Disruption of Inhibition in Area CA1 of the Hippocampus in a Rat Model of Temporal Lobe Epilepsy

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INTRODUCTION

Recent studies have revealed an extensive loss of neurons in layer III of the entorhinal cortex (EC) in patients with temporal lobe epilepsy (TLE) (Du et al. 1993, 1995). Layer III neurons project to the CA1 region of the hippocampus via the temporoammonic (TA) pathway (Steward 1976; Steward and Scoville 1976; Witter 1993), and so the loss of layer III neurons would interrupt one of the important routes of cortical input to the hippocampus.

TA projections terminate as asymmetric (presumed excitatory) synapses on dendrites within the stratum lacunosum-moleculare of CA1 (Desmond et al. 1994; Witter et al. 1992). Physiological studies indicate that the synapses of the TA pathway are excitatory (Colbert and Levy 1992; Leung 1995; Stringer and Colbert 1994). At the same time, however, stimulation of the TA pathway produces powerful inhibition of CA1 pyramidal neurons (Empson and Heinemann 1995; Soltesz and Deschenes 1993). On this basis, it has been proposed that TA projections primarily activate inhibitory interneurons, so that the pathway is a substrate for projected inhibition (Lacaille and Schwartzkroin 1988; Soltesz and Jones 1995). If this is so, loss of the TA pathway in temporal lobe epilepsy could disrupt inhibitory mechanisms in CA1, contributing to the development of seizure-prone circuitry.

An opportunity to test several predictions of this hypothesis in an animal model has come from the discovery that the pattern of neuron loss in layer III of the EC seen in epileptic patients can be reproduced in experimental animals by injecting the indirect excitotoxin, aminoxyacetic acid (AOAA), into the EC (Du and Schwarz 1992). AOAA is termed an indirect excitotoxin because it appears to cause neuronal death by destroying the indirect excitotoxin, aminooxyacetic acid (AOAA). Inhibitory function in the hippocampus was assessed by evaluating the discharge of CA1 neurons in response to stimulation of afferent pathways in vivo. In control animals, stimulation of the temporoammonic pathway leads to heterosynaptic inhibition of population spikes generated by subsequent stimulation of the commissural projection to CA1. This heterosynaptic inhibition was substantially reduced in animals that had received AOAA injections 1 mo previously. Stimulation of the commissural projection also elicited multiple population spikes in CA1 in AOAA-injected animals, and homosynaptic inhibition in response to paired-pulse stimulation of the commissural projection was dramatically diminished. These results suggest a disruption of inhibitory function in CA1 in AOAA-injected animals. To determine whether the disruption of inhibition occurred selectively in CA1, we assessed paired-pulse inhibition in the dentate gyrus. Both homosynaptic inhibition generated by paired-pulse stimulation of the perforant path, and heterosynaptic inhibition produced by activation of the commissural projection to the dentate gyrus appeared largely comparable in AOAA-injected and control animals; thus abnormalities in inhibitory function following AOAA injections occurred relatively selectively in CA1. Electrolytic lesions of the EC did not cause the same loss of inhibition as seen in animals with AOAA injections, indicating that the loss of inhibition in CA1 is not due to the loss of excitatory driving of inhibitory interneurons. Also, electrolytic lesions of the EC in animals that had been injected previously with AOAA had little effect on the abnormal physiological responses in CA1, suggesting that most alterations in inhibition in CA1 are not due to circuit abnormalities within the EC. Comparisons of control and AOAA-injected animals in a hippocampal kindling paradigm revealed that the duration of afterdischarges elicited by high-frequency stimulation of CA3, and the number of stimulations required to elicit kindled seizures were comparable. Taken together, our results reveal that the selective loss of layer III neurons induced by AOAA disrupts inhibitory function in CA1, but this does not create a circuit that is more prone to at least one form of kindling.

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the side of the injection, whereas the layer II stellate neurons that project to the dentate gyrus are relatively spared (Du and Schwarcz 1992). As a result, the TA pathway is largely destroyed, whereas the temporodentate pathway (the perforant path) is preserved.

There are several predictions of the hypothesis that the loss of layer III neurons would disrupt inhibition in CA1, potentially producing a seizure-prone circuit. First, if inhibitory interneurons lose their excitatory drive, there might be a general increase in excitability of CA1 neurons, which would be reflected by a tendency to exhibit greater discharges in response to afferent activation. Second, such changes in excitability should be present in CA1 but not in other hippocampal subfields (since the TA pathway projects selectively to CA1) (Steward and Scoville 1976). Third, complete lesions of the EC should cause the same increases in excitability as AOAA-induced lesions of layer III neurons (in both cases, inhibitory interneurons would lose their excitatory drive). Finally, if the changes lead to a seizure-prone circuit, it should be relatively easier to trigger electrographic and behavioral seizures by stimulating hippocampal afferent pathways.

The present study tests these predictions. Extracellular recording techniques were used to assess responses to afferent stimulation in control animals and animals that had received AOAA injections 30–35 days previously. Experiments were carried out in CA1, which had lost its normal input from the TA pathway, and in the dentate gyrus, in which the projections from the EC were largely intact. Paired-pulse stimulation paradigms were used in which the first (conditioning) pulse produces inhibition that is reflected by decreases in the population spike generated by a second (test) pulse. This analysis revealed that CA1 neurons in AOAA-injected animals exhibited multiple spiking in response to afferent stimulation and substantially decreased paired-pulse inhibition.

To evaluate whether electrolytic lesions that destroyed the EC would produce comparable effects, similar analyses were carried out on animals that had received EC lesions 1 mo previously. These experiments revealed that electrographic lesions of the EC did not cause the same loss of inhibition as AOAA injections. To explore the possibility that the physiological anomalies induced by AOAA were localized within the EC itself, we evaluated the consequences of destroying the EC that had been previously injected with AOAA, and found that these secondary lesions had minimal effect on the hyperexcitability in CA1. To assess whether AOAA-induced disruption of inhibitory function made the hippocampus more prone to seizures, we used a kindling paradigm to evaluate the duration of afterdischarges elicited by high-frequency stimulation of CA3 and the number of stimulations required to elicit fully kindled seizures. These experiments revealed no differences between control and AOAA-injected animals, indicating that disruption of inhibitory processes within CA1 did not enhance seizure susceptibility in this hippocampal kindling paradigm.

Took together, these results indicate that local injections of AOAA into the EC disrupt inhibition in CA1, but this cannot be explained solely by the loss of projected inhibition mediated by the TA pathway. Instead, the abnormalities probably reflect an alteration in inhibitory function within the hippocampus itself. Nevertheless, the disruption of inhibition did not lead to a circuit in the hippocampus that is more prone to kindling. These results provide new insights into the physiological changes that occur following loss of layer III neurons, and provide clues about the possible role that such a selective lesion may play in circuit abnormalities in temporal lobe epilepsy.

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METHODS

Intraentorhinal injection procedure

Male Sprague-Dawley rats (200–300 g) were anesthetized with chloral hydrate (350 mg/kg ip) and positioned in a stereotaxic frame (Kopf, Tujunga, CA). AOAA (Sigma, St. Louis, MO) was dissolved in 0.1 M phosphate-buffered saline (PBS) at a concentration of 100 μg/μl of PBS, pH 7.4. AOAA was injected into the EC of one hemisphere using a 10-μl Hamilton microsyringe at the following stereotaxic coordinates: AP 1.2 mm behind lambda, ML 5.0 mm, with the nose bar set at 3.0 mm and the needle holder set at an angle of 20° posteriorly in the sagittal plane. A total of 0.75 μl of the AOAA solution was delivered over a period of 10 min in 0.25-μl aliquots at each of three dorsoventral levels (3, 4, and 5 mm deep from the cortical surface). Control rats received an injection of the vehicle solution (0.1 M PBS, pH 7.4). The behavior of all animals was observed for at least 4 h post injection. As described previously (Du and Schwarcz 1992), AOAA-injected animals exhibited evidence of limbic seizures (wet dog shakes, chewing, forelimb clonus, and rearing) starting at 2 h post-AOAA administration. None of the control animals exhibited seizures.

Unilateral lesions of the entorhinal cortex

Male Sprague-Dawley rats (200–270 g) were anesthetized with pentobarbital sodium (Nembutal; 50 mg/kg ip) and positioned in a stereotaxic frame, and the entorhinal area was unilaterally ablated as described previously (Loesche and Steward 1977). An electrode was placed in the entorhinal area at the following stereotaxic coordinates: 1.5 mm anterior to the transverse sinus; 3, 4, and 5 mm lateral to the midsagittal sinus, and 2, 4, and 6 mm below the cortical surface with the nose bar set at −2.0 mm and the electrode positioned at an angle of 10° away from the midline. One millimicre of current was passed at each electrode position for a total of 45 s.

Secondary lesions of the entorhinal cortex that had previously been injected with AOAA

For the experiments involving secondary lesions of the EC that had previously been injected with AOAA, male Sprague-Dawley rats (200–300 g) were injected with AOAA as described above. Thirty to 60 days following the injection, animals were anesthetized with urethan (1.2 g/kg ip) for an initial in vivo recording session to assess the hyperexcitability of CA1. Immediately following the recording session, the AOAA-injected EC was ablated as described above. Animals were given subcutaneous injections of 0.9% saline, and a second set of recordings was taken 10–14 h following the electrolytic lesion.

In vivo electrophysiology

A total of 27 animals (7 AOAA-injected, 8 control, 6 EC-lesioned, and 6 AOAA/EC-lesioned animals) was evaluated using in vivo electrophysiological procedures. The control data were pooled from two vehicle-injected and eight naive animals since their responses were virtually identical. Animals were evaluated 30–35 days following the unilateral intra-entorhinal injection of AOAA.

For the physiological experiments, animals were anesthetized with urethan (1.2 g/kg ip) and placed into a stereotaxic frame with the nose...
bar set at 0 mm. Stereotaxic coordinates and physiological stimulation paradigms were as described by Bekenstein et al. (1993) (see also Fig. 1). To activate the entorhinal projection to the hippocampus and dentate gyrus (the “EC pathway”), a bipolar stainless steel electrode (0.01 in. diam) was placed in the angular bundle on the side of the AOAA injection (AP = −8.1 mm from bregma, ML = 4.4 mm, DV = −2.0 mm). This electrode was angled at 20° posteriorly in the sagittal plane. To activate the commissural projection (the “Comm pathway”), a second bipolar stimulating electrode (SNEX 200, tip diameter 100 μm, Rhodes Medical Instruments) was placed in the CA3 region of the dorsal hippocampus, contralateral to the site of the AOAA injection (AP = −3.0 mm from bregma, ML = 3.5 mm, DV = −2.5 mm). A glass capillary microelectrode (2–6 MΩ resistance) filled with 2 M NaCl, and 5% Fast Green was placed in the dorsal hippocampus ipsilateral to the AOAA injection site [AP = −3.0 mm from bregma, ML = 2.0 mm, DV = −2.0 mm for CA1 and DV = −3.1 mm for dentate gyrus (DG)]. The final depths of the recording and stimulating electrodes were adjusted to produce evoked responses of maximal amplitude at minimum stimulus intensities.

Stimuli were applied through a constant voltage stimulus isolation unit controlled by a digital timer (Winston T-10). Data were collected through an amplifier (Biodyne) and in parallel to a computerized data acquisition system (pCLAMP, Axon Instruments, Foster City, CA) and a digital oscilloscope. Data were analyzed using Axograph (Axon Instruments) and Sigma Plot (Jandel). For each response, the amplitude of the population spike evoked by the stimulation was determined automatically by the software.

CA1 recordings and stimulation paradigms

To evaluate afferent-induced inhibition in the CA1 region, two paired-pulse stimulation paradigms were used. The first was a heterosynaptic paradigm, in which the TA pathway was activated, followed by the commissural pathway (EC/Comm protocol). Inhibition resulting from stimulation of the TA pathway is reflected by a decrease in the amplitude of the population spike generated by subsequent stimulation of the commissural pathway (see Fig. 3). The second was a homosynaptic paradigm, in which paired-pulse stimuli were delivered to the commissural pathway.

Input/output curves were established by varying the stimulus intensity from threshold to an intensity that evoked the maximal amplitude population spike for the first (conditioning) response. This produced maximal inhibition of the second (test) response. The interval between the conditioning and test pulse (C-T interval) was then varied from 20 to 1,000 ms to define the time course of the inhibition generated by the first pulse.

DG recordings and stimulation paradigms

To determine whether the AOAA treatment affects inhibitory processes in circuits in which layer III neurons are not involved, we

![Diagram](http://example.com/diagram.png)

**Fig. 1.** Schematic of pathways and stimulation paradigms. The cartoon illustrates the relevant circuitry within the CA1 region of the hippocampus and the dentate gyrus. Recording electrodes were advanced along the long axis of the pyramidal and granule cells. Stimulating electrodes were positioned in the entorhinal cortex ipsilateral to the recording electrode to activate the temporo-ammonic (TA) pathway, which projects to the distal dendritic zone of CA1, and the temporo-dentate pathway (perforant path). The TA pathway originates from pyramidal neurons in layer III [shown here as dashed lines because these are killed by aminooxyacetic acid (AOAA)]; the perforant pathway originates from stellate neurons in layer II. A 2nd stimulating electrode was positioned in the contralateral hippocampus to activate the commissural projection system, including the projections from CA3 to CA1, and the commissural projections to the dentate gyrus, which terminate in the inner molecular layer and activate granule cells and interneurons. CA1, CA1 region of the hippocampus; DG, dentate gyrus; EC, entorhinal cortex.
evaluated paired-pulse inhibition in the DG. Two paired-pulse stimulation paradigms were used. The first was a heterosynaptic paradigm, in which the commissural pathway to the DG was activated, followed by activation of the temporo-dentate projection (Comm/EC protocol). Again, inhibition resulting from activation of the commissural pathway is reflected by a decrease in the amplitude of the population spike generated by the temporo-dentate pathway (Douglas and Goddard 1975). The second was a homosynaptic paradigm in which paired stimuli were delivered to the temporo-dentate pathway (EC/EC protocol).

After all recordings were taken, both DG and CA1 recordings sites were marked by iontophoretic application of Fast Green from the recording electrode using -20 μA of bipolar current for 20 min, and the position of the stimulating electrodes was verified by placement of small electrolytic lesions. Animal body temperature was maintained by a heating pad throughout the experiment.

**Histological analysis**

Animals that were tested using acute neurophysiological techniques received an additional dose of urethan (0.5 g/kg ip) after the recording session and were then perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed and placed in 4% paraformaldehyde in 0.1 M PBS and then in 20% sucrose in 0.1 M PBS at 4°C. The rostral pole of the brain was sectioned coronally on a freezing microtome, and the caudal pole was sectioned horizontally. Serial 30-μm sections were taken, and every third section was collected on a gelatin-subbed slide and stained with thionin. Sections from AOAA-injected and vehicle-injected animals were evaluated by two independent individuals blinded with respect to the experimental protocol.

**Kindling procedure**

A separate group of animals was prepared to evaluate afterdischarge duration and development of kindling resulting from high-frequency stimulation of the CA3 region. One month following either AOAA (n = 8) or a vehicle (n = 7) injection into the EC, animals were anesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg ip) and placed in an AOAA injection (electrode placement: AP -3.6 mm from bregma, ML 4.9 mm, DV -5.0 mm); a ground electrode was placed in the frontal sinus. The electrodes were attached to the skull with jewelers’ screws and dental acrylate.

AOAA-injected and control animals were subjected to a rapid kindling acquisition and retest paradigm beginning 1 wk after electrode implantation (Lothman and Williamson 1994). For kindling acquisition, an alternate-day protocol was used that entailed delivering 12 stimulus trains (at 50 Hz, 10-s train of 1 ms, biphasic, 200-μA peak-to-peak) for 4 days with a stimulus-free day interspersed between each stimulation day. Therefore the complete kindling acquisition period consisted of a total of 7 days with four stimulation days alternating with three intervening stimulus-free days. Another 7 days following the kindling acquisition, a retest paradigm was used that consisted of 12 stimulations with the same stimulus trains used for kindling acquisition.

**SCORING OF KINDLING.** For each stimulus, animals were assigned a behavioral seizure score, based on a 5-point system (Racine 1972): 1 = wet dog shakes, 2 = chewing and nodding, 3 = unilateral clonus, 4 = rearing and forelimb clonus, and 5 = rearing and falling. The behavioral seizure score was assigned by an observer blinded to the experimental protocol.

Electrographic activity was recorded with a Grass 7D amplifier system connected to a computer-assisted system (TL-1 A/D interface, Axotape, Axon Instruments). Primary afterdischarge duration (ADD) was recorded and defined as rhythmic epileptiform activity occurring after the delivered stimulus of amplitude, and greater than or equal to twice baseline electroencephalographic (EEG) activity. Following the retest, animals received an anesthetic overdose and were perfused with 4% paraformaldehyde. Histological analysis was performed as described above.

**RESULTS**

As expected based on previous studies (Du and Schwarcz 1992), there was a conspicuous loss of layer III pyramidal neurons in animals that had received AOAA 30–35 days previously. A representative case is illustrated in Fig. 2. On the injected side, there were few neuronal cell bodies in the medial portion of layer III, and instead, the layer was filled with small cells that had the appearance of glial cells (Fig. 2, A and B). A separate group of animals was prepared to evaluate afterdischarge duration and development of kindling resulting from high-frequency stimulation of the CA3 region. One month following either AOAA (n = 8) or a vehicle (n = 7) injection into the EC, animals were anesthetized with ketamine (50 mg/kg) and xylazine (12.5 mg/kg ip) and placed in a stereotaxic frame with the nose bar set at +5.0 mm. Twisted, insulated bipolar stainless steel (0.01 in. diam) electrodes were implanted into the left ventral hippocampus, ipsilateral to the

![FIG. 2. Cell loss in the EC following AOAA injections. The photomicrographs illustrate Nissl-stained horizontal sections through the EC of a rat injected focally with 75 μg of AOAA 30 days earlier. A and C: the pattern of cell loss ipsilateral to the AOAA injection. B and D: the contralateral (control) side. II and III indicate the cell layers in the medial EC, and the arrows indicate the thickness of layer III. Note the absence of neurons in layer III on the side of the injection, and the presence of small cells (glia). Note also the reduced thickness of the layer on the injected side (D). DG, dentate gyrus; Sub, subiculum; PaS, parasubiculum; RF,rhinal fissure, which is the lateral boundary of the EC.](http://jn.physiology.org/)

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C). In addition to the decrease in neuron density in layer III, the overall thickness of layer III was reduced in comparison to control (compare Fig. 2, C and D). The contralateral side appeared normal in all animals (Fig. 2, B and D). In four animals, neuronal loss was largely restricted to the medial portion of the EC, as in the case illustrated in Fig. 2. In three other animals, there was also a loss of neurons in the lateral part of layer III of the EC, and in two of these animals there was also some loss of layer II neurons and subicular neurons. The neuron loss was more extensive in the ventral portions of the EC, whereas there was typically a greater degree of preservation of neurons in the layer in more dorsal regions.

**Afferent-induced inhibition in CA1**

In normal or vehicle-injected control animals, stimulation of the Comm pathway without prior activation of the EC elicited a field excitatory postsynaptic potential (EPSP) with a single prominent population spike (Fig. 3A, top trace). Stimulation of the EC elicited a small positive response with no apparent population spike (Fig. 3A, bottom trace, 1st response). When Comm stimulation was preceded by stimulation of the EC, the population spike evoked by stimulation of the commissural pathway was substantially reduced or eliminated (Fig. 3A, bottom trace). This is consistent with previous reports that activation of the TA pathway produces substantial inhibition (Colbert and Levy 1992; Empson and Heinemann 1995; LaCaille and Schwartzkroin 1988; Soltesz and Deschenes 1993).

The extent of inhibition of the population spike produced by prior activation of the commissural pathway varied as a function of the intensity of stimulation of the EC. Figure 3C illustrates the ratio of the population spike amplitude evoked without prior activation of the commissural pathway (unconditioned) versus population spike amplitude following prior activation of the commissural system (conditioned). The graph plots this unconditioned versus conditioned response ratio across a range of stimulus intensities in both control and AOAA-injected animals. In control animals, higher intensity stimulation produced greater inhibition. Using such plots, the extent of inhibition produced by EC stimulation could be compared in control animals and animals with the AOAA-induced lesions.

The responses elicited in CA1 by stimulation of the commissural pathway were highly abnormal in animals with AOAA-induced lesions (Fig. 3B). In the first place, multiple population spikes were seen, rather than the single spike seen in control animals. Moreover, paired-pulse inhibition was minimal. Comparison of intensity versus inhibition plots of control and AOAA-injected animals revealed that activation of the EC projection produced less inhibition across the entire range of stimulation intensities (Fig. 3C). This difference in the extent of inhibition was highly significant ($F = 5.23, P < 0.02$).

The extent of heterosynaptic inhibition in CA1 at different conditioning-test (C-T) intervals was determined by varying the C-T interval from 20 to 1,000 ms (Fig. 4). In control animals, heterosynaptic inhibition was seen at C-T intervals between 15 and 100 ms (Fig. 4C). In the example shown in Fig. 4A, the population spike was completely inhibited at a C-T interval of 20 ms. In contrast, in the animals with AOAA-

**FIG. 3.** Decreases in paired-pulse inhibition in CA1 as a result of AOAA-induced lesions. The figure illustrates the consequences of prior stimulation of the EC on population spikes generated by stimulation of the commissural (Comm) pathway in control and AOAA-injected animals. A and B: pairs of traces from a control and an AOAA-injected animal. In the top trace, a single excitatory postsynaptic potential (EPSP) and population spike are shown in response to stimulation of the Comm pathway; the bottom trace illustrates the response elicited following prior stimulation of the EC [15-ms interval between the conditioning and test pulse (C-T interval)]. Note the multiple population spikes and loss of inhibition in the AOAA-injected animal. Also note that the unconditioned response refers to stimulation of only the commissural pathway, while the conditioned response refers to commissural stimulation following activation of the TA pathway. C: ratios of population spikes (conditioned/unconditioned) at varying intensities of EC stimulation. Error bars represent SE. *, Comm pathway stimulation; $S$, EC pathway stimulation.

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induced lesions, heterosynaptic paired inhibition was substantially reduced. In the example shown in Fig. 4B, there was no apparent heterosynaptic inhibition even at a C-T interval of 20 ms, at which inhibition was prominent in control animals. An analysis of covariance of the values over the entire set of C-T intervals (Fig. 4C) revealed that the difference in the population spike ratios between the control and AOAA-injected animals was highly significant ($F = 22.9$, $P < 0.0001$).

The above results demonstrate that, as expected, the physiological effect of stimulation of the TA pathway (heterosynaptic inhibition) is substantially reduced in animals in which the cells of origin of this pathway have been destroyed by AOAA. The physiological responses induced by single-pulse stimulation of the commissural pathway were also highly abnormal, i.e., stimulation elicited multiple population spikes. To further explore this finding, we evaluated responses to paired-pulse stimulation of the commissural pathway.

**Paired-pulse activation of the commissural projection to CA1**

In control animals, paired-pulse activation of the commissural projection to CA1 (Comm/Comm) at high intensities (50–60 V) produced a field EPSP with a prominent population spike in response to the first stimulus. The second (test) stimulus produced a field EPSP with either no or a considerably reduced population spike (Fig. 5A). In this homosynaptic paradigm, AOAA-injected animals also exhibited paired-pulse inhibition at the 20-ms C-T interval (Fig. 5B). The inhibition is not the result of a larger population spike in response to the conditioning pulse in AOAA-injected animals (which would produce greater feedback inhibition) because the maximal population spike amplitude was comparable in AOAA and control animals (Fig. 5C). Nevertheless, evaluation of population spike amplitude across a range of stimulus intensities revealed larger population spikes in AOAA-injected animals at low stimulus intensities (Fig. 5C). These results suggest either enhanced excitability or diminished ongoing inhibition in CA1.

When the extent of inhibition at the 10-ms C-T interval was compared across a range of stimulus intensities, there was no significant difference between the ratios of population spikes elicited by conditioning and test responses in AOAA-injected versus control animals ($F = 1.853$, $P > 0.05$). Nevertheless, at all of the higher stimulus intensities (25 V and above), the population spike ratios were lower in control animals, suggesting a greater degree of inhibition.

Although inhibition did not differ significantly at the 10-ms C-T interval, varying the C-T interval revealed substantial differences in the duration of paired-pulse inhibition in AOAA-injected versus control animals (Fig. 6). In control animals, paired-pulse inhibition was seen at C-T intervals up to 100 ms. In AOAA-injected animals, inhibition was seen only at C-T intervals up to 40 ms. Moreover, at C-T intervals of 20–40 ms, there was less inhibition than in control animals. In fact, at C-T intervals longer than 80 ms, paired-pulse potentiation of the population spike was observed (Fig. 6C), and the amplitude of the second and third spike in the series of multiple spikes increased dramatically (see Fig. 6B, traces at C-T intervals of 200 and 400 ms). This paired-pulse potentiation at long C-T intervals was dramatically different from what was seen in control animals, in which paired-pulse potentiation was seen in some individual control animals (Fig. 6B, C-T interval of 200 ms), but was not evident in the group average of the control animals.

**Afferent-induced inhibition in the DG**

To determine whether the disruption of inhibition occurred selectively in CA1, we evaluated paired-pulse inhibition in the DG. Stimulation of the EC in control animals produced a robust field EPSP and a large population spike (Figs. 7 and 8). When paired-pulse stimuli were delivered at an intensity suf-
sufficient to generate a moderate sized population spike, the population spike elicited by the second stimulus was reduced or eliminated, reflecting paired-pulse inhibition (Fig. 7). In animals that had received an AOAA injection 30–35 days previously, the evoked responses in the DG generated by the EC stimulation did not appear abnormal (there were no multiple population spikes). At the same time, however, the average amplitude of the population spike was smaller across the range of stimulus intensities (Fig. 8C). This may reflect the damage to layer II neurons seen in some animals. Nevertheless, there was no statistically significant difference between AOAA-injected and control animals in the extent of paired-pulse inhibition (Fig. 8D).

These results indicate that there are minimal if any difference in inhibitory function in the dentate gyrus of AOAA-injected animals at the same time that inhibitory function in CA1 is highly abnormal.

**Relationship between AOAA-induced cell loss and hyperexcitability**

As noted above, the AOAA-injected group included four animals in which the loss of layer III neurons was limited to the medial EC, and three animals with larger lesions. To assess whether the degree of hyperexcitability was related to the extent of cell loss, we compared the two measures of excitability (multiple spiking and the loss of paired-pulse inhibition) across the range of stimulus intensities when measuring at a C-T interval of 20 ms (Fig. 7C). This difference was not apparent at longer C-T intervals, however (Figs. 9A and B), and there was no statistically significant difference between control and AOAA-injected animals across the range of C-T intervals.

It has been demonstrated that stimulation of the commissural projection to the DG produces strong inhibition (Steward et al. 1990). Accordingly, when EC stimulation was preceded by activation of the Comm pathway, the population spike generated by the EC stimulation was substantially inhibited (Fig. 8A). Interestingly, the extent of inhibition produced by stimulation of the commissural projection was less in AOAA-injected animals across the range of stimulus intensities when measuring at a C-T interval of 20 ms (Fig. 7C). This difference was not apparent at longer C-T intervals, however (Figs. 9A and B), and there was no statistically significant difference between control and AOAA-injected animals across the range of C-T intervals.

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inhibition) in animals exhibiting different degrees of cell loss. For this analysis, we subdivided the AOAA-injected animals into two groups, one of which was made up of the four animals that exhibited cell loss restricted to the medial portion of layer III, and the other of which included the three animals exhibiting more extensive cell loss (see above). All animals exhibited multiple population spikes in response to commissural stimulation whether the lesions were large or small (data not shown). Also, a comparison of the time course of paired-pulse inhibition revealed that both groups of AOAA-injected animals exhibited reduced inhibition at short C-T intervals (Fig. 10). Interestingly, however, animals with a smaller degree of cell loss did not exhibit the same anomalous paired-pulse potentiation at long C-T intervals as the animals with more extensive cell loss. These data suggest that the anomalous paired-pulse potentiation may be related to the larger lesions. This conclusion should be considered provisional, however, because the number of animals in the group with large lesions were small, and the variability in the measure was high.

![Diagram](image)

**Fig. 6.** Paired-pulse inhibition in CA1 at different C-T intervals. The figure illustrates the consequences of paired-pulse stimulation of the commissural (Comm) pathway in control and AOAA-injected animals. A: recordings from a control animal at different C-T intervals. The control response is the response to the conditioning pulse (C-T interval of 20 ms). B: recordings from an AOAA-injected animal. Note the multiple spiking. C: ratios of population spike amplitudes (test/conditioning) in control and AOAA-injected rats at various C-T intervals (plotted on a semi-logarithmic scale). Error bars represent SE.

**Fig. 7.** Heterosynaptic paired-pulse inhibition in the dentate gyrus. A: top trace shows response of the dentate granule cells following a single pulse to the EC in normal animals; the bottom trace shows inhibition of the population spike with preceding Comm stimulation. C-T interval is 15 ms. B: responses from an AOAA-injected animal are comparable to the control. Both stimulation pulses are at maximal stimulation intensity needed to inhibit the population spike. C: input/output curve showing pooled values of the ratio of population spikes (conditioned/unconditioned) at varying stimulation intensities of the Comm pathway stimulation. Error bars represent SE. *, Comm pathway stimulation; $, EC pathway stimulation.
Electrolytic lesions of the entorhinal cortex do not disrupt inhibitory function in CA1

If the loss of inhibitory function is related to the loss of TA projections, lesions of the EC should produce similar effects. To test this hypothesis, we evaluated whether electrolytic lesions of the EC caused the same disruption of inhibition within CA1 as AOAA injections. One month following a unilateral electrolytic lesion, we recorded from CA1 (ipsilateral to the lesion) and delivered paired stimuli to the commissural pathway. As illustrated in Fig. 11, the responses evoked by stimulation of the commissural pathway in animals with EC lesions were not noticeably different from control (there were no multiple population spikes). Moreover, there was no significant difference in paired-pulse inhibition at any C-T interval. Together, these results demonstrate that a more extensive, electrolytic destruction of the EC (which would also destroy the cells of origin of the TA pathway) does not lead to the same disruption of inhibition as the comparatively minor EC lesion produced by AOAA.

Destruction of the AOAA-injected EC does not eliminate altered inhibitory function in CA1

The fact that electrolytic lesions of the EC do not cause any of the anomalies in CA1 found in the AOAA-injected animals suggested two new hypotheses about the possible mode of action of AOAA. One possibility is that either the cell loss or the acute seizures caused by AOAA might create a circuit abnormality within the EC itself. Alternatively, AOAA-induced neuron loss or seizures might produce a circuit or functional abnormality in the hippocampus. One important prediction of the first hypothesis is that physical destruction of the AOAA-injected EC should eliminate the abnormal responses (multiple population spikes and loss of paired-pulse inhibition) within CA1. The second hypothesis would predict that the abnormalities found in the CA1 region of AOAA-injected animals should persist when the EC is destroyed.

To test these hypotheses, we documented the AOAA-induced abnormal physiological responses in a new group of animals. This group exhibited the multiple population spikes,
loss of paired-pulse inhibition, and anomalous paired-pulse potentiation in response to commissural stimulation described above. After documenting the responses, we electrolytically ablated the EC that had previously been injected with AOAA and reevaluated responses generated by paired-pulse stimulation of the commissural pathway 10–14 h later. As illustrated in Fig. 12, multiple population spikes and the loss of paired-pulse inhibition were still present after electrolytic lesions of the EC. Interestingly, however, the anomalous paired-pulse potentiation that had previously been seen at long C-T intervals was greatly reduced. Together, these results indicate that the anomalous paired-pulse potentiation, but not the other reflections of altered inhibitory function, are dependent on the presence of the EC that had previously been injected with AOAA.

Comparison of afterdischarge duration and the rate of kindling in control and AOAA-injected animals

Because inhibitory processes in CA1 are disrupted in animals that had received AOAA, especially the inhibition that is generated by activation of the commissural pathway from the contralateral CA3 region, the affected hippocampus might be more prone to epileptiform activity, particularly if triggered by stimulation of the CA3 region. Specifically, the loss of inhibition might be expected to increase the duration of the afterdischarge following high-frequency stimulation, and to enhance the rate of kindling as a result of the increased stimulation-induced epileptiform activity. To evaluate these questions, we compared afterdischarge duration elicited by high-frequency stimulation of CA3 and the rate of development of kindling in AOAA-injected and control animals. For these experiments, the stimulating electrodes used to induce kindling were positioned in the CA3 region of the hippocampus ipsilateral to the AOAA injection.

Histological analyses revealed that the animals used for the kindling experiment exhibited lesions that were comparable to those of the group assessed neurophysiologically (data not shown). However, there were no differences between AOAA-treated and control animals in the duration of the afterdischarges elicited by the first 12 stimulations (see Table 1). Moreover, the rate of kindling acquisition was indistinguishable in AOAA-treated and control animals (Table 1). There were also no differences in the duration of the afterdischarges.
across trials, or in the total number of stimulations required to achieve stage 5 seizures, and AOAA-treated animals did not differ from control animals in the retest paradigm (data not shown).

**DISCUSSION**

The present study was undertaken to evaluate whether selective loss of layer III neurons in the EC, which frequently occurs in temporal lobe epilepsy, disrupts inhibitory processes in the hippocampus. Assessment of inhibitory function using paired-pulse stimulation paradigms in the hippocampus in vivo revealed several physiological abnormalities that suggest altered inhibition. Specifically, animals with AOAA-induced EC lesions exhibited 1) multiple population spikes in response to afferent activation, 2) decreased paired-pulse inhibition in response to stimulation of the commissural projection system, and 3) enhanced paired-pulse potentiation of the population spike at long C-T intervals. Inhibitory processes in the DG appeared to be largely unaffected, suggesting that the alterations in inhibitory function occurred selectively in CA1. Despite the alterations in hippocampal inhibitory function, animals with unilateral AOAA-induced lesions did not exhibit longer afterdischarges or more rapid kindling in response to high-frequency stimulation of the affected hippocampus. These findings provide important clues about the possible physiological abnormalities that might result from the loss of layer III neurons.

Our experiments also revealed that complete electrolytic lesions of the EC did not cause any of the changes in inhibition that were seen following AOAA. Also, secondary lesions of the EC that had suffered AOAA-induced damage did not affect two key measures of disrupted inhibition (multiple population spikes and reduced paired-pulse inhibition), but did reduce the anomalous paired-pulse potentiation seen at long C-T intervals. These findings indicate that the principal indexes of disrupted inhibition in CA1 (multiple population spikes and reduced paired-pulse inhibition) are not due to aberrant circuitry within the AOAA-injected EC but may reflect abnormalities intrinsic to CA1. The implications of these findings are discussed below.

**Key indexes of altered inhibitory function in CA1**

Two of the three alterations seen after AOAA strongly suggest a disruption of inhibitory function in CA1: 1) multiple population spikes in response to afferent activation and 2) decrease in paired-pulse inhibition. Multiple population spikes are the expected consequence of any process that disrupts inhibition. In normal animals, single-pulse stimulation of the commissural pathway produces a monosynaptic EPSP in pyramidal cells, which is reflected in extracellular recordings as a “population EPSP.” At sufficiently high stimulus intensities, this EPSP generates action potentials that are reflected by a single population spike. Collaterals from pyramidal cells innervate inhibitory interneurons within the hippocampus, so that the massive discharge of the pyramidal cells produces feedback inhibition (Andersen et al. 1971). Afferents also contact interneurons directly, leading to feed-forward inhibition (Alger and Nicoll 1982). The inhibition produced by afferent stimulation is thought to play an important role in limiting repetitive cell firing, and to be the reason that only one population spike is seen regardless of the intensity of afferent stimulation. Thus when inhibition is intact, only one population spike is generated.
The strong inhibition generated by afferent stimulation also accounts for the decreases in amplitude of the population spike generated by the second of two stimuli in a paired-pulse stimulation paradigm. It is not possible to distinguish whether the inhibition generated by activation of a particular afferent system is mediated by feedback or feed-forward circuits or a combination of both when the stimulation strongly activates the principal neurons (as in the case of stimulation of the commissural pathway). In the case of activation of the TA pathway, however, the stimulation does not strongly activate the pyramidal neurons, so that it is likely that the inhibition is mediated primarily by feed-forward circuitry (see next section).

Consistent with this interpretation, manipulations that disrupt GABAergic inhibition cause multiple spiking and a loss of paired-pulse inhibition. For example, following blockade of GABAergic neurotransmission with bicuculline, a single stimulus generates multiple spikes, and paired-pulse inhibition is eliminated (Kapur et al. 1989; Sloviter and Brismar 1995; Thiels et al. 1994). Inhibitory function in CA1 is also disrupted following prolonged high-frequency stimulation of the perforant pathway, and an important reflection of this is the appearance of multiple population spikes in response to afferent stimulation and a loss of paired-pulse inhibition (Sloviter 1991). Thus the most likely explanation of the multiple population spikes and disruption of paired-pulse inhibition following AOAA injections is that local inhibitory processes in CA1 are disrupted.

Hypothesis of “projected inhibition”

One possible explanation of the alterations in inhibitory function in CA1 derives from the hypothesis that the TA pathway activates hippocampal interneurons and thus mediates projected inhibition. Several physiological studies have shown that the monosynaptic projection from the EC to CA1 produces excitatory responses in CA1 (Andersen et al. 1965; Doller and Weight 1982; Steward et al. 1973; Yeckel and Berger 1990, 1995). Nevertheless, activation of the pathway does not trigger population spikes in CA1 (Leung 1995). Other studies demonstrated that activation of the TA pathway produced only a weak excitatory response that is masked by strong inhibition (Buszaki et al. 1995; Colbert and Levy 1992; Empson and Heinemann 1995; Jones 1995; Soltesz and Deschenes 1993; Stringer and Colbert 1994). An inhibitory effect is also indicated by the fact that activation of the tempo-dentate pathway produces strong inhibition of cell firing in response to subsequent stimulation of other pathways (Colbert and Levy 1992; Empson and Heinemann 1995; Soltesz and Deschenes 1993). This latter effect was confirmed in the present experiments.

If the tempo-dentate pathway mediates projected inhibition, damage to the pathway would reduce the excitatory drive to hippocampal interneurons, producing disinhibition. The idea of a loss of excitatory drive of interneurons is similar to the “dormant basket cell hypothesis” proposed by Sloviter (1991) to account for the loss of inhibition following prolonged high-frequency stimulation of the perforant path. This idea was advanced because there was no detectable loss of interneurons in CA1 following prolonged perforant path stimulation despite the loss of inhibition. Hence it was proposed that the basket cells were present but “dormant.” If inhibitory interneurons in AOAA-injected animals are dormant because of a loss of afferent drive, the resulting disinhibition could account for two of the three response abnormalities discussed above, namely the multiple population spikes in response to afferent activation and the decrease in paired-pulse inhibition.

An important prediction of the disinhibition hypothesis is that physical destruction of the EC should produce the same disruption of inhibition in CA1 as seen following AOAA-induced degeneration of layer III neurons, because such lesions would also remove the excitatory drive provided by layer III neurons. However, our results demonstrated that electrolytic EC lesions do not lead to detectable alterations in inhibitory function in CA1.

Possible mechanisms at the circuit level

There are several possible explanations for our findings that are based on circuit abnormalities that might result from the lesions. Injections of AOAA into the EC result in the loss of layer III pyramidal neurons while layer II neurons (the cells of origin of the perforant pathway) are largely preserved (Du and Schwarcz 1992; Du et al. 1998). Thus the physiological abnormalities observed in the present study could be due to a disruption of the balance between the inhibition generated by the TA pathway and polysynaptic excitation of CA1 pyramidal cells (via the trisynaptic circuit).

A related possibility is that the tempo-dentate pathway might have two components, one of which projects to interneurons, and another that projects directly to pyramidal neurons in CA1. In this scenario, AOAA might preferentially kill those layer III neurons in the EC that project to the interneurons that mediate projected inhibition, and spare those neurons that give rise to direct excitatory projections to CA1 pyramidal cells. Again, this would disrupt the balance between projected inhibition and excitation of CA1 pyramidal cells. In either of the above cases, complete electrolytic lesions of the EC would not have the same effect as a selective lesion because both projected inhibition and polysynaptic excitation would be lost.

A third possibility is that the loss of layer III neurons in the EC could create a circuit abnormality within the EC itself.
These neurons probably participate importantly in intra-entorhinal circuitry, and their loss could disrupt overall functional activity. The loss of layer III neurons could also trigger synaptic reorganization within the EC, modifying circuit function. In this regard, it is of interest that physiological studies of AOAA-treated EC slices reveal important physiological abnormalities (Scharfman et al. 1998) (see AOAA model below). Also, multiple spiking and a loss of inhibition in CA1 is seen as early as 7 days after the AOAA injections (Denslow et al. 1995a,b), indicating that the alterations in inhibitory function can occur rather rapidly after the lesions.

An important prediction of all of these hypotheses is that secondary lesions of the EC that had previously been injected with AOAA should eliminate the physiological indexes of disrupted inhibition. This was not the case, however. Indeed, both multiple population spikes and diminished paired-pulse inhibition were still seen in the animals after secondary electrolytic lesions of the AOAA-injected EC. Only the late anomalous paired-pulse potentiation was disrupted by secondary lesions of the AOAA-injected EC. Thus anomalous paired-pulse potentiation could be related to circuit abnormalities within the EC, whereas the multiple spiking and loss of paired-pulse inhibition most likely reflects a process intrinsic to CA1.

Possible alterations in CA1

The explanation that is most consistent with the present data is that the multiple population spikes and loss of paired-pulse inhibition reflect a disruption of inhibitory mechanisms within CA1 itself. Possible mechanisms include 1) decreased release of an inhibitory neurotransmitter (see, for example, Gibbs et al. 1996), 2) altered receptor function, 3) a loss of inhibitory synapses, and 4) loss of a population of inhibitory interneurons. It remains to be established whether such changes do occur and if they are in fact triggered by intra-entorhinal AOAA injections. Such alterations could be a consequence of AOAA-induced acute seizures or deafferentation, and/or synaptic reorganization within CA1.

AOAA model

One of the strengths of the “AOAA model” is that hyperexcitability in CA1 can be examined with little direct pathology in the hippocampus (Du and Schwarz 1992). This is distinct from other models of epilepsy, such as those induced by kainic acid or prolonged hippocampal stimulation, in which there is extensive neuron death in the hippocampus, as well as the parahippocampal region, and other extrahippocampal areas. In those models it is therefore more difficult to identify the cause of altered function that makes the circuit more prone to seizures. The AOAA model involves a localized lesion (layer III of the EC) and a resulting hyperexcitability in a region (CA1) downstream from the affected site.

A disadvantage of the present model is that it is difficult to assess the dorso-ventral gradient of the alterations in inhibition because it is complicated to evaluate the ventral hippocampus in vivo. Therefore further studies are needed where hippocampal slices are taken from AOAA-injected animals along the dorsal-ventral extent of the hippocampus to determine whether the more ventral hippocampus also exhibits disrupted inhibition.

Although a circuit abnormality within the EC cannot account for the alterations in inhibitory function in CA1 (see above), this does not imply the absence of an important circuit abnormality within the AOAA-injected EC. Indeed, intracellular recordings from neurons in EC slices from animals that had received AOAA in vivo revealed that surviving neurons in superficial layers exhibited abnormal, prolonged repetitive EPSPs in response to white matter stimulation (Scharfman et al. 1998). Similar excitatory repetitive responses were seen in vivo following stimulation of the CA1/subicular border. The circuitry that mediates these responses has not been defined, and the neurons in superficial layers that exhibit abnormal repetitive activity are likely to be those that project to the dentate gyrus rather than to CA1 (Steward 1976). Nevertheless, similar excitatory activity may occur in other neuronal populations in the EC, including any surviving cells of origin of the TA pathway. Conceivably, abnormal physiological responses within the EC due to the loss of layer III neurons could play a key role in triggering seizure activity in temporal lobe epilepsy (see next section).

It is noteworthy that Scharfman et al. (1998) also described hyperexcitability in the EC contralateral to the AOAA-injected hemisphere, and we have also observed evidence of disrupted inhibition in CA1 contralateral to the AOAA injection (Denslow et al. 1995a). Although the cellular basis of these evoked responses was not completely characterized, those abnormalities resembled the multiple population spikes described in the present study. Given the commissural projections between the entorhinal cortices in the two hemispheres (Blackstad 1956), there may be a common mechanism underlying the abnormal activity in structures that receive synaptic input from the AOAA-injected EC.

Implications for seizure susceptibility

Given the disruption of inhibition in CA1, especially the inhibition generated by stimulation of the commissural pathway, the absence of detectable differences in afterdischarge duration or in the rate of development of kindled seizures in animals with AOAA-induced lesions was surprising. In many situations, disruption of inhibition is associated with an increase in seizure susceptibility. For example, prolonged electrical stimulation of the hippocampus creates an epilepsy-prone state in which spontaneous electrographic seizures continue after the stimulation has ceased (Lothman et al. 1995). Animals that exhibit such spontaneous seizures also exhibit physiological changes similar to those described here, including multiple population spikes in response to afferent stimulation, and diminished paired-pulse inhibition (Lothman et al. 1995; Mangai et al. 1995). At the same time, however, there is evidence that a disruption of GABAergic inhibition does not invariably lead to seizures. Apparently, inhibition must be disrupted to some threshold level for spontaneous seizures to occur (Kapur et al. 1989). The loss of inhibition, which results from the AOAA-induced lesion, may be below this threshold, or there may be mechanisms that compensate for the loss of inhibition and thus decrease susceptibility to seizures, at least in CA1. It is also important to note that the AOAA-induced lesions in the present experiments were unilateral. It remains to be seen whether there are differences in afterdischarge duration or in
the rate of development of kindled seizures when the kindling involves pathways more closely related to the affected EC.

Possible significance of loss of layer III neurons in temporal lobe epilepsy

Studies from patients with TLE provide evidence implicating the EC as the source of seizure generation in the hippocampal region. For example, EEG studies of patients with TLE suggest that the EC may be the site of seizure initiation (Spencer and Spencer 1994). Also, in patients with epilepsy resulting from tumors in the EC, seizures cease when the EC with the tumor is removed.

Our evaluations of afterdischarge threshold and propensity to kindling suggest that the loss of layer III neurons in the EC produced by focal AOAA injections is not sufficient to lead to a seizure-prone circuit in the hippocampus. Nevertheless, increased excitability in the EC (Scharffman et al. 1998) could serve as a focus for seizure initiation that could then propagate through hippocampal circuits in which inhibition was diminished. Thus, although loss of layer III neurons is not sufficient to promote kindled seizures in the hippocampus, it may still be an important component in the propagation of seizure activity generated in the EC. In any case, our results suggest that a loss of layer III neurons such as occurs in TLE does have important consequences on hippocampal function. Because these changes appear to be intrinsic to the hippocampus, it should be possible to test for their presence in tissue that has been surgically removed for seizure management.

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