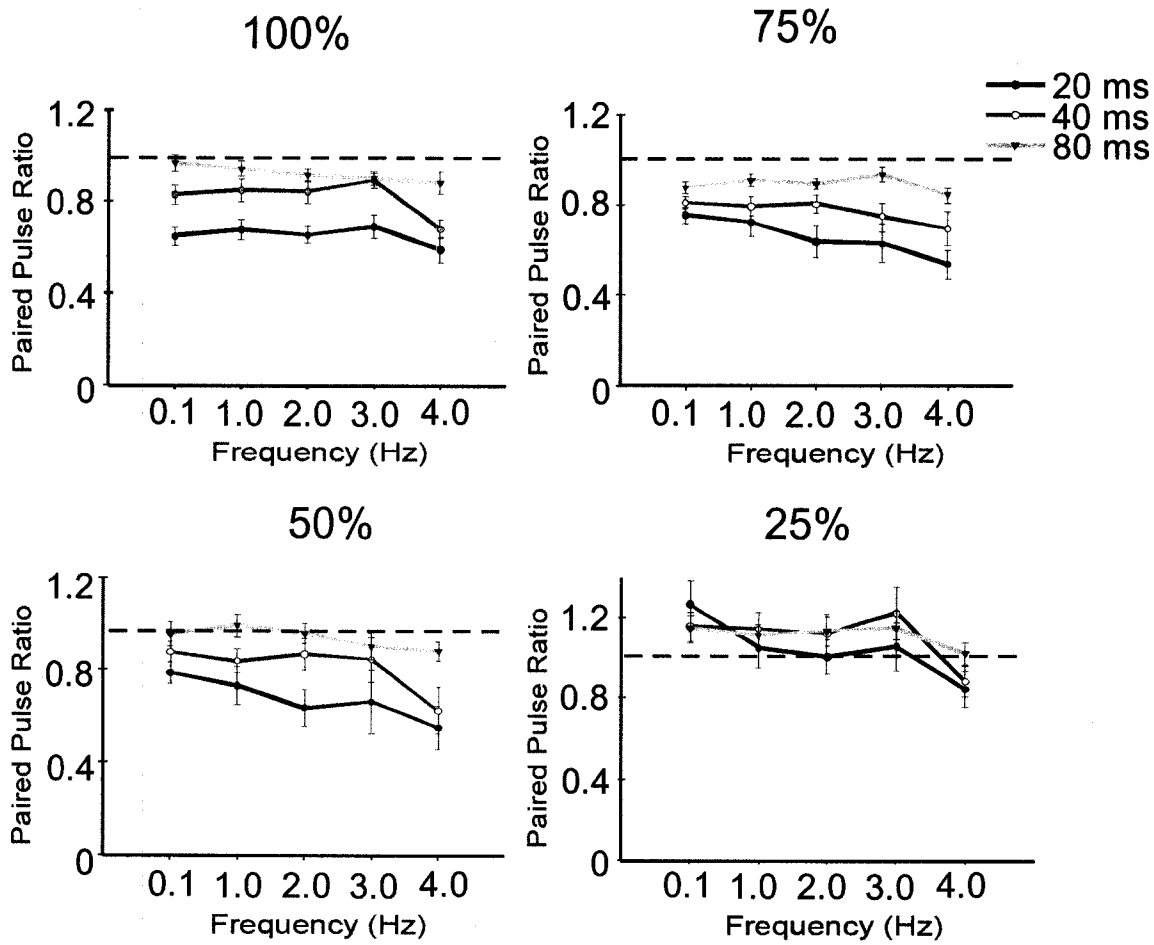


Fig. 4.10 Paired-pulse suppression typically increased as stimulus frequency increased at all stimulation intensities. Plots of the paired-pulse ratio from averaged responses to repetitive paired-pulse stimulation at frequencies of 0.1-4.0 Hz and all stimulus intensities tested (n=8).

little change in suppression was observed between 0.1-4.0 Hz, with the largest difference present using an interpulse interval of 40 ms. For stimulus intensities that evoked responses from 25% to 75% of the maximal amplitude, the paired-pulse ratio decreased, particularly when using short interpulse intervals. These data indicate that stimulating repetitively with pairs of pulses at low-frequency showed little difference in the level of paired-pulse suppression compared to

stimulating with single pairs of pulses alone, and that considerable increases in suppression only became evident at higher frequencies ≥2.0 Hz.

CHAPTER 5

EFFECTS OF MEMBRANE EXCITABILITY AND REDUCED $GABA_A$-RECEPTOR MEDIATED INHIBITION ON PAIRED-PULSE SUPPRESSION

5.1 Paired-pulse suppression under conditions of low membrane excitability

Hippocampal $[K^+]_o$ and $[Ca^{++}]_o$ are altered during neuronal activation, seizures, and in epileptic tissue (Frankenhaeuser & Hodgkin 1957; Moody et al. 1974; Heinemann et al. 1977; Krnjevic et al. 1980; Somjen & Giacchino 1985) influencing membrane excitability by affecting a neuron's threshold level for firing action potentials (Hodgkin 1957). To determine the effects of membrane excitability on paired-pulse suppression, $[K^+]_o$ and $[Ca^{++}]_o$ were modified during paired-pulse experiments. A decrease in membrane excitability was accomplished by increasing $[Ca^{++}]_o$ from 1.3 mM to 2.5 mM. Figure 5.1 shows evidence of paired-pulse facilitation when membrane excitability was decreased at interpulse intervals of 20 to 80 ms, as indicated by increased amplitude population spikes from the test pulse relative to those of the conditioning pulse. At a range of stimulus intensities and interpulse intervals of 20 to 80 ms, paired-pulse suppression was abolished and facilitation observed when membrane excitability was decreased (Fig. 5.2). Differences in the paired-pulse ratio were

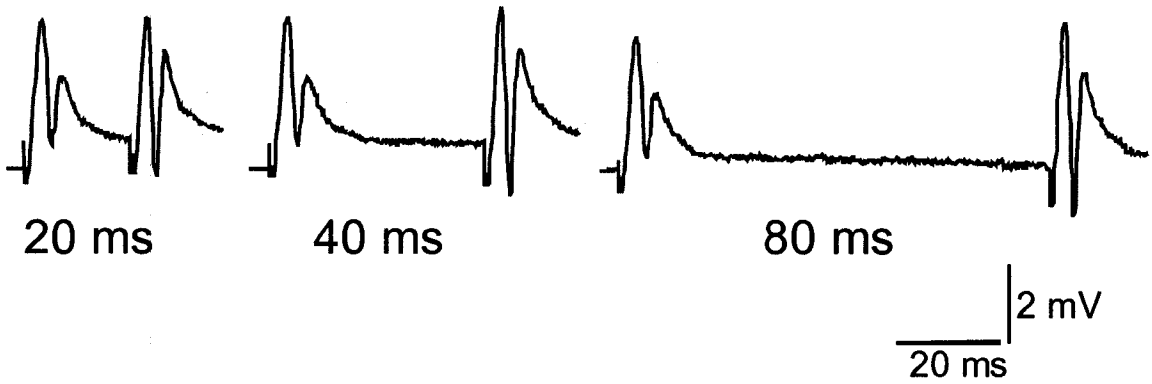


Fig. 5.1 Electrophysiological responses showing the presence of paired-pulse facilitation when membrane excitability was lowered. Single paired-pulse responses at interpulse intervals of 20-80 ms under conditions of decreased membrane excitability (3.0 mM $[K^+]_o$, 2.5 mM $[Ca^{++}]_o$). Stimulus intensity was 75% of maximal amplitude. Traces are an average of 10 individual responses.

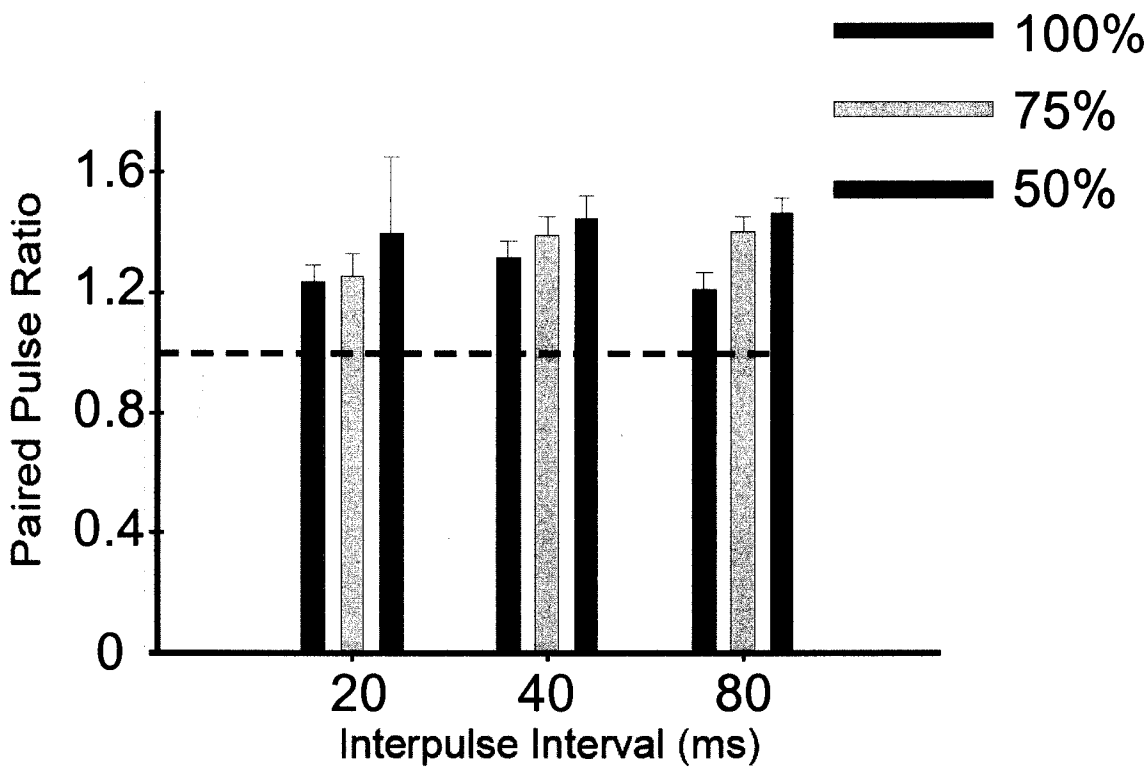


Fig. 5.2 Summary data showing paired-pulse facilitation when membrane excitability was decreased. Histogram of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of 50%-100% of the maximum population spike amplitude and interpulse intervals of 20-80 ms in conditions of decreased membrane excitability (3.0 mM $[K^+]_o$, 2.5 mM $[Ca^{++}]_o$) (n=8).

statistically significant between physiological ACSF and conditions of low membrane excitability at all intensities and interpulse intervals with the exception of 50% of maximum using an interpulse interval of 20 ms ($p<0.05$) (Fig 5.3).

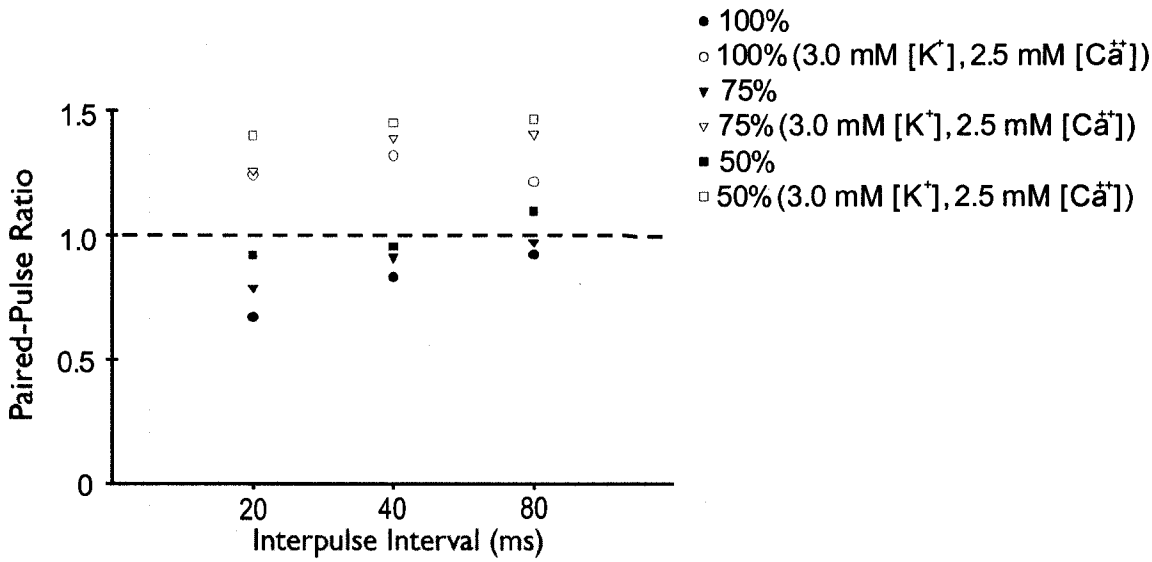


Fig. 5.3 Summary data comparing single paired-pulse suppression in physiological ACSF and conditions of decreased membrane excitability. Plot of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of 50%-100% of the maximum population spike amplitude and interpulse intervals of 20-80 ms in physiological ACSF and conditions of decreased membrane excitability (3.0 mM $[K^+]_o$, 2.5 mM $[Ca^{++}]_o$).

These data indicate that when membrane excitability was decreased by raising $[Ca^{++}]_o$, paired-pulse suppression was eliminated and facilitation produced.

5.2 Paired-pulse suppression under conditions of high membrane excitability

In order to investigate the effects of increased membrane excitability on paired-pulse suppression, $[K^+]_o$ was increased from 3.0 mM to 6.0 mM, and $[Ca^{++}]_o$ was decreased from 1.3 mM to 1.0 mM. Under these conditions, single paired-pulse suppression increased compared to responses under physiologic ACSF (3.0 mM $[K^+]_o$, 1.3 mM $[Ca^{++}]_o$) as indicated by a smaller amplitude population spike in response to the test pulse (Fig. 5.4). Increased suppression

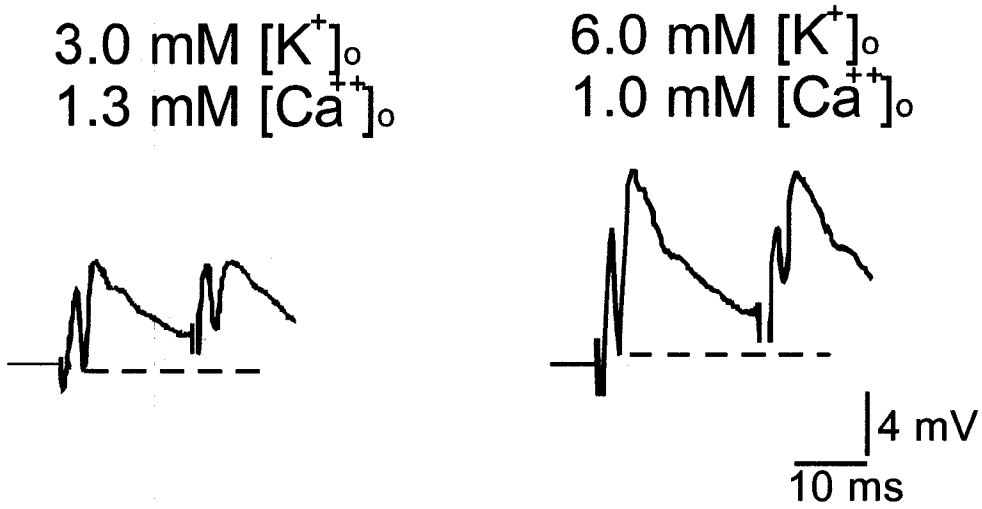


Fig. 5.4 Electrophysiological responses showing increased paired-pulse suppression when membrane excitability was increased. Single paired-pulse responses under conditions of physiological ACSF (3.0 mM $[K^+]_o$, 1.3 mM $[Ca^{++}]_o$) and conditions of increased membrane excitability (6.0 mM $[K^+]_o$, 1.0 mM $[Ca^{++}]_o$), respectively. Traces are an average of 10 individual responses.

under conditions of high membrane excitability was maintained at all stimulus intensities and interpulse intervals with the exception of 25% of maximum amplitude, and 50% of maximum amplitude using an interpulse interval of 40 ms

(Figs. 5.5, 5.6). A statistically significant difference in the paired-pulse ratio between physiological ACSF and conditions of increased membrane excitability

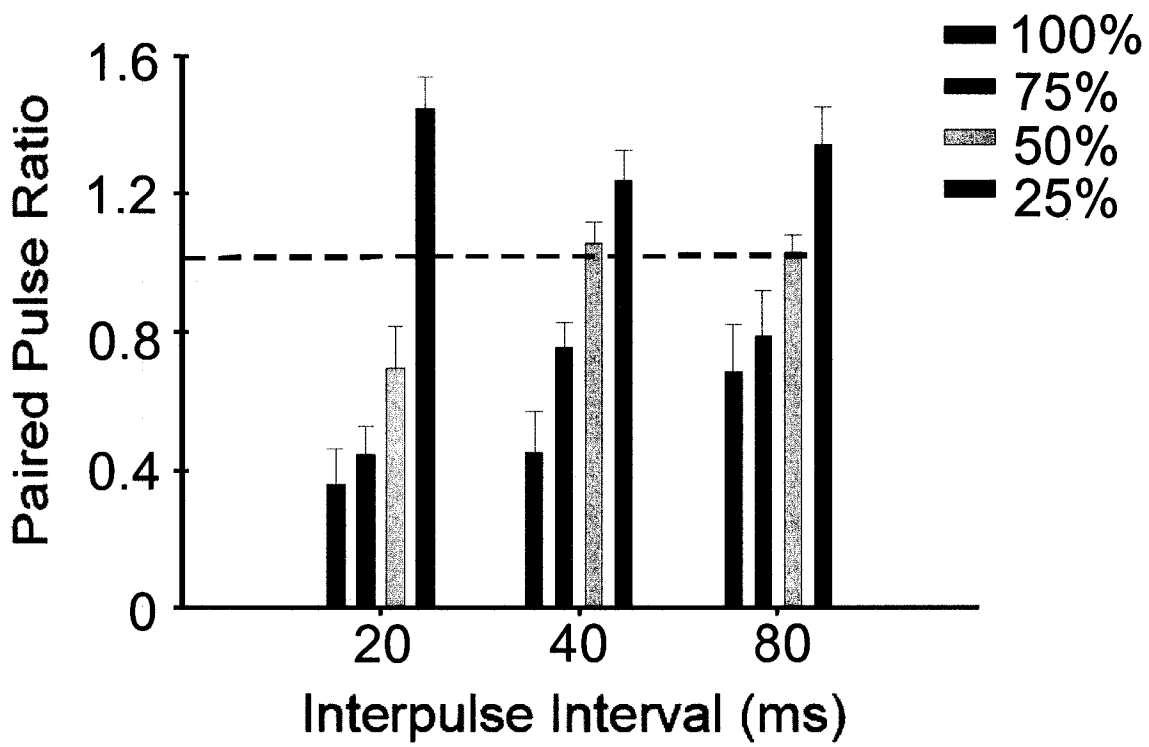


Fig. 5.5 Summary data showing increased single paired-pulse suppression under conditions of increased membrane excitability. Histogram of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of 25%-100% of the maximum population spike amplitude and interpulse intervals of 20-80 ms under conditions of increased membrane excitability (6.0 mM $[K^+]_o$, 1.0 mM $[Ca^{++}]_o$), (n=6).

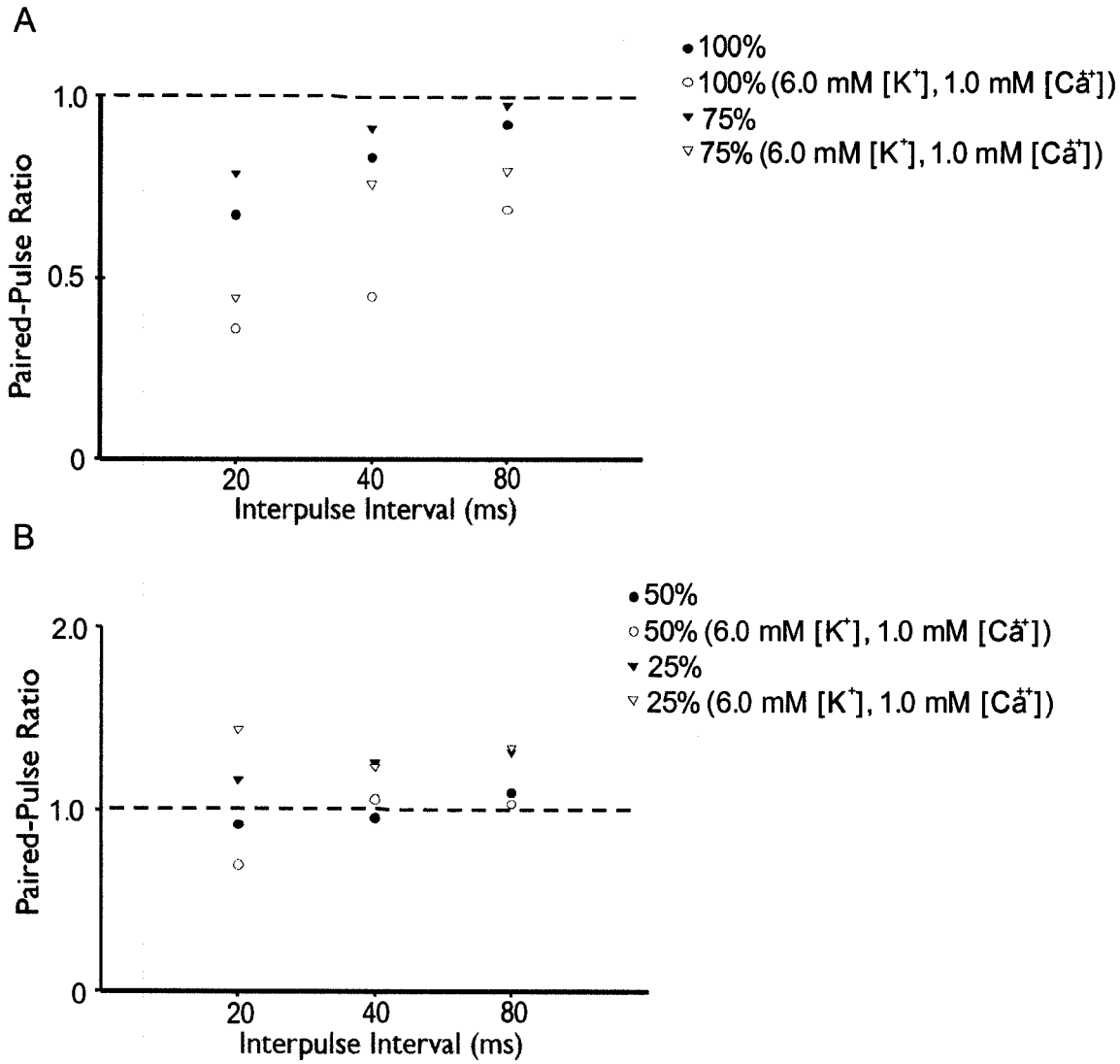


Fig. 5.6 Summary data comparing single paired-pulse suppression in physiological ACSF and conditions of increased membrane excitability. Plot of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of (A) 75%-100% of the maximum population spike amplitude and (B) 25%-50% of the maximum population spike amplitude with interpulse intervals of 20-80 ms in physiological ACSF and under conditions of increased membrane excitability (6.0 mM $[K^+]_o$, 1.0 mM $[Ca^{++}]_o$).

occurred at 100% of maximum intensity with an interpulse interval of 20 ms ($p<0.05$) (Fig. 5.6). Figure 5.7 shows data indicating that an increase in population spike amplitude in response to the conditioning pulse was present when membrane excitability was increased. These data indicate that paired-

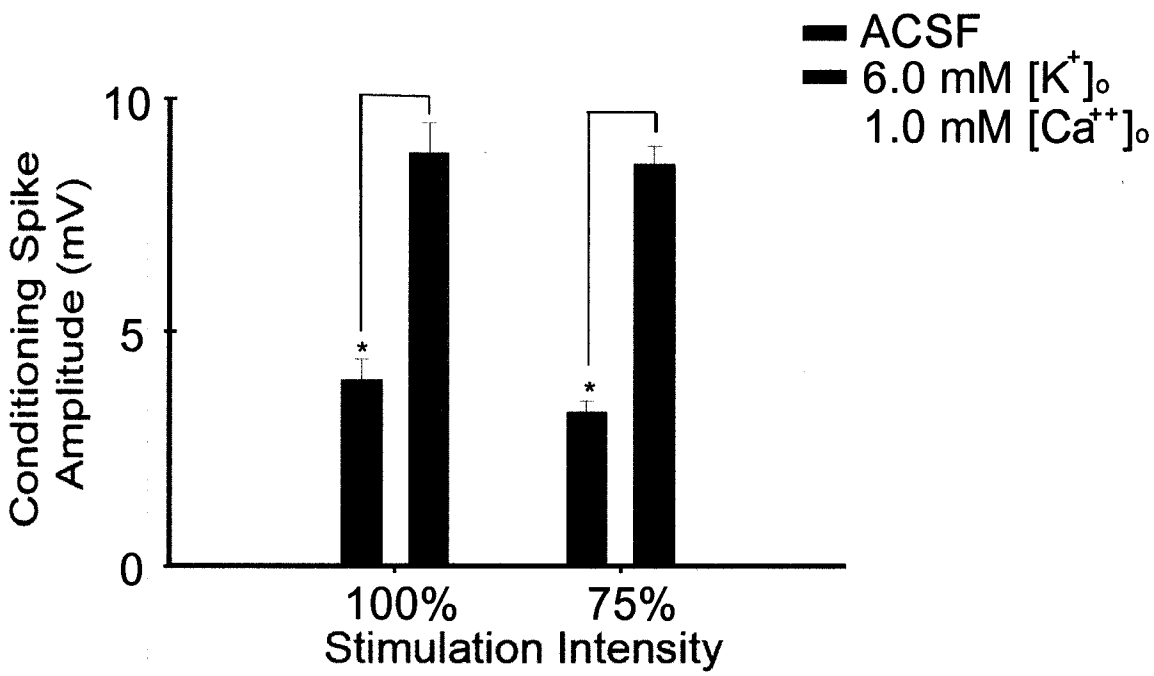


Fig. 5.7 Summary data showing an increase in the population spike amplitude to the conditioning pulse under conditions of increased membrane excitability. Histogram of the averaged single paired-pulse population spike amplitudes in response to the conditioning pulse under conditions of physiological ACSF and increased membrane excitability. *p< 0.01, n=7.

pulse suppression increased when membrane excitability was increased, and an increase in the amplitude of the population spike to the conditioning pulse accompanied increases in membrane excitability.

Paired-pulse suppression was assessed at the first, fifth, and tenth pairs in a train of ten consecutive pairs under conditions of increased membrane excitability to investigate changes during repetitive stimulation. Figure 5.8 shows that at higher stimulus intensities of 75%-100% of maximum amplitude, suppression decreased at the fifth pair in a train and then increased by the tenth pair. At lower stimulus intensities of 25%-50% of maximum amplitude,

suppression typically showed little change or increased by the tenth pair in a train. These data indicate that during repetitive stimulation, paired-pulse suppression increased more when membrane excitability was increased compared to physiological ACSF, although the patterns of suppression during a train were similar.

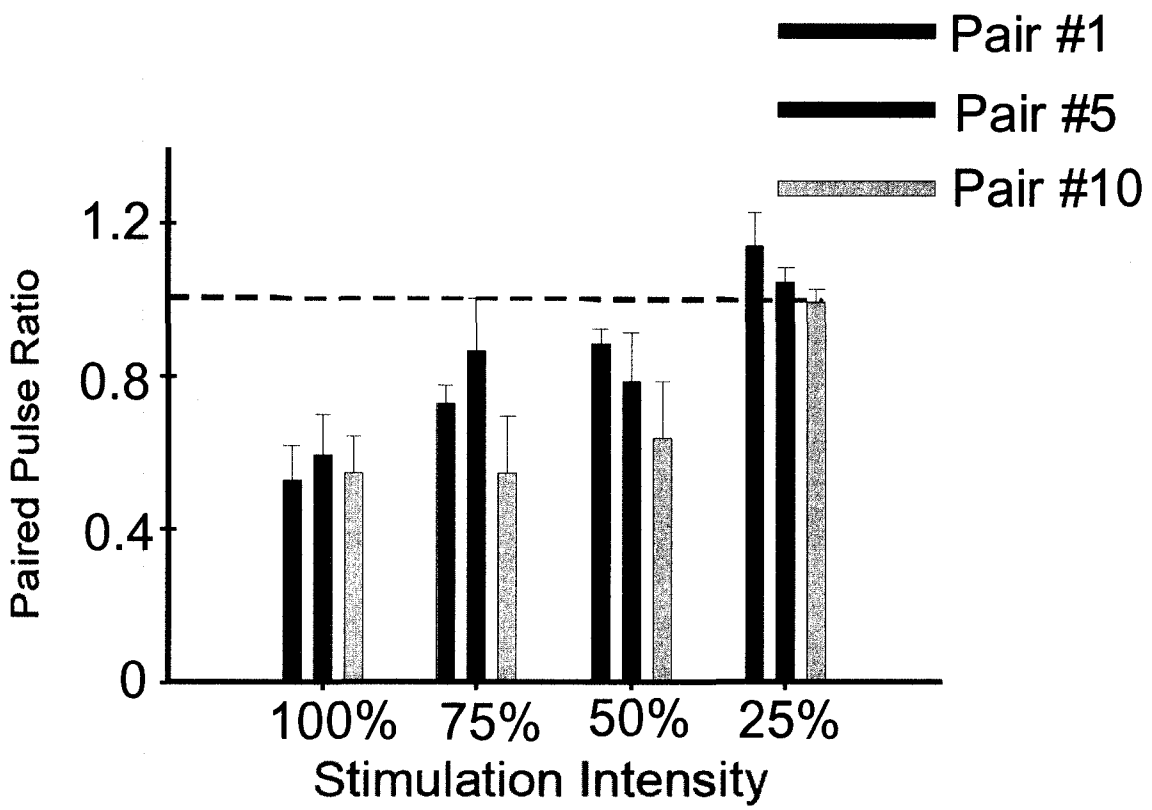


Fig. 5.8 Summary data showing changes in the level of paired-pulse suppression during a repetitive train of 10 consecutive paired pulses under conditions of increased membrane excitability. Histogram of the paired-pulse ratio for averaged responses at the first, fifth, and tenth pairs in a repetitive train from 25%-100% of maximal population-spike amplitude, 2.0 Hz, and 40 ms interpulse interval (n=6).

The level of paired-pulse suppression using single pairs of stimuli showed little difference from that produced by using low-frequency repetitive pairs of stimuli of 0.1-1.0 Hz when membrane excitability was increased (Fig. 5.9).

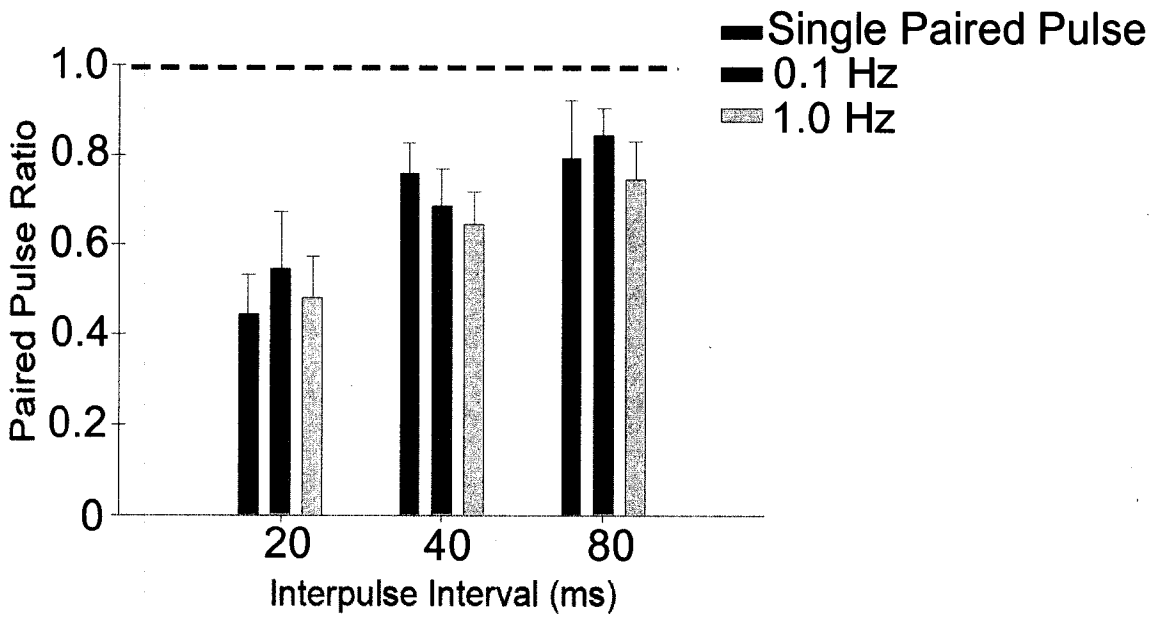


Fig. 5.9 Responses to single and repetitive paired pulses showed similar levels of paired-pulse suppression at low frequencies when membrane excitability was increased. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 0.1 Hz and 1.0 Hz and interpulse intervals of 20-80 ms under conditions of increased membrane excitability (6.0 mM $[K^+]_o$, 1.0 mM $[Ca^{++}]_o$ Intensity was 75% of maximum population-spike amplitude.

As the stimulus frequency was increased to 4.0 Hz, a larger degree of suppression was observed when compared to single pairs of stimuli (Fig. 5.10). Levels of suppression were increased under conditions of increased membrane excitability compared to physiological ACSF at all frequencies tested. At high stimulus intensities, when frequency was increased from 0.1-4.0 Hz, paired-pulse suppression increased (Fig. 5.11). Low stimulus intensity responses were inconsistent when frequency was increased from 0.1-4.0 Hz and little change in suppression was observed. These data indicate that stimulating repetitively with pairs of pulses at low-frequency showed little difference in the level of paired-pulse suppression compared to stimulating with single pairs of pulses alone, and

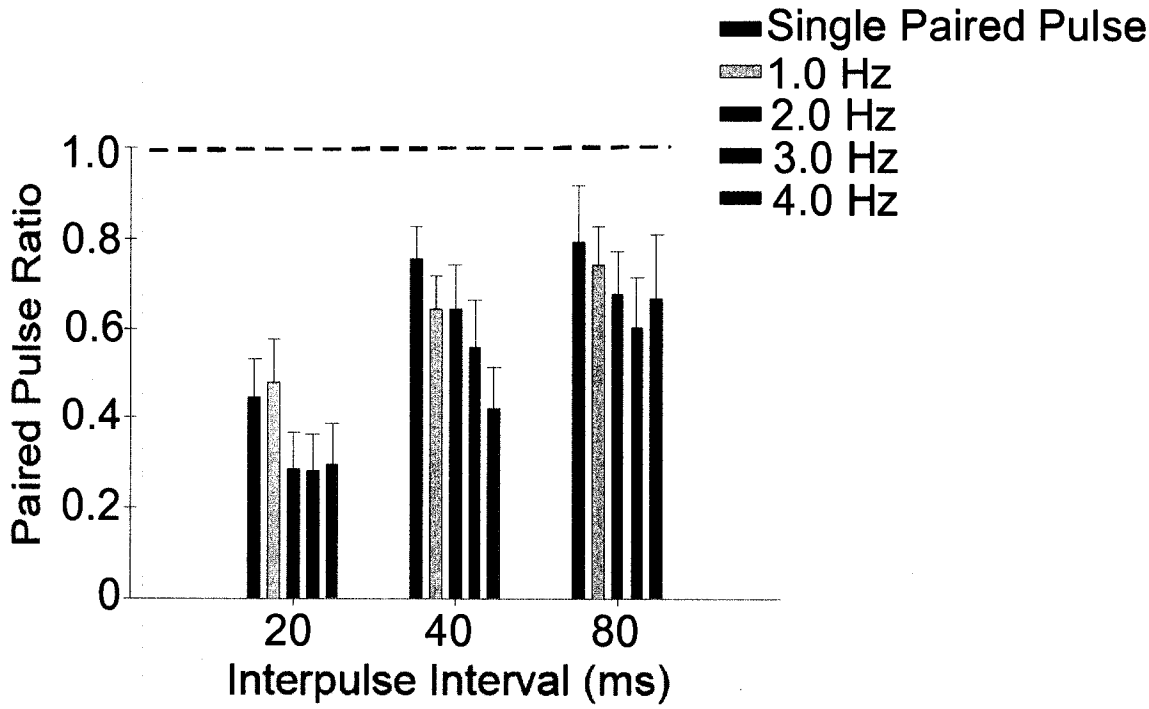


Fig. 5.10 Paired-pulse suppression increased at higher frequencies of stimulation when membrane excitability was increased. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 1.0-4.0 Hz and interpulse intervals of 20-80 ms under conditions of increased membrane excitability. Intensity was 75% of maximum population spike amplitude.

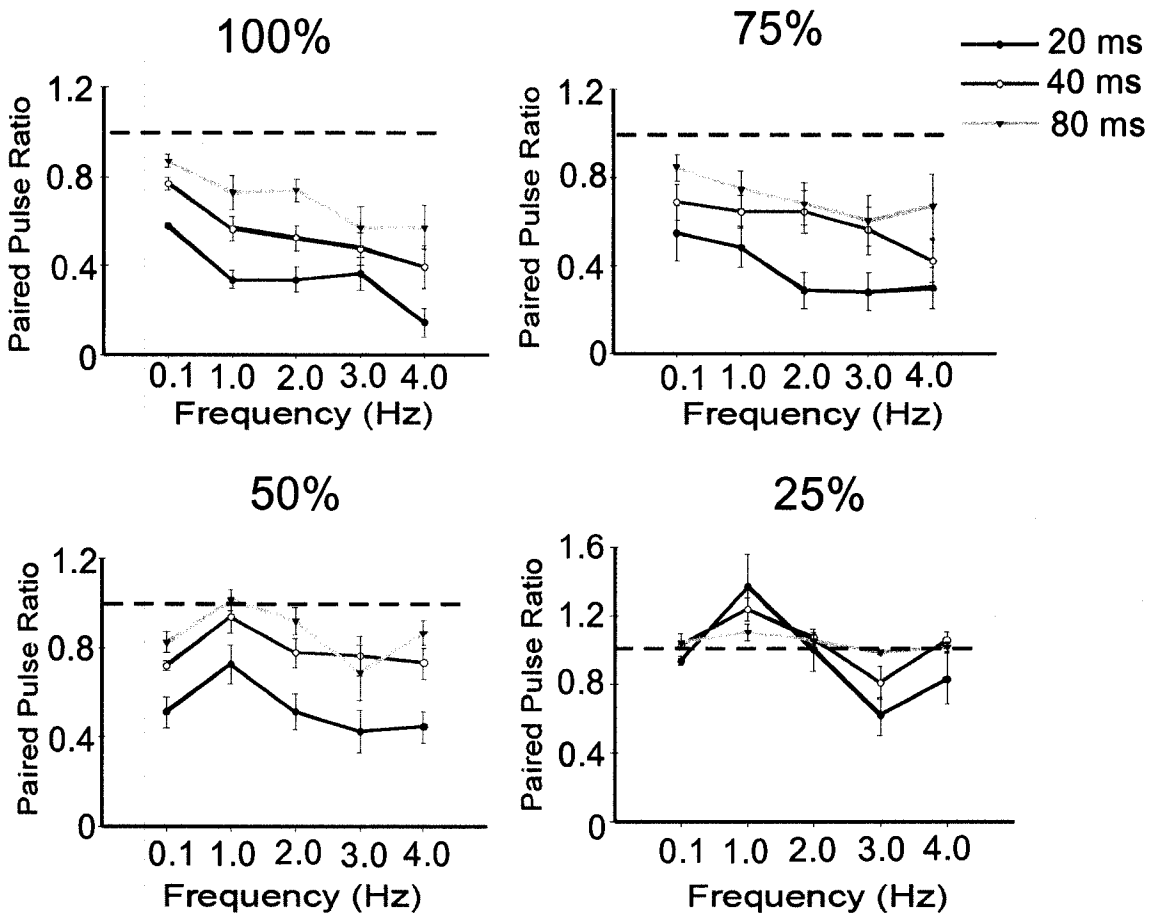


Fig. 5.11 Paired-pulse suppression increased as stimulus frequency increased at high intensities. Plots of the paired-pulse ratio from averaged responses to repetitive paired-pulse stimulation at frequencies of 0.1-4.0 Hz and all stimulus intensities tested.

that considerable increases in suppression only became evident at higher frequencies $\geq$2.0 Hz when stimulating with high intensities.

5.3 Single paired-pulse suppression under conditions of reduced $GABA_A$-receptor mediated inhibition

The hypothesis was tested that slightly reduced $GABA_A$-receptor mediated inhibition results in an increase in paired-pulse suppression. The $GABA_A$-receptor antagonist, SR-95531 (GABAzine), was used due its specificity for the GABA

recognition site of the receptor-channel complex and action as an allosteric inhibitor. A concentration of 0.3 μM was chosen to achieve only a minor reduction in $GABA_A$-receptor function without producing multiple population spiking. Theoretically, a reduction in $GABA_A$-receptor mediated inhibition should produce a decrease in paired-pulse suppression. Figure 5.12 shows that in response to single pairs of pulses at two separate intensities, an increase in paired-pulse suppression was observed when GABAzine was applied as indicted by relatively smaller amplitude responses to the test pulses. The summary data for these

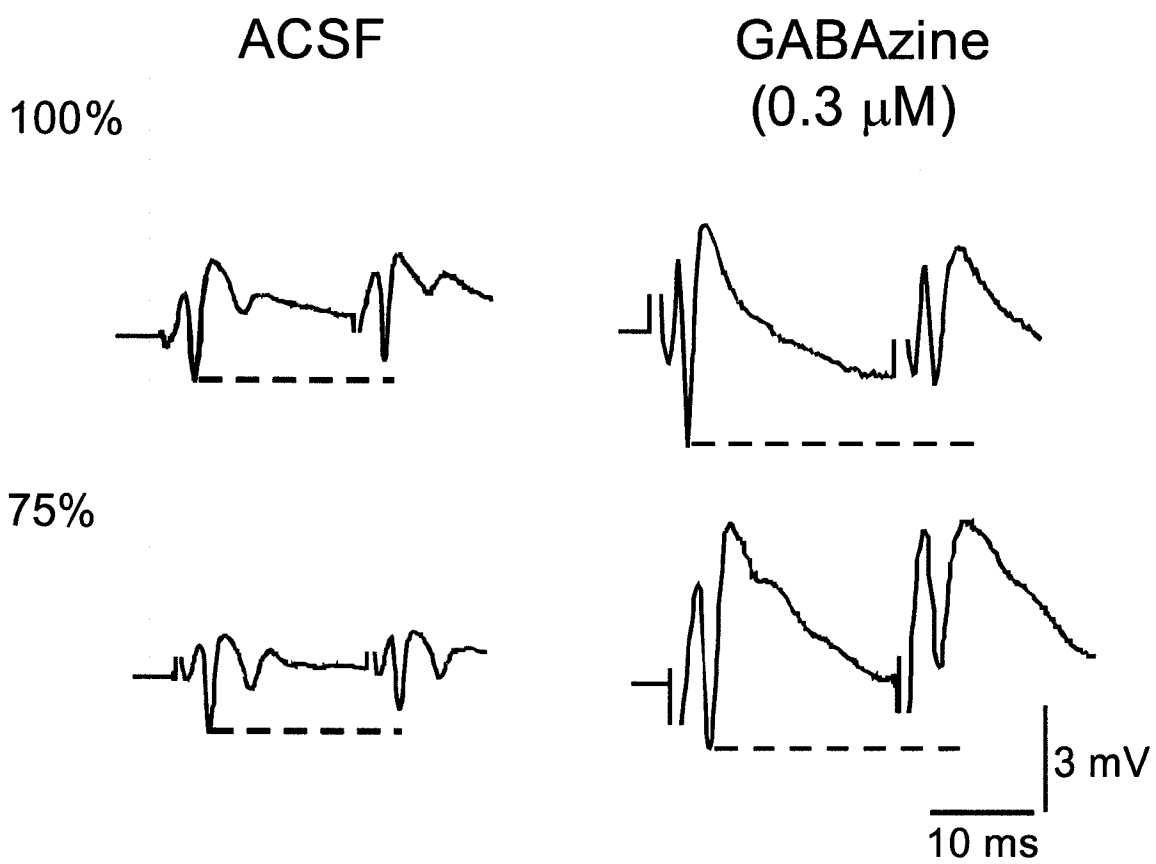


Fig. 5.12 Paradoxical enhancement of single paired-pulse suppression when $GABA_A$-receptor mediated inhibition was reduced. Single paired-pulse responses from the granule cell layer to stimulation of the perforant path at 100% and 75% of maximal population spike amplitude in physiological ACSF and 0.3 μM GABAzine. Traces are an average of 10 individual responses.

experiments demonstrate that suppression decreased as intensity decreased and interpulse interval increased when $GABA_A$-receptor mediated inhibition was reduced. An overall increase in paired-pulse suppression occurred under conditions of 0.3 μM GABAzine when compared to identical experiments in physiological ACSF (Figs. 5.13, 5.14). This difference was statistically significant

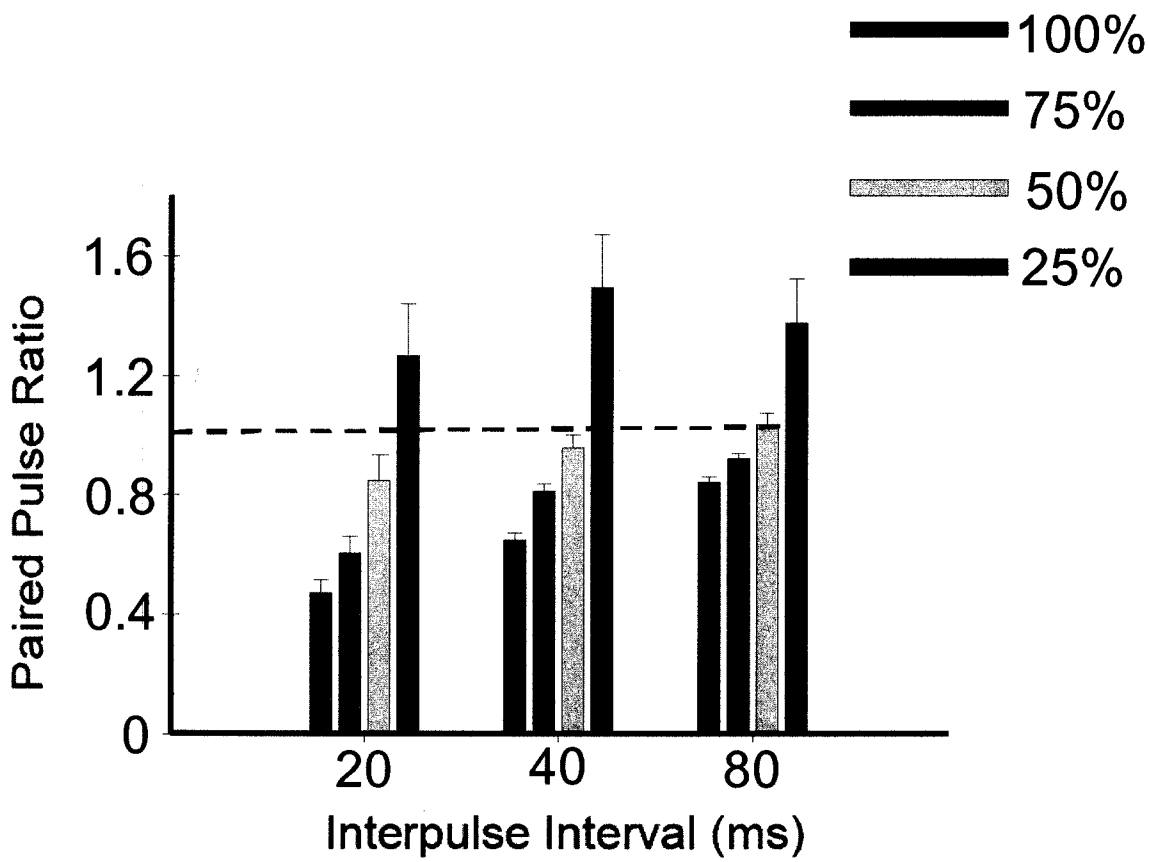
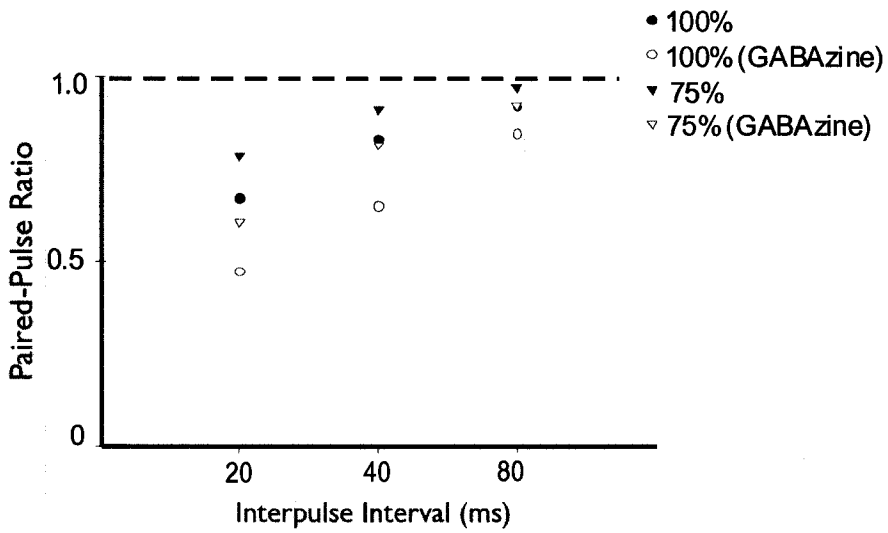


Fig. 5.13 Summary data showing an increase in paired-pulse suppression when $GABA_A$-receptor mediated-inhibition was reduced. Histogram of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of 25%-100% of the maximum population spike amplitude and interpulse intervals of 20-80 ms in conditions of decreased membrane excitability $GABA_A$ mediated-inhibition (0.3 μM GABAzine) (n=8).

A



B

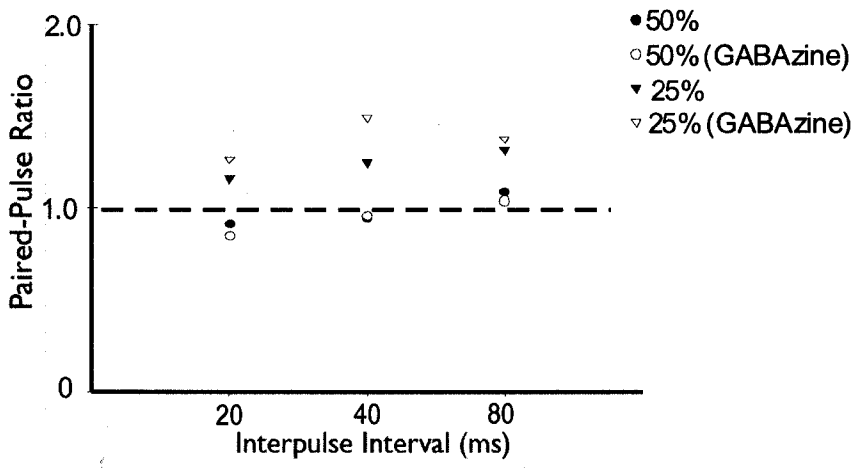


Fig. 5.14 Summary data comparing single paired-pulse suppression in physiological ACSF and when $GABA_A$-receptor mediated inhibition was decreased. Plot of the paired-pulse ratio of averaged single paired-pulse responses to stimulus intensities of (A) 75%-100% and (B) 25%-50% of the maximum population spike amplitude and interpulse intervals of 20-80 ms in physiological ACSF and conditions of decreased $GABA_A$-receptor mediated inhibition.

at 100% of maximum amplitude using interpulse intervals of 20 and 40 ms ($p<0.05$). Increases in suppression were more pronounced at high stimulus intensities of 75%-100%, however, with a breakdown in suppression at 25% of maximum population spike amplitude. Figure 5.15 shows that an increase in the amplitude of the population spike to the conditioning pulse occurred under conditions of 0.3 μM GABAzine. These data demonstrate that under conditions of

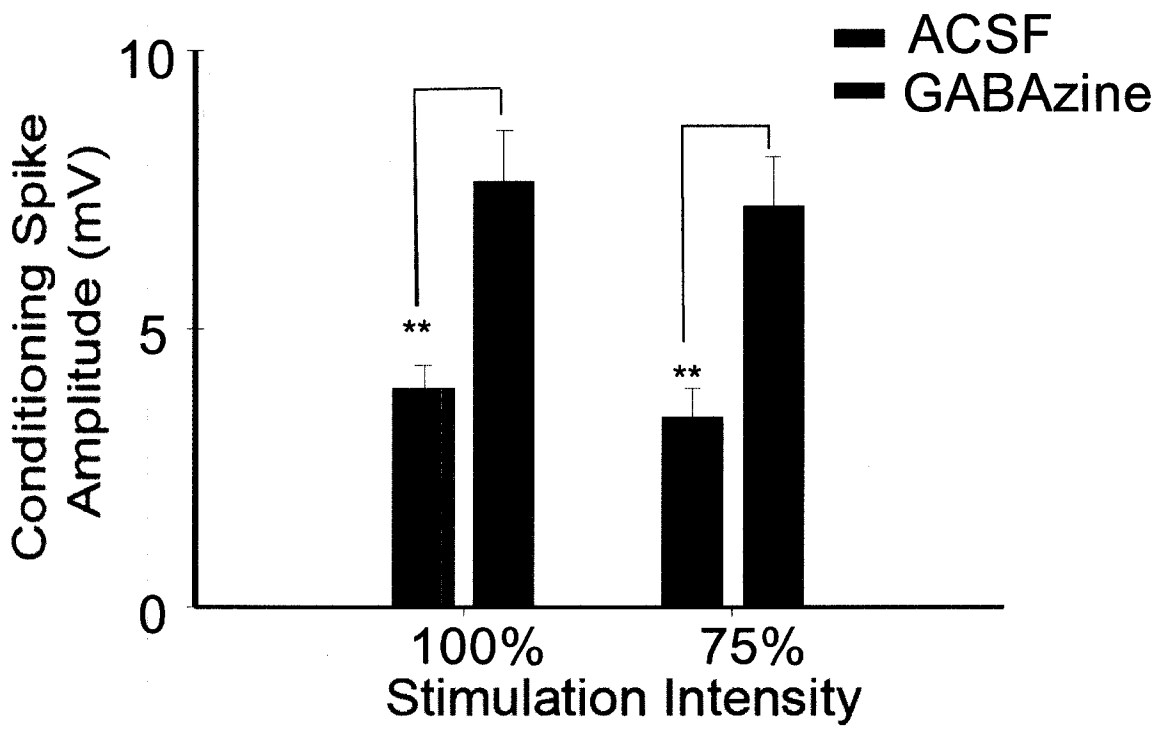


Fig. 5.15 Summary data showing an increase in the population spike amplitude to the conditioning pulse under conditions of reduced $GABA_A$-receptor mediated inhibition. Histogram of the averaged single paired-pulse population spike amplitudes in response to the conditioning pulse under conditions of physiological ACSF and 0.3 μM GABAzine. **$p< 0.01$ (n=5).

reduced $GABA_A$-receptor mediated inhibition, when paired-pulse suppression should theoretically decrease, a paradoxical increase in paired-pulse suppression was observed. Additionally, an increase in the amplitude of the population spike to the conditioning pulse was observed when $GABA_A$-receptor mediated inhibition was reduced.

5.4 Repetitive paired-pulse suppression under conditions of reduced $GABA_A$-receptor mediated inhibition

Levels of suppression during repetitive paired-pulse stimulation were assessed at the first, fifth, and tenth pairs in a train under conditions of reduced $GABA_A$-receptor mediated inhibition. Figure 5.16 shows that levels of suppression remained relatively consistent during a train. Overall levels of suppression increased compared to identical experiments in physiological ACSF, however. The level of paired-pulse suppression using single pairs of stimuli showed little difference from that produced by using low-frequency repetitive pairs of stimuli of 0.1-1.0 Hz (Fig. 5.17). Increases in suppression were more pronounced, however, than in physiological ACSF. As the stimulus frequency was increased to 4.0 Hz, a progressively larger degree of suppression was seen when compared to single pairs of stimuli (Fig. 5.18). At all stimulus intensities when frequency was increased from 0.1-4.0 Hz, paired-pulse suppression typically increased (Fig. 5.19). The greatest degrees of suppression were

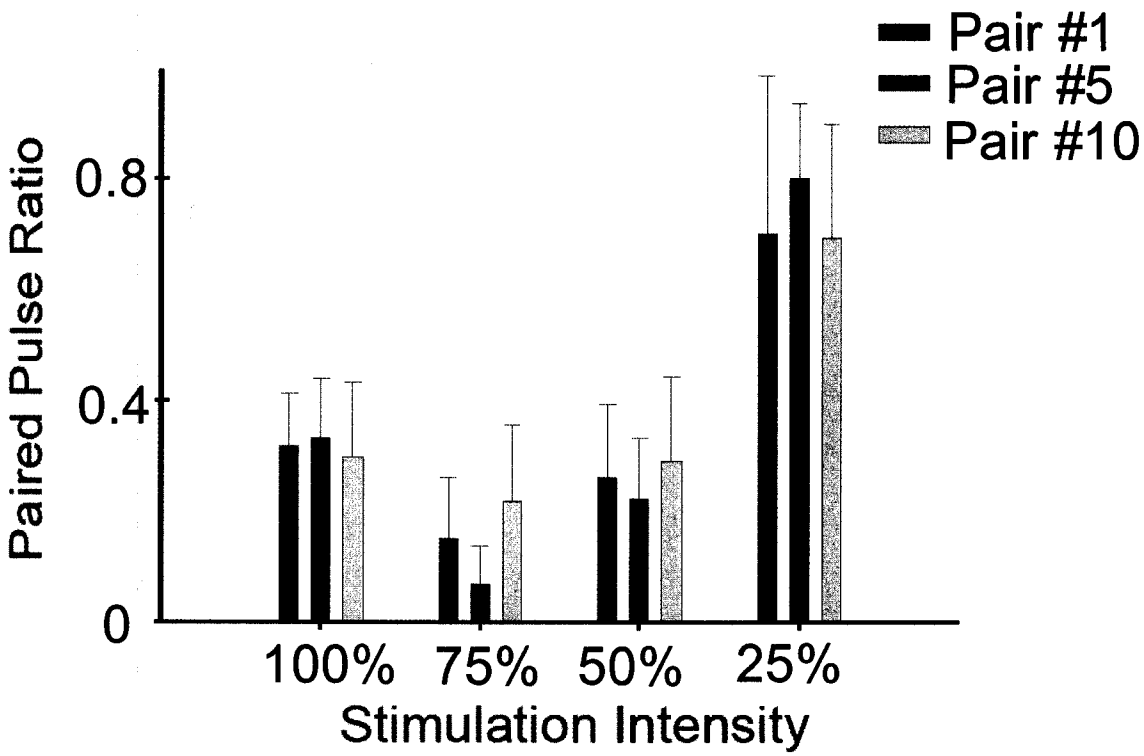


Fig. 5.16 Summary data showing changes in the level of paired-pulse suppression during a repetitive train of 10 consecutive paired pulses when $GABA_A$-receptor mediated inhibition was reduced. Histogram of the paired-pulse ratio for averaged responses at the first, fifth, and tenth pairs in a repetitive train from 25%-100% of maximal population-spike amplitude, 4.0 Hz, and 20 ms interpulse interval (n=8).

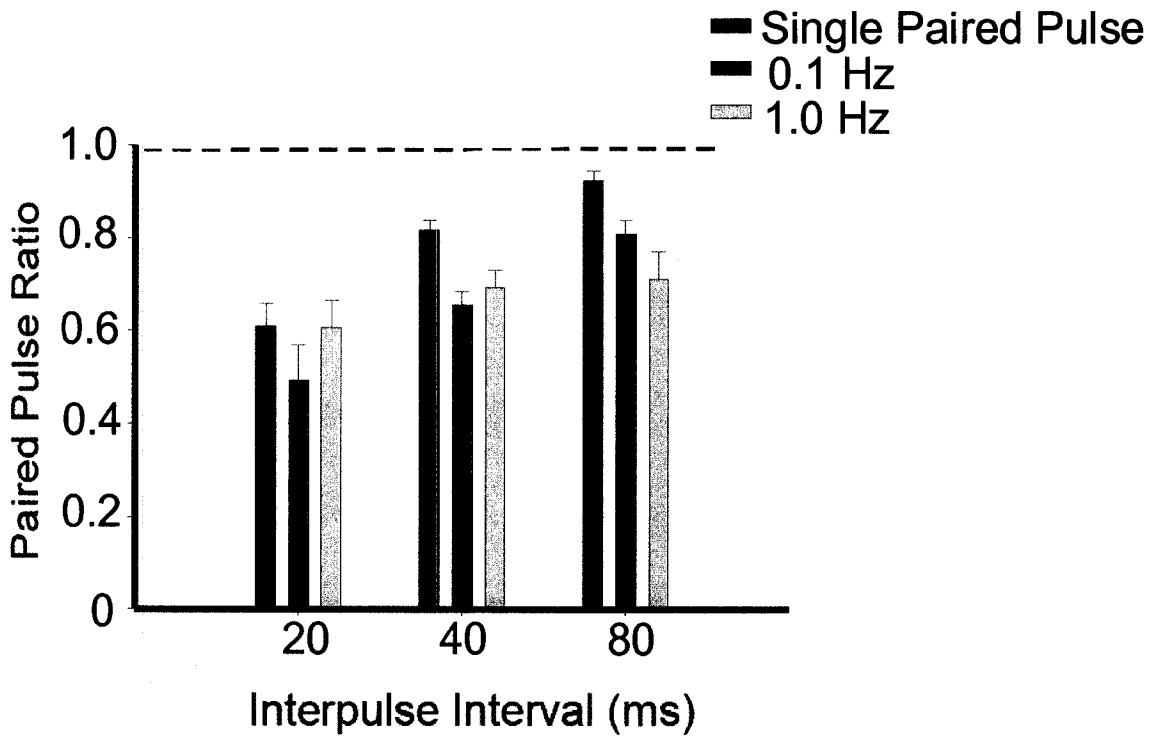


Fig. 5.17 Responses to single and repetitive paired pulses showed similar levels of paired-pulse suppression at low frequencies. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 0.1 Hz and 1.0 Hz and interpulse intervals of 20-80 ms (n=8). Intensity was 75% of maximum population-spike amplitude.

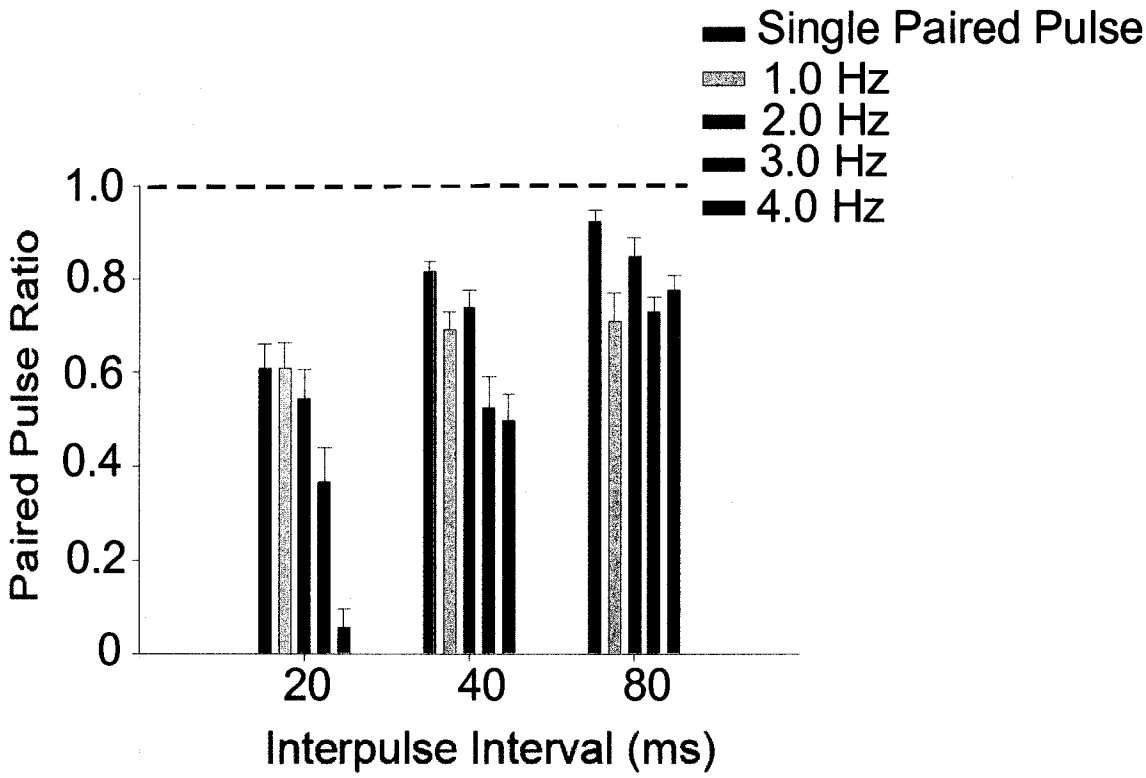


Fig. 5.18 Paired-pulse suppression increased at higher frequencies of stimulation. Histogram of the paired-pulse ratio from averaged single paired-pulse versus repetitive paired-pulse stimulation at 1.0-4.0 Hz and interpulse intervals of 20-80 ms. Intensity was 75% of maximum population-spike amplitude (n=8).

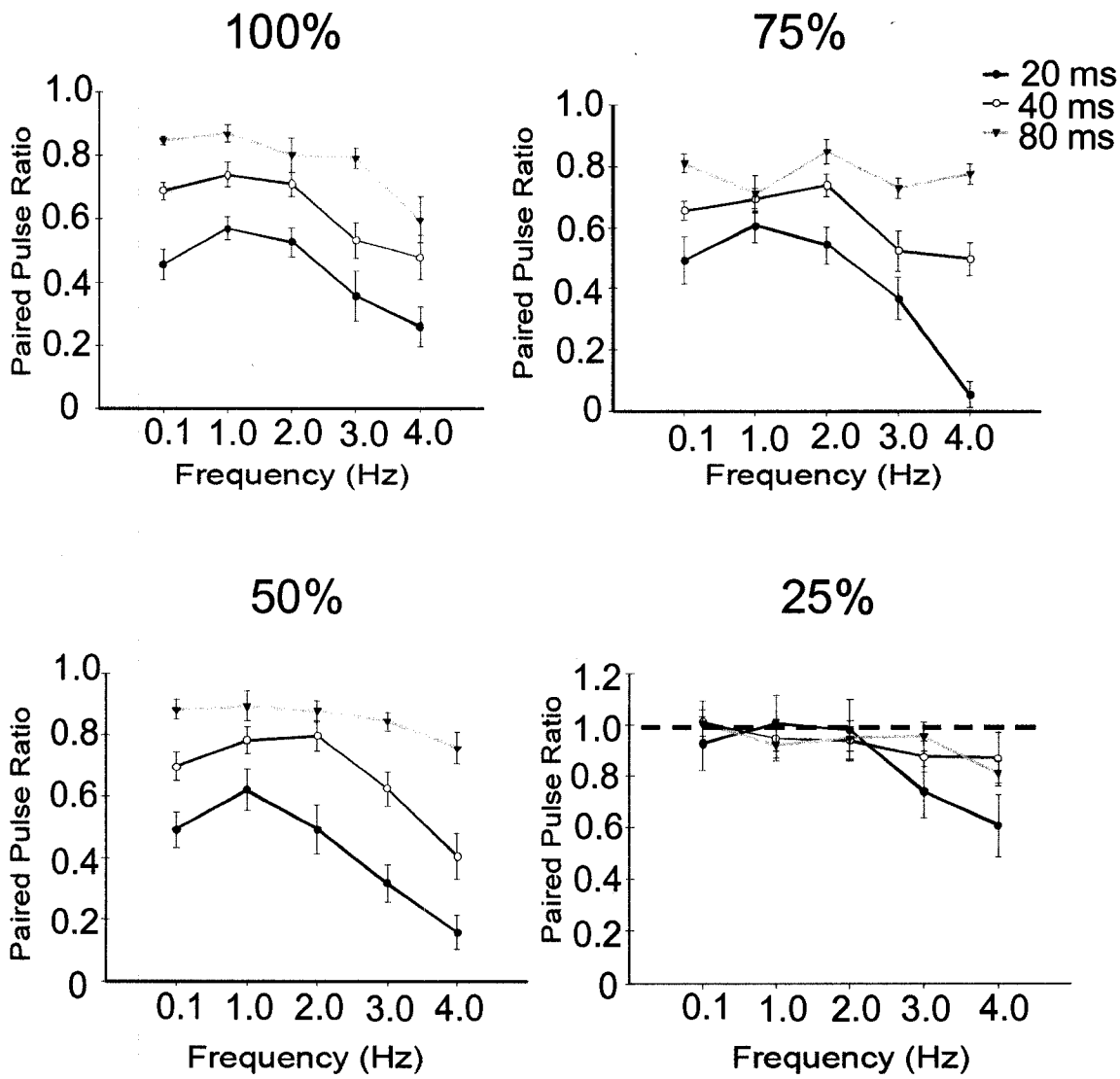


Fig. 5.19 Paired-pulse suppression typically increased as stimulus frequency increased at all stimulation intensities. Plots of the paired-pulse ratio from averaged responses to repetitive paired-pulse stimulation at frequencies of 0.1-4.0 Hz and all stimulus intensities tested (n=8).

observed at 4.0 Hz using a 20 ms interpulse interval at each stimulus intensity tested. These data demonstrate that increased suppression resulted from increased stimulus frequency under conditions of decreased $GABA_A$-receptor mediated inhibition, and that levels also increased compared to parallel experiments in physiological ACSF.

CHAPTER 6

DISCUSSION

The objective of this study was to investigate the paired-pulse technique as a measure of synaptic inhibition in the dentate gyrus. Single and repetitive paired-pulse stimulus parameters, membrane excitability, and reduced $GABA_A$-receptor mediated inhibition were assessed to determine their affects on paired-pulse suppression as a maker of inhibitory function. The results indicate that responses from single and repetitive paired-pulse stimuli are highly dependent on stimulus intensity and interpulse interval, whereby high intensity and short interpulse intervals produced the greatest levels of paired-pulse suppression. During repetitive trains of ten consecutive pairs of pulses, the level of paired-pulse suppression increased during the train at all but the lowest intensity in vivo. The amplitude of the population spike produced by the conditioning pulse increased during repetitive paired-pulse trains at higher frequencies. As the frequency of stimulation increased from 0.1 to 4.0 Hz, the level of suppression increased. When $[Ca^{++}]_0$ was altered to decrease membrane excitability, paired-pulse suppression was abolished. When $[K^+]_o$ and $[Ca^{++}]_0$ were altered to increase membrane excitability, paired-pulse suppression increased. When $GABA_A$-receptor mediated inhibition was reduced pharmacologically, paired-pulse suppression paradoxically increased rather then decreased. These findings

indicate that levels of suppression, as measured by the paired-pulse technique, are highly influenced by the choice of stimulus parameters, membrane excitability, and have the potential for generating erroneous results when used as a marker of inhibitory function.

6.1 Importance of stimulus intensity to measurements of paired-pulse suppression

The effects of stimulus intensity on levels of paired-pulse suppression were similar in experiments conducted in vivo and in hippocampal slices. However, the effect was less pronounced in a slice, presumably due to the lack of intact inhibitory circuits that had been severed by the slice preparation. As stimulus intensity increased, the level of suppression progressively increased. The effect of intensity on paired-pulse suppression has been reported to partly determine the duration of the inhibitory response (Matthews et al. 1981; Tuff et al. 1983). Andersen (1966) reported that a threshold stimulation level resulting in a population spike was necessary to activate inhibitory interneurons, and that the inhibitory force must be powerful enough to overcome the force of potentiation that is also present with orthodromic stimulation. As stimulus intensity increases, a larger number of perforant path fibers and granule cells are recruited for activation, leading to a larger amplitude population spike to the conditioning pulse. This in turn activates a greater number of GABAergic basket cells via feedback mechanisms and increases the level of tonic inhibitory control. Many interneurons involved in feed-forward inhibitory circuits have been reported to respond earlier than principal cells, and they too are increasingly activated as

stimulus intensity increases Buzsaki and Eidelberg 1982; Freund and Buzsaki 1996). Some investigators have reported that relatively low intensity orthodromic stimulation can have an effect on paired pulse suppression (Lynch et al. 1981), while others found suppression to be highly variable at low stimulus intensities but constant above a threshold value (Kapur et al. 1989). The results reported here indicate that stimulus intensities as low as 25% of maximum can have an effect on paired-pulse suppression in vivo, but only produce paired-pulse facilitation in a hippocampal slice. This contradictory result may be due to the severing of inhibitory circuits by the slice preparation, particularly in light of the fact that interneurons innervate thousands of pyramidal cells (Li et al. 1992; Buhl et al. 1994; Sik et al. 1995). All types of "artificial" stimulation are inherently problematic because recruitment of inhibition and excitation are nonlinearly coupled, and physiological patterns are not mimicked accurately. However, it is clear from this study and the results from other laboratories that as stimulus intensity increases, so too does the level of paired-pulse suppression.

For studies using the paired-pulse technique to compare levels of "inhibition" in groups of animals or preparations, it is critical that stimulus intensity remains consistent so that comparable responses to the conditioning stimulus can be evoked so that changes in levels of suppression of the response to the test stimulus can be determined. This is especially the case in epileptic tissue where pathological changes such as mossy fiber sprouting, loss of inhibitory interneurons, and receptor subunit changes may influence how the tissue responds to differences in stimulus intensity. Any increase or decrease in the

amplitude of the population spike to the conditioning pulse, which is determined by stimulus intensity, will alter the measured levels of paired-pulse suppression. It still remains unclear, however, how to consistently control for the effect of stimulus intensity across preparations and animals. Attempts have been made to remove intensity as a variable by choosing an intensity sufficient to reach a constant paired-pulse index (Kapur et al. 1989), matching conditioning pulse population spike amplitudes (Stringer & Lothman 1989), and stimulating at a percent of maximum population spike (Uruno et al. 1994; Haas et al. 1996; Swanson et al. 1998; Hellier et al. 1999) or population postsynaptic potential (pPSP) amplitude (Wilson et al. 1998). Buckmaster and Dudek (1997) attempted to control for stimulus intensity by determining a minimum stimulus for population spike threshold when using paired pulses following kainate-induced epilepsy, however, an increase in the amplitude of the population spike to the conditioning pulse was evident in epileptic animals and a minimal amount of paired-pulse suppression was reported in control experiments. These methods still do not rule out the potential for variability, however, and make results difficult to compare due to the existence of various protocols. The result reported here that a non-linear relationship exists between stimulus intensity and spike amplitude at higher intensity stimulation further suggests that variability could exist between preparations. The choice of a single stimulus intensity when performing the paired-pulse technique (Sloviter & Damiano 1981a, 1981b; Sloviter 1983) without first determining where on an intensity-response curve it lies, or failing to report a stimulus intensity altogether (Maru & Goddard 1987; Bragin et al. 2005; Harvey &

Sloviter 2005), makes results even more difficult to reproduce and interpret. Thus, the importance of a consistent stimulus intensity that has been defined on an input-output curve when utilizing the paired-pulse technique is critical to obtaining reliable and reproducible results. Yet, even under these conditions, it is difficult if not impossible to know that the normalization-across-preparations protocol is actually valid.

Paired-pulse suppression is often equated directly to GABAergic inhibition even though other mechanisms may be affecting levels of suppression. For example, increases in calcium-dependent potassium conductance, changes in receptor subunits (Buhl et al. 1996; Macdonald and Kapur 1999), ephaptic interactions (Turner et al. 1984), and progressive depolarization inactivation. During repetitive stimulation, IPSP's decrease (Thompson and Gahwiler 1989; McCarren and Alger 1985), conditioning pulse population spike amplitudes increase, and paired-pulse suppression increases even though GABAergic inhibition has not increased. When using the paired-pulse technique to measure levels of excitability in the perforant path to granule cell synapse of the dentate gyrus there are a variety of sites in the pathway that all potentially influence levels of suppression. The initial conditioning pulse stimulus produces an action potential dependent response in the perforant path fibers, whose strength is graded by fiber number activation. This response produces a postsynaptic EPSP and action potential dependent population spike whose amplitude is graded by the number of synchronously firing granule cells. The presynaptic action potential of the granule cell response is an all or none response not graded by cell

number, which subsequently excites inhibitory interneurons, namely basket cells, through axon collaterals involved in feedback inhibition. The interneurons produce a postsynaptic EPSP and action potential in response to this excitation, as well as a presynaptic action potential response that acts back on the granules cells. The action of the interneuron is then to produce a graded IPSP response on the granule cells. The subsequent test pulse stimulus then occurs after a given interpulse interval that may or may not coincide with the $GABA_A$ or $GABA_B$-receptor mediated response, depending on the length of the interval. This second stimulus produces all of the same types of responses as described for the conditioning stimulus. This potential for large variability within the circuit, and the presence of non-GABA related mechanisms across preparations or in epileptic tissue could compromise paired-pulse results leading to misinterpretations about the state of "inhibition."

6.2 Influence of interpulse interval on paired-pulse suppression

The second factor affecting the level of paired-pulse suppression similarly in vivo and in a hippocampal slice was interpulse interval. Three distinct responses in the level of hippocampal paired-pulse suppression are known to occur depending on the interpulse interval: a strong early suppression phase (20-50 ms), a progressively lower level of suppression and facilitation phase (50-150 ms), and a late suppression phase (>150 ms) (Racine 1983; Tuff et al. 1983; Joy and Albertson 1987). Stimulation of afferent fibers elicits biphasic IPSP's in principal cells. The early suppression response corresponds to the time course

for $GABA_A$-receptor mediated recurrent inhibition (Lomo 1971; Thalmann and Ayala 1982), and the late suppression response corresponds to $GABA_B$-receptor mediated inhibition whereby K^+ flux occurs through G-protein linked channels. When using single pairs of pulses, the present study confirms this pattern and the results reported by Kapur et al. (1989) and others by observing that paired-pulse suppression was strongest at short interpulse intervals and progressively decreased as the interval increased.

6.3 Frequency as a factor in determining paired-pulse suppression

Results from this study indicated that paired-pulse suppression increased as stimulus frequency increased. Low-frequency stimulation between 0.1-1.0 Hz showed little difference in the level of suppression than single pairs of pulses, presumably because the changes that occur with higher frequency stimulation were not produced at such a low rate. One hypothesis for the observation that suppression increases with increased frequency is based on the activation of frequency-dependent feedback and/or feed-forward inhibitory circuits (Sloviter 1991a), however there is a lack of quantitative evidence to support this idea. According to this study, low-frequency stimulation < 2.0 Hz only activates feedback inhibition while stimulation ≥ 2.0 Hz activates both feedback and feed-forward circuits. Frequency dependent paired-pulse suppression could also conceivably be due to a number of plastic alterations at many sites within the activated circuit, such as increased output of granule cells leading to increased activation of basket cells. Frequency potentiation could increase the number of

granule cells involved in a synchronized discharge leading to an increase in feedback inhibition (Andersen & Lomo 1967). It has also been reported that potentiation in inhibitory interneuron responses during repetitive stimulation can occur in vitro (Lee et al. 1980). Based on the previous pattern of repetitive stimulation, interneurons may undergo modifications such as presynaptic changes of excitatory terminals, modification of the postsynaptic sites, presynaptic modification of GABA release, and changes in postsynaptic sensitivity to GABA (Marty & Llano 1995). The spontaneous firing of interneurons has also been reported to increase as a result of tetanization (Stelzer et al. 1994; Poncer et al. 1995). Additionally, states of prolonged depolarization could lead to activity-dependent decreases in GABA release (Mott et al. 1993), disinhibition, and further abnormal activation of hippocampal circuits. Levels of suppression could be influenced by an increase in calcium-dependent potassium conductance, thus producing after-hyperpolarizations, altered thresholds resulting from slow conductance changes, abnormal levels of membrane excitability due to increased $[K^+]_o$ and decreased $[Ca^{++}]_o$, and shunting inhibition (Staley and Mody 1992). A decrease in the level of existing facilitation could also lead to misinterpretations about increased levels of suppression. Even under conditions when all stimulus parameters are properly controlled, the use of a repetitive paired-pulse protocol cannot discriminate between these possibilities, and only confounds the potential for variability already present when using a single paired-pulse protocol.

6.4 Pitfalls of repetitive paired pulses in the assessment of synaptic inhibition

Results from this study indicate that the amplitude of the population spike to the conditioning pulse increased during repetitive paired-pulse trains at higher frequencies. The effect of this observation can be equated to results observed when stimulus intensity is increased and can account for the increased suppression reported during repetitive paired-pulse stimulation. Increased amplitude of the population spike to the conditioning pulse could conceivably be due to a breakdown in GABAergic inhibition. However, the paired-pulse technique indicates an increase in suppression under these conditions. The fact that levels of suppression vary depending on when in the train measurements are taken stresses the importance of consistency and adherence to stimulus parameters. However, many studies using repetitive paired-pulse trains to measure levels of suppression fail to take this into account (Sloviter 1991a; Haas et al. 1996; Swanson et al. 1998; Wilson et al. 1998; Martin and Sloviter 2001; Sloviter et al. 2006). This begs the question, can repetitive paired-pulse be relied upon as an accurate measure of inhibition? Hence, the use of single stimulation or single paired-pulse may provide more precise measurements of suppression than repetitive paired-pulse stimulation.

A clear rationale for the advantages of using repetitive paired pulses to assess cellular inhibition remains vague (Sloviter 1991a; Swanson et al. 1998; Wilson et al. 1998; Harvey and Sloviter 2005). Results from the present study indicate that paired-pulse suppression increased during a repetitive train in vivo. However, IPSP's have been shown to decrease when stimulated with repetitive

trains in vivo (Ben-Ari et al. 1979) and in vitro (Miles and Wong 1987). Extracellular stimulation in vivo has the potential for variability by exciting networks outside those theoretically being isolated. Repetitive stimulation may heavily activate these and other circuits resulting in increased paired-pulse suppression. Other alterations during repetitive stimulation may also occur that do not involve GABAergic mechanisms. For example, increases in $[K^+]_o$ (Thalmann & Ayala 1982), decreases in $[Ca^{++}]_o$ (Krnjevic et al. 1980), changes in receptor sensitivity, resting membrane potential, voltage-dependent channels, and neurotransmitter depletion. Additionally, the effect of progressive depolarization inactivation can lead to a failure of somatodendritic action potentials. Na^+ channel inactivation leads to a decrease in the amplitude and number of action potentials. As the channels return to an active state, the action potentials will increase again, leading to the potential for results to be misinterpreted as an increase in suppression. Thus, the repetitive paired-pulse technique has even more potential pitfalls than single paired-pulse stimulation: (1) increased paired-pulse suppression occurs during stimulation frequencies >1.0 Hz, even though $GABA_A$-receptor mediated inhibition is known to decrease under these conditions, (2) changes in the amplitude of the population spike to the conditioning pulse produce variable measurements of suppression, and (3) activity-dependent changes in physiological mechanisms that are independent of GABA-mediated inhibition are likely to influence the level of paired-pulse suppression at frequencies >1.0 Hz.

6.5 Membrane excitability determines levels of paired-pulse suppression

In the hippocampus of normal and epileptic tissue, increases in $[K^+]_o$ and decreases in $[Ca^{++}]_o$ occur that influence neuronal membrane excitability by increasing or decreasing the threshold level for action potential generation (Frankenhaeuser & Hodgkin 1957; Hodgkin 1957; Moody et al. 1974; Fisher et al. 1976; Fritz & Gardner-Medwin 1976; Heinemann et al. 1977; Benninger et al. 1980; Krnjevic et al. 1980; Somjen & Giacchino 1985) and produce prolonged field bursts in the hippocampus and dentate gyrus (Snow and Dudek 1984; Korn et al. 1987; Roper et al. 1992; Schweitzer et al. 1992; Patrylo et al. 1994). $[K^+]_o$ has been reported to increase from physiological levels of 3 mM toward a ceiling of 12 mM, and unbound $[Ca^{++}]_o$ to decrease by 0.5 to 1.0 mM from physiological levels of 1.3 mM (Krnjevic et al. 1980; Benninger et al. 1980; Krnjevic et al. 1982; Somjen & Giacchino 1985). Interictal spiking produces increases in $[K^+]_o$ and decreases in $[Ca^{++}]_o$ (Moody et al. 1974; Fisher et al. 1976; Heinemann et al. 1977), thereby increasing the amplitude of the population spike to the conditioning pulse and leading to increased paired-pulse suppression. Repetitive afferent stimulation also increases $[K^+]_o$ and decreases $[Ca^{++}]_o$ (Krnjevic et al. 1982) creating an increase in paired-pulse suppression even though no change in GABAergic inhibition has taken place. Additionally, increased $[K^+]_o$ is known to result in a decrease in the amplitude of IPSP's, thus reducing levels of inhibition (Korn et al. 1987). Under all of these conditions in which excitability is increased, paired-pulse suppression measurements increase indicating an increase in "inhibition." This observation may partly account for the increased paired-pulse

suppression observed in experimental and human epileptic tissue, from which it was concluded that inhibition had increased after epilepsy-associated synaptic reorganization (Tuff et al. 1983; King et al. 1985; de Jonge & Racine 1987; Stringer & Lothman 1989; Milgram et al. 1991; Haas et al 1996; Buckmaster & Dudek 1997; Swanson et al. 1998; Wilson et al. 1998; Harvey & Sloviter 2005; Sloviter et al. 2006). Hence, the use of the paired-pulse technique in the assessment of hippocampal synaptic inhibition may lead to erroneous conclusions regarding epilepsy-associated changes in levels of excitability.

6.6 The "hyperinhibition" hypothesis following epilepsy-associated synaptic reorganization in the hippocampus

Changes in the level of hippocampal inhibition are a common finding in experimental and human epileptic tissue, but the fundamental problem of whether inhibition is increased or decreased remains unresolved. The evidence supporting a decrease in inhibition has been largely based on the loss of inhibitory interneurons (Ribak et al. 1979; Obenaus et al. 1993; Houser & Esclapez 1996), the loss of excitatory drive onto inhibitory interneurons (Sloviter 1991b; Doherty & Dingledine 2001), excitatory recurrent mossy fiber connections (Tauck &Nadler 1985; Franck et al. 1995; Wuarin & Dudek 1996), changes in receptor subunits (Macdonald & Kapur 1999), and a decrease in mIPSC's (Shao & Dudek 2005). The evidence supporting the "hyperinhibition" hypothesis is based on the survival of GABAergic interneurons (Babb et al. 1989), the sprouting of mossy fibers onto inhibitory interneurons resulting in increased excitatory drive (Sloviter 1992; Buhl et al. 1996; Kotti et al. 1997; Sloviter et al.

2006), synaptic connections between inhibitory interneurons (Cobb et al. 1997; Bausch 2005), changes in receptor subunits (Otis et al 1994; Gibbs et al. 1997; Nusser et al. 1998), and an increase in mIPSC amplitude following kindling (Otis et al. 1994). In the present study, a paradoxical increase in paired-pulse suppression was observed when GABAergic inhibition was slightly reduced with a specific antagonist for $GABA_A$-receptors. In parallel, Uruno et al. (1994) reported an enhancement of dentate granule cell paired-pulse "depression" by bicucilline in response to antidromic paired-pulse stimulation, and Scharfman (1994) reported a transient enhancement of dentate granule cell IPSP's during pharmacological blockade of $GABA_A$-receptors. However, in experiments with inhibition blocked, a gradual reduction of IPSP's and a concomitant increase in EPSP size and duration has been reported (Dingledine & Gjerstad 1979). Most of the electrophysiological support for an increase in inhibition following epilepsy-associated synaptic reorganization comes from the paired-pulse technique (Tuff et al. 1983; King et al. 1985; de Jonge & Racine 1987; Stringer & Lothman 1989; Milgram et al 1991; Haas et al. 1996; Buckmaster & Dudek 1997; Swanson et al. 1998; Wilson et al. 1998; Harvey & Sloviter 2005; Sloviter et al. 2006), where paired-pulse suppression is often equated to GABAergic inhibition. Other mechanisms may be affecting levels of suppression, however, such as increases in calcium-dependent potassium conductance, ephaptic interactions (Turner et al. 1984), and progressive depolarization inactivation. During repetitive stimulation, IPSP's decrease (McCarren & Alger 1985; Thompson & Gahwiler 1989), conditioning pulse population spike amplitudes increase, and paired-pulse

suppression can be influenced by non-GABAergic mechanisms. The presence of all of these mechanisms across preparations or in epileptic tissue could compromise paired-pulse results. The present result that paired-pulse suppression increased under conditions where excitability is known to increase, casts further doubt on the reliability of the paired-pulse technique as an accurate measure of synaptic inhibition.

Under the most ideal conditions, GABAergic inhibition can be difficult to measure and compare across preparations. The use of the paired-pulse technique increases these difficulties and has the potential to produce artifactual results. The data presented here oppose the notion that the paired-pulse technique, particularly with a repetitive protocol, is a reliable and accurate means of assessing GABAergic synaptic inhibition. Contrasting levels of suppression may be obtained using this technique depending on how the parameters of interpulse interval, stimulation intensity, and frequency are chosen and used together. Further increases in paired-pulse suppression can be measured even though no change has occurred in the level of synaptic inhibition based on changes in $[K^+]_o$ and $[Ca^{++}]_o$. Additionally, slight pharmacological decreases in $GABA_A$-receptor mediated inhibition result in an increase rather then decrease in paired-pulse suppression. This study indicates that the paired-pulse technique is not only a poor measure of synaptic inhibition, whereby variable levels of suppression are obtainable based on the interplay of stimulus parameters, but it can actually produce results that are in direct contradiction to known levels of excitability. These results strongly challenge the assumption that paired-pulse

suppression directly equates to GABAergic inhibition, and confronts much of the electrophysiological data in support of an increase in inhibition following epilepsy-associated synaptic reorganization in the hippocampus.

CHAPTER 7

SUMMARY AND FUTURE DIRECTIONS

7.1 Summary

This study investigated the paired-pulse technique as a measure of synaptic inhibition in the dentate gyrus. Variability in the measure of paired-pulse suppression was assessed using single and repetitive paired-pulse stimulation, alterations in $[K^+]_o$ and $[Ca^{++}]_0$ affecting membrane excitability, and reduced $GABA_A$-receptor mediated inhibition. High intensity and short interpulse intervals produced the greatest levels of paired-pulse suppression to single and repetitive stimulation. During repetitive paired-pulse experiments, the level of paired-pulse suppression and the amplitude of the population spike produced by the conditioning pulse increased. Paired-pulse suppression also increased with increased frequencies of stimulation. When membrane excitability was decreased, paired-pulse suppression was abolished and facilitation observed. When membrane excitability was increased, paired-pulse suppression increased. When $GABA_A$-receptor mediated inhibition was slightly reduced, paired-pulse suppression paradoxically increased rather then decreased. These findings indicate that the paired-pulse technique, and studies that have used it as evidence for increased inhibition following epilepsy-associate synaptic reorganization in the hippocampus, have the potential to be the result of artifact

and lead to misinterpretations concerning alterations in synaptic inhibition in epilepsy.

7.2 Targeted neurotoxin approach

Targeted neurotoxins bind to specific receptors in a neuronal population and eliminate those neurons containing those receptors without affecting other cell types. Mantyh et al. (1997) demonstrated that neurokinin-1 receptors, or substance-P receptors (SPR's), expressed on neurons of the spinal cord could be selectively targeted by a neurotoxic conjugate of substance-P and the ribosome inactivating protein, saporin. When the substance-P portion of the conjugate binds to the substance-P receptor, both are internalized leading to cell death by protein synthesis inhibition. This study showed that a demonstrable network pathophysiology could be achieved in vivo using a neurotoxic approach. Within the hippocampus, SPR's are expressed exclusively by GABAergic inhibitory interneurons (Nakaya et al. 1994; Acsady et al. 1997; Sloviter et al. 2001). Martin and Sloviter (2001) reported that the neurotoxic conjugate of substance-P and saporin could selectively lesion hippocampal inhibitory interneurons without producing the principal cell loss, widespread brain damage, reactive gliosis, and other effects produced by prolonged seizures. Using this method, it may be possible to determine the role of a selective and focal loss of inhibitory interneurons in epileptogenesis.

Tests of inhibition, such as paired-pulse stimulation of the perforant path, suggest increased rather than decreased synaptic inhibition months after treatments that lead to chronic epilepsy in animal models, although somatostatin-

GABAergic and some parvalbumin-GABAergic inhibitory interneurons are killed. These lost interneurons form regions of reduced or non-existent levels of inhibition that have been corroborated with immunocytochemistry as well as electrophysiologically. These regions may form an epileptogenic zone that is capable of generating interictal spikes that may spread from this site. Under conditions whereby $[K^+]_o$ is increased, these events may turn into actual seizures. It is unknown how focal lesions of inhibitory interneurons may contribute to chronic epileptogenesis. Several lines of evidence from multiple laboratories suggest that mossy fiber sprouting, the formation of new axon collaterals from granule cells, leads to new recurrent excitatory circuits. New inhibitory circuits may also play a role in the synaptic reorganization and chronic epilepsy that follows SE. The high level of inhibition present in the dentate gyrus of animal models and humans with epilepsy is thought to mask these new local excitatory circuits. It is unknown whether a focal lesion of inhibitory interneurons, in conjunction with mossy fiber sprouting after SE, will unmask the effects of the new local excitatory circuits and generate localized interictal spikes and seizure-like activity. The use of the substance-P saporin conjugate has the potential for development as a chronic model of epilepsy that could elucidate the respective roles of focal loss of inhibitory interneurons and recurrent excitatory connections to aid in our understanding of the mechanisms of epileptogenesis.

7.3 Immunocytochemical analysis of SSP-saporin induced lesions

A majority of GABAergic interneurons in the hippocampus contain substance-P receptors on their dendrites and somata, and are vulnerable to the effects of SSP-saporin. All other cell types not containing substance-P receptors, including granule cells and pyramidal cells, are spared the effects of the neurotoxin. Figure 7.1 shows an image of a naïve rat stained with NeuN to visualize the normal distribution of neurons in the hippocampus. Figure 7.2 shows substance-P receptor staining in a naïve rat to visualize the normal distribution of these receptors in the hippocampus. The majority of positive staining for substance-P receptors was localized to the hilus, with relatively little staining scattered throughout the other hippocampal layers. Initially, injections of SSP-saporin were performed with 0.1% methylene blue to confirm the delivery of the neurotoxin to the dorsal blade of the dentate gyrus (Fig. 7.3). Following injections of SSP-saporin, slices were prepared and stained for substance-P receptors to visualize the extent of the lesion produced (Figure 7.4). As indicated in Fig. 7.4C, a complete ablation of hilar neurons did not occur in SSP-saporin injected animals, and could be the result of the binding sites of surviving neurons being too deep in the tissue to be reached by the neurotoxin.

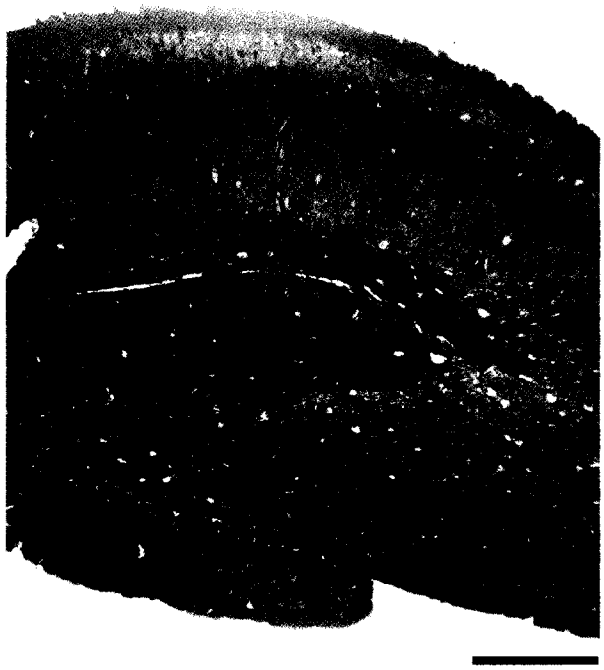


Fig. 7.1 Normal distribution of neurons in the hippocampus. NeuN staining in the hippocampus. Scale 500 μm.

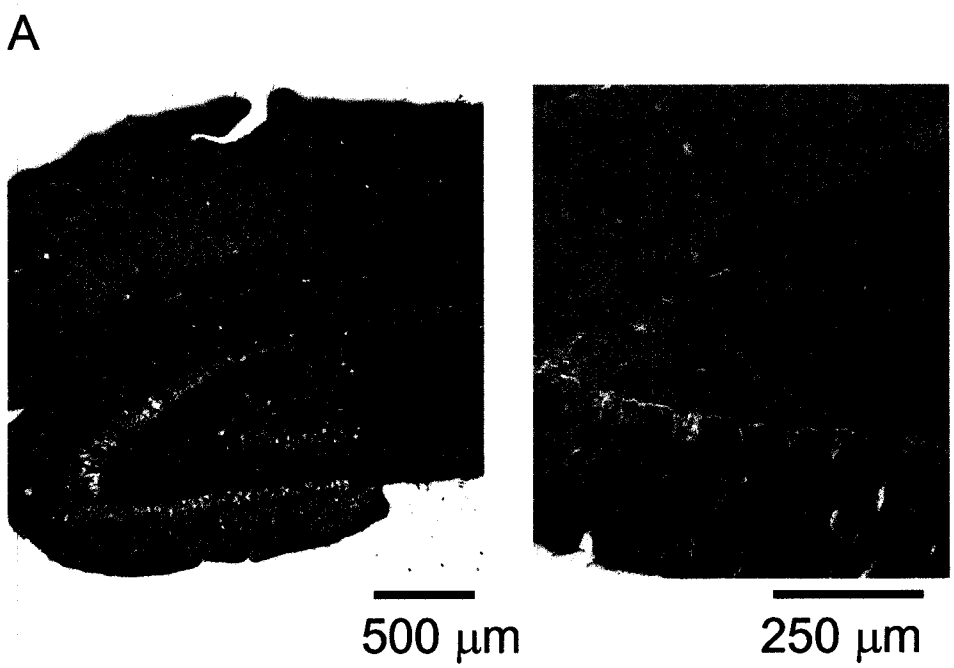


Fig.7.2 Localization of substance-P receptors to the hilus. A: Neurokinin-1 receptor staining in a naïve rat shows the majority of staining within the hilus. B: Negative control for neurokinin-1 receptors with the primary antibody left out. Arrows indicate the dorsal blade of the dentate gyrus.

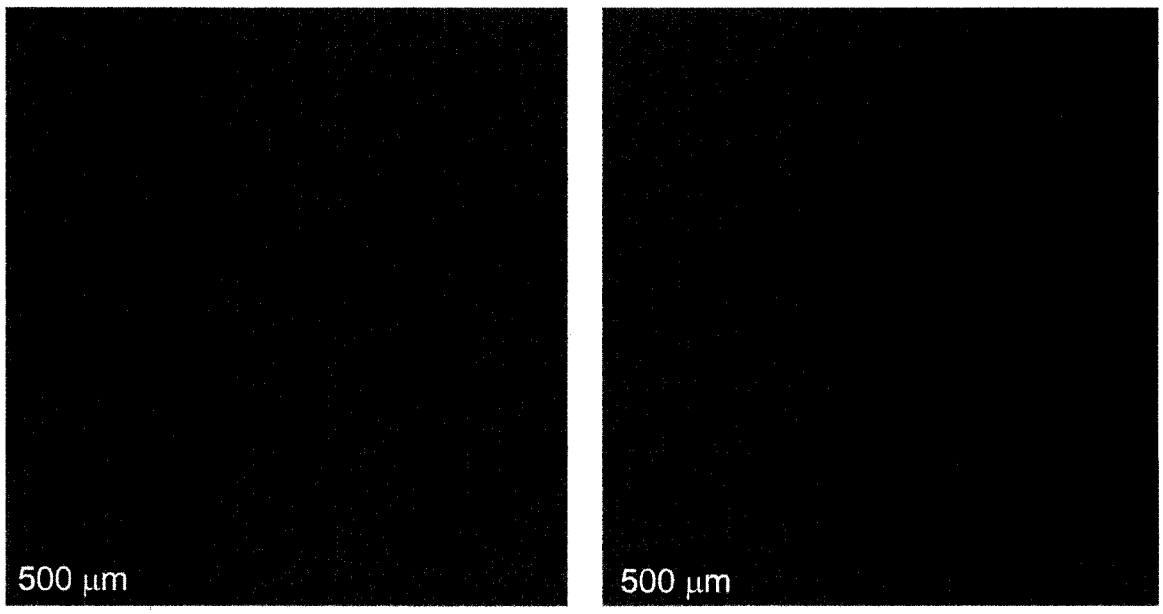


Fig.7.3 SSP-saporin injection sites. Light microscopic images of the hippocampi of two rats that received injections of SSP-saporin containing 0.1% methylene blue. The large arrow points to the injection site, the small arrows point to the dorsal blade of the dentate gyrus.

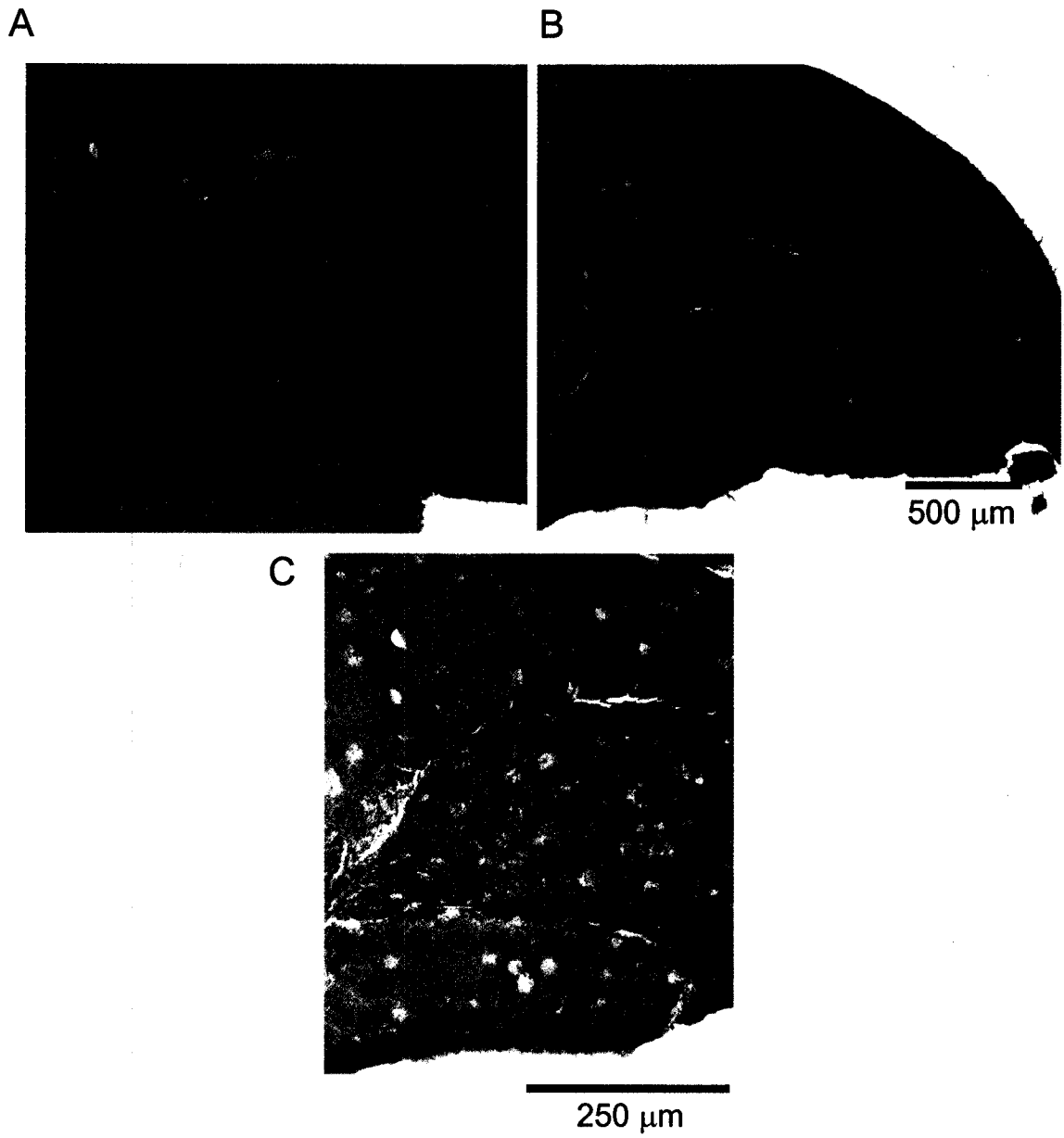


Fig.7.4 A reduction of substance-P receptor staining within the SSP-saporin injection zone. Substance-P staining (A) outside of the injection zone, (B) within the injection zone, and (C) at higher magnification within the injection zone.

7.4 Electrophysiological analysis of SSP-saporin induced lesions

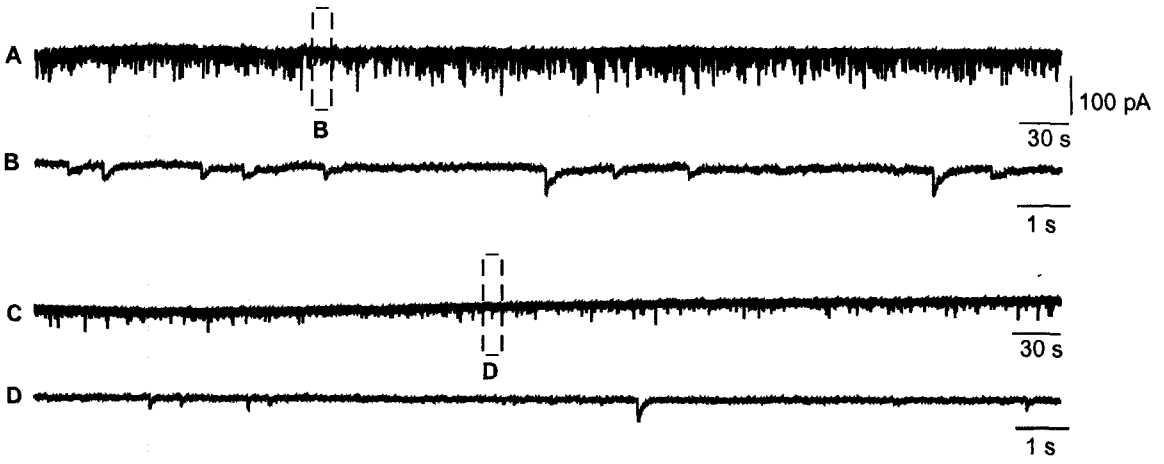
Functional loss of substance P-receptor containing GABAergic interneurons was characterized by recording IPSC's from SSP-saporin induced

lesion sites. A reduction in the number of GABAergic inhibitory interneurons within the injection zone should theoretically produce a reduction in the frequency of IPSC's compared to areas outside of the injection zone. Figure 7.5 shows whole cell patch clamp recordings of sIPSC's from granule cells in a hippocampal slice prepared from a rat which received an injection of SSP-saporin. Although not quantified, the data preliminarily indicate a lower frequency of sIPSC's within

7.5 Examples of sIPSC's recorded in dentate granule cells from a SSP-saporin injected rat. A: Numerous sIPSC's recorded outside of the injection zone shown at a fast time scale. B: Expanded time scale of the boxed area shown in A. C: Relatively fewer sIPSC's recorded within the injection zone shown at a fast time scale. D: Expanded time scale of the boxed area shown in C.

the SSP-saporin injection zone. A higher frequency of IPSC's in the injection zone could possibly arise through synaptic input from axons of SPR-containing inhibitory interneurons with somata beyond the boundary of the effects of the neurotoxin, which would still release transmitter spontaneously onto the recorded granule cells. Another possibility is the presence on non-SPR-containing

inhibitory interneurons that survived the treatment near the recorded neuron. In order to distinguish between these two possibilities, whole-cell recordings were performed in the presence of tetrodotoxin (TTX, 1 μM) to isolate mIPSC's. A decrease in frequency and amplitude of IPSC's in TTX would suggest the presence of non-SPR-containing interneurons in the slice, while no change would indicate the events are from cut axons. Figure 7.6 shows whole-cell recordings of mIPSC's from granule cells in a hippocampal slice prepared from an animal which received an injection of SSP-saporin. An apparent decrease in IPSC's



7.6 Examples of mIPSC's recorded in dentate granule cells from a SSP-saporin injected rat. A: Numerous mIPSC's recorded outside of the injection zone shown at a fast time scale. B: Expanded time scale of the boxed area shown in A. C: Relatively fewer mIPSC's recorded within the injection zone shown at a fast time scale. D: Expanded time scale of the boxed area shown in C.

occurred in the injection zone indicating the functional loss of SPR-containing GABAergic interneurons, but a quantification of these events needs to be performed to judge the extent of the lesion and distinguish between the cells types remaining in the injection zone. However, the data so far suggests that a

functional loss of GABAergic interneurons takes place within the SSP-saporin injection zone.

7.5 Future Experiments

A key question to be answered by future experiments is whether a focal lesion of inhibitory interneurons, in conjunction with mossy fiber sprouting after SE, will unmask the effects of the new local excitatory circuits and generate localized interictal spikes and seizure-like activity. If not, what is required to produce interictal spikes and electrographic seizures in the isolated dentate gyrus? What role do modest elevations of $[K^+]_o$ play in evoking epileptiform activity under these conditions? Further studies will use SSP-saporin and the kainate model of temporal lobe epilepsy to produce mesial temporal sclerosis, mossy fiber sprouting, and chronic epilepsy in rats. Experiments will use this method of inducing focal death of interneurons to test the hypothesis that the combined effects of chronic focal interneuron loss and the formation of new recurrent excitatory circuits associated with mossy fiber sprouting leads to an increased propensity for spontaneous interictal spikes and seizures in the isolated dentate gyrus from rats with kainate-induced epilepsy. This method has many advantages over blocking inhibition pharmacologically, because it allows a permanent and focal ablation that can be performed (and later studied) in an intact animal. It also simulates the actual loss of interneurons, which is known to occur in some regions of epileptic tissue. Small and graded shifts in $[K^+]_o$ will be used as a physiological method to test for alterations in seizure susceptibility.

The existence of just one factor alone (be it a loss of inhibition or increased excitatory circuits) hypothetically does not result in a comparable alteration in seizure susceptibility. By creating focal lesions of inhibitory interneurons in rats with kainate-induced epilepsy, the aim is to produce areas of the dentate gyrus in which inhibition is reduced or eliminated focally and new excitatory circuits have been formed through mossy fiber sprouting. With inhibition depressed focally, the excitatory effects of mossy fiber sprouting should be unmasked with less of an elevation of $[K^+]_o$. This dual form of synaptic reorganization will hypothetically enhance the susceptibility to generate interictal spikes and seizure-like activity. These experiments should shed light on the long-standing problem of how multiple alterations in local hippocampal circuits may underlie epileptogenesis.

Intracellular analyses of GABA-mediated inhibition has been undertaken in vivo from chronically epileptic rats (Buckmaster & Dudek 1997, 1999). However, most research has necessarily involved the paired-pulse technique with field-potential recordings (Sloviter 1992), particularly when recording from freely behaving animals for prolonged periods (Hellier et al. 1998). Although chronic recordings from freely behaving animals are necessary to understand the phenomena associated with chronic epilepsy, in vivo field-potential recordings are difficult to interpret mechanistically and inherently involve many connections and networks that may obscure the events under investigation whether dealing with an awake or anesthetized animal. In vitro experiments on hippocampal slices will allow patch-clamp analysis of specific local circuits in isolation from the rest of the brain. Martin and Sloviter (2001) showed that SSP-saporin eliminated

SPR-containing inhibitory interneurons in a radius of about 1 mm around the injection site, with minimal tissue damage. Again using repetitive paired-pulse perforant-path stimulation and field-potential recording in anesthetized rats, Martin and Sloviter (2001) observed apparent disinhibition and hyperexcitability, which was characterized by multiple population spikes and a lack of paired-pulse inhibition with 2 Hz repetitive stimulation. The hyperexcitability and depressed repetitive paired-pulse inhibition was only present within the region of damaged interneurons. However, it is known that repetitive stimulation in the intact brain can decrease levels of inhibition due to the wide range of connections that are activated and the effects of other structures. The purpose of the future experiments will be to evaluate with whole-cell recording the synaptic interactions that occur after selective lesion of inhibitory interneurons alone and in combination with kainate-induced mossy fiber sprouting in hippocampal slices, allowing the dentate gyrus to be studied in isolation from the rest of the brain.

There is much debate about whether the loss of inhibition alone can account for the presence of interictal spikes and seizure-like activity in the dentate gyrus. It seems that a combination of factors must exist at least transiently at the onset of an epileptic seizure, hypothetically including reduced inhibition, increased local excitation, and elevated $[K^+]_o$. The chronic loss of inhibitory interneurons alone does not fully account for either the histopathological data on epileptogenic tissue or the recordings of seizure activity in chronic epilepsy. With pharmacological treatments of hippocampal slices, blockade of $GABA_A$-receptors alone only produces a mild "hyperexcitable" state

in the isolated dentate gyrus from normal animals, characterized by one or only a few population spikes after single stimuli to the perforant path (Fricke & Prince 1984; Buckmaster &Dudek 1997; Patrylo et al. 1999). Neither interictal spikes nor electrographic seizures are generated under these conditions. By injecting SSP-saporin into the dentate gyrus of normal rats, the aim is to investigate how a small focal lesion of GABAergic interneurons alters this critical neural circuit. Furthermore, the aim is to determine if chronic loss of inhibitory interneurons alone generates epileptiform activity in normal $[K^+]_o$.

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