Published in final edited form as:

Epilepsia. 2009 April ; 50(4): 904–916. doi:10.1111/j.1528-1167.2008.01939.x.

Single and repetitive paired-pulse suppression: a parametric analysis and assessment of usefulness in epilepsy research

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Abstract

Purpose—The paired-pulse technique has been widely used as a convenient but indirect measure of “inhibition” in hippocampal circuits of normal and epileptic animals. Most investigators have used a single paired-pulse protocol, while others have utilized repetitive paired pulses. This study investigated which parameters influence results from paired-pulse tests, focusing on the repetitive paired-pulse technique; it aims to assess how it may be used in an unbiased and quantitative manner across animal preparations for comparisons of control and experimental epileptic animals.

Methods—The perforant path was stimulated while field potentials were recorded from the granule cell layer under isoflurane anesthesia. Paired-pulse suppression was analyzed as a function of stimulation intensity, interpulse interval and frequency.

Results—Paired-pulse suppression was greater with increased stimulus intensity and decreased interpulse interval (20–100 ms). During repetitive protocols, stimulation frequencies ≤ 1.0 Hz produced paired-pulse suppression similar to single paired-pulse responses, but caused more paired-pulse suppression between 1.0 and 4.0 Hz at all but the lowest intensities. The amplitude of the population spike produced by the conditioning pulse progressively increased during stimulation at higher frequencies (1.0–4.0 Hz).

Discussion—The single paired-pulse technique is highly dependent on stimulation parameters, as is the repetitive paired-pulse protocol, which is more variable. To generate reliable, consistent, and unbiased data in comparisons of control and experimental epileptic groups, all parameters should be specified and controlled across experiments. Paired-pulse suppression is susceptible to alterations in many mechanisms, and thus represents a circuit response rather than an assay of GABAergic inhibition in epilepsy research.

Keywords

dentate gyrus; GABA; inhibition; hippocampus

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Disclosure Statement

None of the authors has any conflict of interest to disclose.

Introduction

Inhibition of neuronal activity is critically important for normal brain function and for the control of epileptic seizures. A relatively small number of heterogeneous interneurons that use gamma-aminobutyric acid (GABA) as their neurotransmitter (Mohler et al., 1996) mediate a particularly important form of synaptic inhibition in the hippocampus, neocortex, and other areas of the brain. GABA acts in the hippocampus and neocortex on two pharmacologically distinct types of receptors (i.e., GABA_A and GABA_B), which are responsible for fast and most slow inhibitory postsynaptic potentials (IPSP's), respectively (McCormick, 1989). Other forms of "inhibition" arise from activation of intrinsic currents, such as the potassium currents that result from action potential activity, and from neuromodulators that act through second-messenger systems. These forms of inhibition can be difficult to measure and compare across normal and epileptic preparations. For in vivo studies, few methods are available to study changes in the state of "inhibition" of neuronal circuits across time and animal preparations, and studies aimed at isolating "inhibition" mediated by intrinsic mechanisms, GABA, and various neuromodulators are even more problematic. 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 in vivo, are sensitive to shifts in membrane potential, and are generally possible in vivo only in anesthetized animals.

The paired-pulse suppression technique has been used extensively in normal and epileptic animals to study overall "inhibition" in hippocampal neuronal circuits (i.e., GABA-mediated inhibition, plus intrinsic and neuromodulatory mechanisms) because of its ability to measure activity of large neuronal populations in a relatively stable manner (Andersen et al., 1966; Tuff et al., 1983; Stringer and Lothman, 1989; Hellier et al., 1999). Paired-pulse suppression in the dentate gyrus was interpreted to be caused by GABAergic inhibition resulting from the recurrent activation of basket-shaped interneurons (i.e., recurrent or feedback inhibition), as well as interneurons activated directly by afferent stimulation (feedforward inhibition) (Andersen et al., 1964). Since GABAergic inhibition, particularly the inhibition mediated by GABA_A receptors, is particularly powerful, paired-pulse suppression has often been equated to GABAergic inhibition. In awake animals, it is practically 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 overall "inhibition" (Andersen et al., 1966), but the mechanisms that contribute to paired-pulse suppression are complex and the effects of different experimental variables and the interplay of these variables are often not considered in studies with this technique. 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 and Sloviter, 2005; Sloviter et al., 2006).

Epilepsy-associated changes in the level of "inhibition" – particularly GABA-mediated inhibition - in the hippocampus are a common finding (Prince, 1968; Ribak et al., 1979; Babb et al., 1989; Davenport et al., 1990; Sloviter, 1991b), but whether various mechanisms of inhibition are decreased or increased is highly controversial. Furthermore, most hypotheses concerning "inhibition" as a mechanism for epilepsy invoke GABAergic mechanisms, so it is understandable that paired-pulse data would tend to be interpreted in terms of GABAergic inhibition. The fact that the paired-pulse technique has been used extensively to assess levels of "inhibition" raises the question of how alterations in the parameters of electrical stimulation affect the results. Although it has been argued that repetitive stimulation is needed to measure the full strength of inhibition (Harvey and Sloviter, 2005), GABA_A-receptor mediated inhibition is known to fade during repetitive

activation of GABAergic synapses (Ben-Ari et al., 1979; McCarren and Alger, 1985; Thompson and Gahwiler, 1989). The strong dependence of the evidence in support of augmented GABAergic inhibition in the hippocampus following epilepsy-associated synaptic reorganization on this technique (Tuff et al., 1983; King et al., 1985; de Jonge and Racine, 1987; Stringer and Lothman, 1989; Milgram et al., 1991; Haas et al., 1996; Buckmaster and Dudek, 1997; Swanson et al., 1998; Wilson et al., 1998; Harvey and Sloviter, 2005; Sloviter et al., 2006) justifies a detailed analysis of the parameters that affect the outcome. This study aims to assess how the parameters of stimulation intensity, interpulse interval, and frequency used in the paired-pulse technique (i.e. single and repetitive paired pulses) affect the results in the dentate gyrus of anesthetized rats.

Methods

Surgery Preparation

All procedures described in this study were approved by the Colorado State University and University of Utah Institutional Animal Care and Use Committees. Adult male Sprague-Dawley rats (180-250 g; Harlan, Indianapolis, IN) were used for all experiments. All animals were pre-treated with dexamethasone (4 mg/kg) and atropine (2.0 mg/kg) to prevent cardiorespiratory complications before being anesthetized with 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. Using bregma as a reference, five small holes were drilled into the skull using a Dremel and #105 bit. A ground electrode made of exposed Teflon-coated steel wire (127 μ m diameter) (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 a frontal lobe support screw with dental cement. A Teflon-coated platinum-iridium recording electrode (76 μ m diameter) was stereotaxically positioned 2.5 mm lateral and 4.0 mm caudal to bregma, and a bipolar stimulating electrode (114 μ m diameter) positioned 4.2 mm lateral and 8.0 mm caudal to bregma.

In Vivo Electrophysiology

The recording electrode was lowered into the hippocampus using audio monitoring of spontaneous spikes until the dorsal blade of the granule cell layer was confirmed. The stimulating electrode was then lowered into the ipsilateral angular bundle and low-frequency stimulation (duration 0.01 ms) was applied until the response showed a population spike of maximum amplitude. The responses were filtered (0.5 Hz-10 kHz), amplified (100 $\times$), displayed on an oscilloscope, digitized (Neuro-corder, NeuroData Instruments; NYC, NY) and stored on video tape. Once a response consisting of a stable, short-latency excitatory post-synaptic potential (EPSP) and population spike was confirmed, the minimum intensity needed to produce the largest population spike to the conditioning stimulus was determined and defined as 100% intensity (Fig. 1A). 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 (Fig. 1B) and interpulse intervals of 20, 40, 60, 80, 100, and 200 ms (Fig. 1C). 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.

Analysis

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 population spike to the test pulse by that of the population spike to the conditioning pulse. Values <1.0 indicated paired-pulse suppression, and values >1.0 indicated facilitation. One-way ANOVA was used to determine statistically significant differences.

Results

Single paired-pulse stimulation

The lowest stimulus voltage that produced the largest granule-cell population spike was defined as 100%, and changes in stimulus intensity were calculated as percent changes from this value. A stimulus-response curve was plotted to determine how changes in relative intensity of the stimulus affected the amplitude of the population spike produced by the conditioning pulse. The curve showed that the conditioning-spike amplitude was not a linear function of stimulus voltage; rather, stimulus intensities of 50%-100% produced responses progressively smaller in amplitude than expected from a linear function of spike amplitude versus stimulus voltage (i.e., saturated).

Single paired stimuli were used to assess the effects of stimulus intensity and interpulse interval on levels of paired-pulse suppression (Fig. 1). Interpulse intervals of 20, 40, and 80 ms were analyzed most intensely due to findings suggesting GABAergic recurrent inhibition is strongest at intervals ≤ 100 ms (Andersen et al., 1966) and that alterations using GABA_A-receptor agents were restricted to intervals ≤ 100 ms (Tuff et al., 1983). Figure 1B 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. As the stimulus intensity increased, more paired-pulse suppression became evident. As reported by other investigators (Kapur et al., 1989), a short interpulse interval of 20 ms produced the highest level of paired-pulse suppression, as indicated by a low-amplitude population spike in response to the test pulse (Fig. 1B,C). As the interpulse interval was increased, the population spike produced by the test pulse increased in amplitude, and the level of paired-pulse suppression decreased (Fig. 1B,C). When stimulations were given 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, which is presumably during the slow GABA_B-receptor mediated IPSP, the late phase of the GABAergic response (Tuff et al., 1983) (Fig 2B). 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) (Fig 2A). Differences in the averaged paired-pulse ratios were statistically significant between intensities using an interpulse interval of 20 ms, and between interpulse intervals at stimulus intensities of 50% to 100% with the exception of 20 and 40 ms using an intensity of 50% (one-way ANOVA, $p < 0.05$). These data indicate that stimulus intensity played a direct role in determining the amplitude of the conditioning-pulse population spike, and hence the level of measured paired-pulse suppression. Furthermore, when utilizing a single paired-pulse protocol, stimuli of higher intensities and shorter interpulse intervals produced the greatest levels of paired-pulse suppression (Fig. 2).

Stimulation trains of 10 repetitive paired pulses

To determine how the parameters of the paired-pulse technique affect the levels of measured suppression during repetitive paired-pulse stimulation, interpulse intervals of 20, 40, and 80

ms were used with stimuli of 100%, 75%, 50%, and 25% intensity and frequencies of 0.1, 1.0, 2.0, 3.0, and 4.0 Hz. The level of paired-pulse suppression during a repetitive train was assessed at the first, fifth, and tenth pair in a train of ten consecutive paired stimuli. Paired-pulse suppression 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 (Fig 3A). This effect was evident at all stimulation intensities (Fig 3B) except 25% where, presumably, the stimulation was too weak to produce an adequate activation of the granule cells and interneurons, thus leading to little or no inhibitory effect (data not shown). The same pattern was evident at all stimulus frequencies tested, with the exception of 0.1 Hz where little to no change was observed during the train, regardless of the stimulus intensity. These data 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.

The level of paired-pulse suppression using low-frequency repetitive pairs of stimuli of 0.1-1.0 Hz showed little difference from that produced by single pairs of stimuli (Fig 4A). 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. 4B). An increase in the level of paired-pulse suppression was evident at all but the lowest intensity tested as the frequency of stimulation increased from 0.1-4.0 Hz (Fig 5). 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 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.

Variability in the amplitude of the population spike to the conditioning pulse during repetitive paired-pulse stimuli

In order for measurements of paired-pulse suppression to be reproducible and accurate during a repetitive train, the amplitude of the population spike produced by the conditioning pulse must remain constant. To determine if this was the case, 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 6A shows a repetitive paired-pulse train resulting from stimulation of 75% intensity at 3.0 Hz. The population spike to the conditioning pulse from the first, fifth and tenth pairs are shown at a larger scale in Figure 6B, 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. Low-frequency stimulation of 0.1 Hz did not produce any substantive differences in conditioning-pulse population-spike amplitudes during a train, as indicated by an amplitude ratio consistently close to 1.0 (Fig. 7). At 1.0 Hz, the amplitude ratio initially dropped below 1.0 and then progressively increased when using a stimulus intensity of 75% (Fig. 7). As stimulus frequency was increased from 2.0 to 4.0 Hz, the amplitude ratios progressively increased, with the largest values and degree of variability present at 4.0 Hz (Fig. 7). Differences were statistically significant at 2.0 Hz using interpulse intervals of 40 and 80 ms, at 3.0 Hz using an 80 ms interpulse interval, and at 4.0 Hz using interpulse intervals of 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 was 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 high intensities of 75%-100%.

Discussion

Overview of results

The goal of this study was to determine how the parameters used in the paired-pulse technique in vivo affect the levels of paired-pulse suppression in the dentate gyrus. This technique has been widely used in epilepsy research, generally with quite different stimulation parameters across studies, and sometimes without specification of the stimulation parameters. The present study shows that the degree of paired-pulse suppression from single and repetitive paired-pulse stimuli is highly dependent on the combination of stimulus intensity and interpulse interval; high stimulus intensity and short interpulse intervals produced the greatest levels of paired-pulse suppression. During repetitive pairs of stimulus pulses at 1.0 to 4.0 Hz, the level of paired-pulse suppression increased during the train at all but the lowest intensity. Importantly, the amplitude of the population spike produced by the conditioning pulse increased and was variable in amplitude during repetitive paired-pulse trains at higher frequencies. These data show that the measured levels of paired-pulse suppression, particularly with repetitive pairs of pulses, are highly dependent on the choice of stimulus parameters. Therefore, consistent and detailed information on the stimulus parameters, with special attention paid to stimulus intensity, are essential for data from paired-pulse experiments to be both reliable and reproducible in comparisons of normal and epileptic tissue.

Single paired-pulse stimulation: influence of interpulse interval

The most obvious factor affecting the level of paired-pulse suppression to single pairs of stimuli was the interpulse interval. Three distinct effects on the level of paired-pulse suppression are known to occur depending on the interpulse interval: (1) a strong early suppression phase, (2) a progressively lower level of suppression and facilitation phase, and (3) a late suppression phase (Tuff et al., 1983; Racine and Milgram, 1983; Joy and Albertson, 1987). Early paired-pulse suppression is thought to correspond primarily to the time course of the intracellularly recorded GABA_A-receptor mediated fast IPSP (e.g., McCormick, 1989). Late paired-pulse suppression is thought to reflect the time course of the intracellularly recorded GABA_B-receptor mediated slow IPSP (e.g., McCormick, 1989). When using single pairs of pulses, the present study confirms the results reported by Kapur et al. (1989) by observing that paired-pulse suppression was strongest at short interpulse intervals and progressively decreased as the interval increased, thus representing the transition from fast GABA_A-receptor to slow GABA_B-receptor mediated IPSPs.

Influence of stimulus intensity

The second, and often overlooked, factor determining levels of paired-pulse suppression was stimulus intensity. It has been previously reported that the duration and magnitude of the suppression is partly determined by the stimulus intensity (Matthews et al., 1981; Tuff et al., 1983; Kamphuis et al., 1992). As the stimulus intensity increases, more granule cells or pyramidal cells are excited, which then activates more GABAergic basket cells via feedback mechanisms (Fig. 8). Raising the stimulus intensity also increases the number of excitatory afferent fibers that directly activate interneurons that participate in feed-forward inhibition (Fig. 8). Thus, the level of paired-pulse suppression increases with stimulus intensity. For studies using the paired-pulse technique to compare groups of animals or preparations, it is thus critical that the level of stimulus input remains consistent across groups so that comparable responses to the conditioning stimulus can be evoked, thus allowing changes in the levels of paired-pulse suppression to be determined with the same conditioning stimulus.

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. Thus, issues such as the relative distance between the stimulating and recording electrodes can have a large effect on intensity-dependent population-spike amplitude, and adjusting the intensity of conditioning and test pulses independently may simplify interpretation. It remains unclear, however, how to consistently control for the effect of stimulus intensity across preparations and animals. This is especially the case in epileptic tissue, where pathological changes may influence how the tissue responds to stimuli of different intensity. Attempts have been made to reduce or remove intensity as a variable in studies on animal models of epilepsy by choosing an intensity sufficient to reach a constant paired-pulse index (Kapur et al., 1989), matching conditioning-pulse population-spike amplitudes (Stringer and Lothman, 1989), determining a minimum stimulus for population spike threshold (Buckmaster and Dudek, 1997), and stimulating at a percent of maximum population-spike amplitude (Isokawa-Akesson et al., 1989; Uruno et al., 1994; Haas et al., 1996; Swanson et al., 1998; Hellier et al., 1999; Sayin et al., 2001) or population postsynaptic potential (pPSP) amplitude (Wilson et al., 1998). The variability in the choice of stimulus intensity leads to the potential for variability in the data, and makes the results from different studies difficult to compare. The choice of a single stimulus intensity when performing the paired-pulse technique (Sloviter and 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 and Goddard, 1987; Bragin et al., 2005; Harvey and Sloviter, 2005), makes results even more difficult - if not impossible - to reproduce and interpret. Thus, the importance of a consistent stimulus intensity, which has been defined on an input-output curve, is critical to obtaining reliable and reproducible results when utilizing the paired-pulse technique.

Influence of frequency on paired-pulse suppression

During repetitive stimulation, paired-pulse suppression increased as stimulus frequency was raised beyond about 1 Hz. One proposal to account for this observation is based on differences in the activation of frequency-dependent feedback and/or feed-forward inhibitory circuits (Sloviter, 1991a), although there seems to be a lack of direct evidence to support this hypothesis. 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 (i.e., an increase in synaptic transmission during high-frequency stimulation) could increase the number of granule cells involved in a synchronized discharge (Andersen and Lomo, 1967), leading to an increase in feedback inhibition. It has also been reported that potentiation in the responses of inhibitory interneurons during repetitive stimulation can occur in vitro (Lee et al., 1980). The level of paired-pulse suppression, particularly during repetitive stimulation, could be influenced by increases in calcium-dependent potassium conductance and associated after-hyperpolarizations, altered threshold resulting from other slow conductance changes, and changes in membrane excitability due to increased $[K^+]_o$ and decreased $[Ca^{++}]_o$. In addition to traditional inhibition from hyperpolarizing IPSPs, shunting inhibition could contribute to paired-pulse suppression (Staley and Mody, 1992). Even under conditions when all stimulus parameters are properly controlled, the use of a repetitive paired-pulse protocol likely invokes these possible mechanisms, thus adding to the potential for variability that is already present when using a single paired-pulse protocol.

Conditioning pulse responses during repetitive paired-pulse stimulation

Results from this study indicate that the amplitude of the population spike to the conditioning pulse increased and was variable during a repetitive paired-pulse train at higher

frequencies (i.e., >1 Hz). This effect is similar to increased stimulus intensity, which can account at least in part 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 gradual depression of GABAergic inhibition during the stimulus train. However, the paired-pulse technique shows an increase in suppression under these conditions. Thus, the use of single paired-pulse stimuli may provide more reliable and interpretable measurements of suppression than repetitive paired-pulse stimulation.

Rationale for the repetitive paired-pulse protocol

There does not appear to be a clear rationale for using repetitive paired pulses to assess “inhibition” (Sloviter, 1991a; Swanson et al., 1998; Wilson et al., 1998; Harvey and Sloviter 2005; Sloviter et al., 2006). Although the results from the present study indicate that paired-pulse stimulation becomes stronger during a repetitive train, 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). Furthermore, repetitive extracellular stimulation in vivo has the potential for progressively recruiting networks outside those being targeted for electrical stimulation, which is much less likely with single paired-pulse stimuli. Other alterations during repetitive stimulation may also occur that do not involve GABAergic or other inhibitory mechanisms (also see above). For example, in addition to increases in $[K^+]_o$ (Thalman and Ayala, 1982) and decreases in $[Ca^{++}]_o$ (Krnjevic et al., 1980), changes in receptor sensitivity, membrane potential, voltage-dependent channels may occur; plus, neurotransmitter depletion is likely to be an increasingly important factor during a repetitive stimulation protocol. Thus, the repetitive paired-pulse technique has more potential pitfalls than single paired-pulse stimulation in the assessment of “inhibition” in normal and epileptic animals: (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 and other forms of “inhibition” are likely to influence the level of paired-pulse suppression at frequencies >1.0 Hz.

Synaptic influences on paired-pulse suppression in the dentate gyrus

A multiplicity of synaptic connections in the perforant path to granule cell circuit can account for a decrease in the amplitude of the population spike produced by the test pulse, and thus potentially influence levels of paired-pulse suppression in the dentate gyrus. Similar types of local circuitry exist in other areas of the hippocampus and neocortex, and these mechanisms could influence the degree of paired-pulse suppression in these other structures (Fig. 8). The initial conditioning and subsequent test pulse each produce a compound action potential in the perforant path and other afferent fiber pathways of the hippocampus, whose overall amplitude is graded by the number of fibers activated. As a result of the release of glutamate from pre-synaptic terminals, a post-synaptic EPSP and population spike are produced in granule cells and pyramidal cells (Fig. 8A), whose amplitude is also graded by the number of synchronously firing neurons. Activated granule cells and their mossy fibers in the dentate gyrus and pyramidal cell axon collaterals in the hippocampus subsequently excite inhibitory interneurons (Fig. 8B), which produce post-synaptic EPSPs and action potentials in the interneurons. The inhibitory interneurons then cause a graded IPSP in the hippocampal granule and pyramidal cells (Fig 8B). Those inhibitory interneurons that are simultaneously activated directly by afferent perforant path fibers (i.e., feedforward inhibition) also produce a graded IPSP response in the granule and pyramidal cells (Fig 8A,B). The test pulse stimulus, delivered after a given interpulse interval, would coincide with the GABA_A- or GABA_B-receptor mediated IPSP, and thus

produce population spikes of variable amplitude. These synaptic responses, however, will be influenced by the presence of non-GABA-related mechanisms across preparations or in epileptic tissue, which may actually underlie the paired-pulse results and lead to misinterpretations about the state of “inhibition.”

Increased vs. decreased inhibition after epilepsy-associated synaptic reorganization

Data from experimental and human epileptic tissue has been used to support hypotheses of both an increase and a decrease in the level of GABAergic inhibition following epilepsy-associated synaptic reorganization in the dentate gyrus of the hippocampus. A selective loss of some GABAergic inhibitory interneurons is well-established, which should cause a decrease in hippocampal inhibition (Ribak et al., 1979). Some investigators have argued that GABAergic interneurons are relatively spared in epileptic tissue, and there may be a larger loss of glutamatergic cells (Babb et al., 1989; Davenport et al., 1990). A small and selective loss of inhibitory interneurons, however, may have a large effect on the neuronal network (Prince, 1968). It has been difficult, nonetheless, to discern physiological changes arising from different degrees of inhibitory interneuron loss. The “dormant basket cell” hypothesis states that a loss of excitatory mossy cell synapses on inhibitory basket cells accounts for a lack of inhibitory drive in the epileptic hippocampus (Sloviter, 1991b). Other investigators have proposed that alterations in GABA_A receptors may account for changes in GABA-mediated inhibition (e.g., Kapur and MacDonald, 1997; Brooks-Kayal et al., 1998) in chronic epilepsy. A decrease in the frequency (but not amplitude) of miniature inhibitory post-synaptic currents (mIPSC's) in the kainate model of epilepsy (Shao and Dudek, 2005) suggests a persistent decrease in GABA-mediated inhibition arising from a selective loss of GABAergic interneurons rather than an alteration of GABA_A receptors, at least in this brain region of this animal model of chronic epilepsy. The formation of new recurrent mossy fiber connections among granule cells (Tauck and Nadler, 1985; Franck et al., 1995; Wuarin and Dudek, 1996) in the kainate model of epilepsy has also suggested increased excitation. Others have proposed an increase in GABA-mediated inhibition in the dentate gyrus based largely on studies suggesting aberrant mossy fiber sprouting onto inhibitory interneurons (Sloviter, 1992; Kotti et al., 1997; Sloviter et al., 2006; see Ribak and Peterson, 1991 for evidence of such connections in normal hippocampus) and new synaptic connections between surviving inhibitory interneurons (see Bausch, 2005 for a review). In isolated neurons, epilepsy-associated alterations in GABA receptor subunit expression and current density have been reported (Gibbs et al., 1997a,b; Brooks-Kayal et al., 1998). In the kindling model of epilepsy, increased excitatory drive onto interneurons, increased granule cell evoked IPSC's, changes in post-synaptic receptors, and a decreased autoinhibition of GABA release have been reported (Otis et al., 1994; Titulaer et al., 1995; Buhl et al., 1996; Nusser et al., 1998). However, a majority of electrophysiological data supporting an increase in inhibition is a result of measured increases in paired-pulse suppression (Tuff et al., 1983; King et al., 1985; Oliver and Miller 1985; de Jonge and Racine, 1987; Stringer and Lothman, 1989; Milgram et al., 1991; Haas et al., 1996; Buckmaster and Dudek, 1997; Swanson et al., 1998; Wilson et al., 1998; Harvey and Sloviter, 2005; Sloviter et al., 2006), which has generally been equated to increases in GABAergic inhibition. However, conclusions regarding alterations to GABAergic inhibition may be misleading based solely on the relative interaction of arbitrarily chosen paired-pulse stimulus parameters, and in some studies, the repetitive nature of the paired-pulse stimulation.

Conclusions

In experimental and human epileptic tissue from the hippocampus, contrasting levels of “inhibition” have been reported ranging from a “hyperexcitable” to a “hyperinhibited” state. Electrophysiological evidence derived from the paired-pulse technique has been used to support alterations to inhibition in this tissue, with many studies not specifying the

stimulation parameters and/or using a repetitive paired-pulse protocol. Results presented in this study have direct implications for the interpretation of these and other data using the paired-pulse technique to make conclusions regarding levels of neuronal circuit inhibition, and hence on the current understanding of the relationship of altered states of excitability during epileptogenesis. 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. Thus, the need for stringent, and possibly uniform, standards in setting stimulus parameters is necessary to reduce the potential for variable results and misinterpretations regarding GABA-mediated inhibition. That this technique directly reflects changes in GABA-mediated inhibition, versus any or all of several other possible mechanisms, is highly unlikely. Hence, the paired-pulse technique can be used as a measure of an overall neural circuit response when comparing treatments, but only if precise specifications of stimulus parameters, extensive replication, and quantitative analyses are used and made consistent across preparations.

Acknowledgments

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. This work was supported by NIH grants NS-45465 (SW) and NS-16883 (FED).

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Fig. 1.

Electrophysiological responses showing that paired-pulse suppression depends heavily on stimulus intensity and interpulse interval. A: Individual conditioning population spikes from single paired-pulse responses at stimulus intensities between 25 and 100% of maximum. B: Single paired-pulse responses to stimulus intensities of 25%-100% of maximum with an interpulse interval of 20 ms. C: Single paired-pulse responses with interpulse intervals of 20-80 ms and stimulus intensity of 100% of maximum. D: Single paired-pulse responses with interpulse intervals of 20-80 ms and stimulus intensity at 50% of maximum. Note that the level of paired-pulse suppression decreased as stimulus intensity decreased and interpulse interval increased, with relatively little suppression evident with an interpulse interval of 80 ms. Traces represent an average of 10 individual responses.

Fig. 2.

Summary data showing that paired-pulse suppression depends on stimulus intensity and interpulse interval. A: Bar graph 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. One-way ANOVA, $*p < 0.05$, $**p < 0.01$ ($n=7$). B: Bar graph 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 ($n=7$). Paired-

pulse suppression decreased as the interpulse interval increased from 20 ms to 80 ms, with apparent paired-pulse facilitation at 100 ms and a return to suppression at 200 ms.

Fig. 3.

The level of paired-pulse suppression increased progressively during a repetitive train of 10 consecutive paired pulses. A: 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. Note the decreased amplitude of the population spike from the test pulse during the train, indicating increased paired-pulse suppression. B: Bar graph 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. One-way ANOVA, $*p < 0.05$, ($n=7$). Data not shown for 25%.

Fig. 4.

Responses to single and repetitive paired pulses showed similar levels of paired-pulse suppression at low frequencies, but suppression progressively increased at higher frequencies of stimulation. A: Bar graph 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. For a particular stimulus intensity and interpulse interval, the amount of paired-pulse suppression was relatively independent of stimulation <1.0 Hz. B: Bar graph 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. Note that paired-pulse suppression increased considerably at ≥ 1.0 Hz stimulation. Intensity was 75% of maximum population-spike amplitude. One-way ANOVA, * $p < 0.05$, ** $p < 0.01$ ($n=7$).

Fig. 5.

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).

Fig. 6. 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 (including the population spike) became progressively larger during the stimulus train.

Fig. 7.

Population-spike amplitude in response to the conditioning stimulus increased at higher stimulation frequency. A: 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. B: Conditioning-pulse population-spike amplitudes increased during a 10-pulse repetitive train at high-frequency stimulation. The averaged 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, ($n=7$). Intensity of stimulation was 75% of maximum population spike amplitude.

Fig.8.

Diagram illustrating the synaptic connections from a typical *en passage* afferent pathway (such as the perforant path) to a population of principal neurons (such as dentate granule cells or hippocampal pyramidal cells), which would potentially affect measures of paired-pulse suppression. A: An afferent fiber forms an excitatory glutamatergic synapse to the dendrite of a principal neuron (left panel) and to a GABAergic inhibitory interneuron (feed-forward inhibition; right panel). B: An axon collateral of a principal neuron synapses on a GABAergic inhibitory interneuron (left panel), and a GABAergic inhibitory interneuron synapses onto a principal neuron (feedback inhibition; right panel). C: The multi-synaptic connections from an afferent pathway to a population of principal neurons. The blue neurons represent principal neurons (e.g., granule or pyramidal cells), while the red neurons represent GABAergic inhibitory interneurons, and green fibers represent an afferent pathway.