Electrophysiological Evidence Using Focal Flash Photolysis of Caged Glutamate that CA1 Pyramidal Cells Receive Excitatory Synaptic Input from the Subiculum

Running title: Subicular-CA1 circuits

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ABSTRACT

The hippocampus sends efferent fibers to the subiculum, which projects to the entorinal cortex. Previous studies suggest that the hippocampal CA1 area may receive a projection back from the subiculum. This hypothesis was tested using whole-cell recording from CA1 pyramidal cells while subicular neurons were selectively stimulated with focal flash photolysis of caged glutamate, which avoids stimulation of fibers of passage. Control experiments showed that focal flash stimulations caused direct glutamate-mediated depolarizations and bursts of action potentials in the recorded CA1 pyramidal cells, but only when the stimulation targeted the somatodendritic regions of a neuron, not the axons. To block GABA_\text{A}-mediated inhibition and isolate local excitatory circuits, bicuculline was applied to minislices containing only the isolated CA1 area and the subiculum. Of 24 CA1 pyramidal cells, 25% (6 of 24) consistently generated repetitive excitatory postsynaptic currents (EPSCs) in response to flash stimulation in the subiculum. The responsive neurons were located 200–500 \mu m from the distal end of CA1 and 400–1100 \mu m from the stimulation sites in subiculum, suggesting excitatory synaptic projections from the subicular neurons to CA1 pyramidal cells. This study provides new electrophysiological evidence that CA1 pyramidal cells receive excitatory synaptic input from the subiculum. Thus, a reciprocal excitatory synaptic circuit connects the subiculum and the CA1 area in the normal adult rat.

Key words: neuronal circuitry; whole-cell recording; EPSC; backward projection; hippocampus
INTRODUCTION

The hippocampus receives input from the entorhinal cortex (EC), and sends output back to EC via the subiculum, a transition region between the hippocampus and EC where the majority of hippocampal efferents terminate (for review, see O’Mara et al, 2001). The information flow within the classical EC-hippocampus-EC circuit is generally considered to be unidirectional (i.e., from EC to the dentate to Cornu Ammonis (CA) to subiculum, and back to EC), and the projection from hippocampal CA1 to the subiculum has been well documented (Amaral and Witter, 1989; Amaral et al, 1991). However, several studies have provided evidence for a bidirectional connection between CA1 and the subiculum. Using retrograde tracer techniques with horseradish peroxidase (HRP), neurons in rabbit subiculum were consistently labeled when HRP was injected in the CA1 area (Berger et al., 1980). Another study, using the anterograde neuronal tracer phaseolus vulgaris leucoagglutinin (PHA-L), observed subicular projections to the hippocampal CA1 area (Kohler, 1985) when PHA-L was injected into the rat subiculum. More recent research has provided further evidence for this hypothetical subicular-CA1 projection. One study has reported that nitric oxide-containing pyramidal cells in the subiculum might innervate the adjacent CA1 area (Seress et al., 2002). Another study using extracellular recordings showed that “EPSP-like” field potentials could be evoked in the CA1 area when the subiculum was electrically stimulated (Commins et al., 2002). Harris and Stewart (2001) used intracellular staining with Neurobiotin and provided direct anatomical evidence that the axons of subicular pyramidal cells project to the CA1 apical dendritic region, and epileptiform events that originated in subiculum appeared to
propagate into CA1. These studies support the hypothesis of a backward projection from the subiculum to CA1, but more direct physiological evidence for a subicular-CA1 projection has been lacking, and the functional nature of this hypothetical circuit is unknown. To address this issue, we used the approach of focal flash photolysis of caged glutamate (Callaway and Katz, 1993; Dalva and Katz, 1994; Wieboldt et al., 1994) to excite a small group of subicular neurons, independent of fibers of passage, while recording the whole-cell synaptic responses of CA1 pyramidal cells. We postulated that if CA1 pyramidal cells receive excitatory synaptic inputs from subicular neurons, focal activation of subicular neurons by flash photolysis of caged glutamate would be expected to evoke EPSCs in CA1 pyramidal cells. We found that glutamate-mediated excitation of small groups of subicular neurons caused robust excitatory postsynaptic currents (EPSCs) in CA1 pyramidal cells, indicating that CA1 pyramidal cells receive excitatory synaptic input from the subiculum, thus demonstrating the presence of reciprocal hippocampal-subicular circuits. Hence, these data provide new electrophysiological evidence that CA1 receives an excitatory backward projection from the subiculum.

**METHODS**

*Slice preparation*

Male Sprague-Dawley rats (Harlan) of 3-4 months were euthanized with halothane and decapitated. Their brains were dissected out and placed in partially frozen oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 26 NaHCO3, 1.4 NaH2PO4, 1.3 CaCl2, 1.3 MgSO4 and 11 glucose. Transverse
hippocampal slices (i.e., parallel to the base of the brain, 300 µm thick) were prepared with a vibroslicer (Lancer series 1000, Vibratome, St. Louis, MO). The CA3/CA2 areas of the slices were isolated by a knife cut (see Fig. 3A) to prevent the propagation of potential spontaneous burst activity in CA3/CA2 that could develop in bicuculline. The isolated slices were then submerged in a storage chamber containing oxygenated ACSF at 30 °C for at least 2 h to recover.

**Experimental procedure and data acquisition.**

During recording, the slices were transferred into a submerged chamber perfused with oxygenated ACSF. All recordings were conducted at room temperature and in the presence of the GABA<sub>A</sub> receptor antagonist bicuculline methiodide (Sigma, 30 µM) to suppress inhibitory synaptic transmission. Whole-cell patch-clamp recordings were performed with glass pipettes (tip diameter of 1-3 µm, resistances of 2-5 MΩ when filled with K-gluconate based internal solution), which were pulled from borosilicate glass capillaries (OD 1.65 mm, ID 1.2 mm, Garner Glass, Claremont, CA), using a P-87 Flaming-Brown puller (Sutter Instruments, Novato, CA). Patch pipettes were filled with conventional intracellular solution composed of (in mM): 130 K-gluconate, 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 2 ATP and 5 biocytin. The pH was adjusted to 7.2 by 5 M KOH. Signals were amplified with an Axopatch 1D amplifier (Axon Instruments, Foster city, CA), low-pass filtered at 2 kHz and sampled at 10 kHz, and recorded online with pClamp 8.0 software (Clampex, Axon Instruments, Foster city, CA) through a digitizer (Digidata-1320A, Axon Instruments, Foster city, CA). A 5-mV, 30-ms hyperpolarizing voltage command was used to estimate the series resistance of the whole-
cell configuration and the input resistance, which was $10.5 \pm 0.7$ and $167 \pm 24 \, \text{M}\Omega$, respectively. Series resistance was uncompensated and monitored during each experiment. Data without significant change in the series resistance during the experiment were accepted for analyses.

**Flash photolysis of caged glutamate**

A xenon lamp (Chadwick-Helmuth, El Monte, CA) was used to produce the UV flash for photolysis of caged glutamate. The flash was then transmitted through an epifluorescence attachment to a microscope (Optiphot, Nikon) that was inverted, and the light beam was focused by a high-numerical aperture, oil-immersion objective ($\times 40$, Nikon). The intensity and duration of each flash was 100–200 watt-sec and 0.5 ms, which was determined by a Strobex power supply (model 238, Chadwick-Helmuth, El Monte, CA). Repeated stimulations were given once every 20 s, using an electronic timer (Winston Electronics). A monochrome charge-coupled device (CCD) camera (Cohu, San Diego, CA) was employed to view the slices and a HeNe laser (Oriel instruments, CT) was used to indicate the location of the focal flash stimulation. Photostimulations were aimed at different sites in the subiculum (see Fig. 3A). Caged glutamate was purchased from Molecular Probes, Inc. (Eugene, OR).

**DIRECT NEURONAL EXCITATION.** In order to validate the approach of focal flash photolysis of caged glutamate for selective stimulation of neurons independent of fibers of passage, we first tested the responses of CA1 neurons ($n = 4$ neurons from 4 slices) to different types of control stimulations (an actual light flash versus sham flash with and without shutter closed; and in the presence and absence of caged glutamate). As
expected, when caged glutamate was absent, focal flash stimulation alone at this intensity (100–200 watt-sec, 0.5 ms) failed to excite the recorded CA1 pyramid cell when aimed at the soma (Fig. 1A, arrow). After caged glutamate (250 µM) was bath applied to the preparation, the same flash stimulation at the same neuron caused direct depolarization and a burst of action potentials (Fig. 1B). At this time, when the shutter was closed to block the pathway of the light flash (i.e., sham flash), stimulation evoked no response (Fig. 1C), just as with the absence of caged glutamate. These data show that both the presence of caged glutamate in the bath and a light flash of adequate strength were required, but neither of them alone was able to excite a neuron, which validates the rationale of the experimental design to use focal flash photolysis of caged glutamate to activate neurons. The effectiveness of caged glutamate was tested by somatic flash stimulation at the end of each experiment. Sometimes the flashes at the soma failed to generate an obvious depolarization, possibly due to the limit of UV transmission into the slice. Only the neurons that displayed a direct depolarization and/or action potentials in response to somatic flash stimulation were included for analyses. With the stimulus intensity (100–200 watt-sec, 0.5 ms) and caged glutamate concentration (250 µM) used in the present study, an estimated effective spatial resolution of flash stimulation in the CA1 area was 150-200 µm (based on the appearance of inward currents in the recorded neurons induced by uncaging of glutamate in the presence of 2 µM TTX, data not shown), which is larger than in dentate gyrus (i.e., ~100 µm, Wuarin and Dudek, 2001), probably because CA1 pyramidal cells have more extensive dendrites than dentate granule cells.
ANATOMIC SPECIFICITY OF EXCITATION. Another important prerequisite for using the approach of focal flash photolysis of caged glutamate to study synaptic circuits is that glutamate excite the somadendritic region of neurons selectively (i.e., versus axons-of-passage or axon terminals). To test this assumption, flash stimulations aimed at the somata of CA1 pyramidal cells evoked a direct depolarization with a burst of action potentials (Fig. 2; n=6 neurons from 5 slices). A similar result was obtained when flash stimulation was directed at the apical dendritic area (stratum radiatum) or basal dendritic area (stratum oriens), but not when the presynaptic axons (stratum radiatum, ~400 µm apart) were stimulated via focal flash photolysis of caged glutamate. These data confirm that glutamate only excites the somato-dendritic regions of CA1 neurons but not their presynaptic axons, similar to its effects on CA3 pyramidal cells (Christian and Dudek, 1988a) and dentate granule cells (Wuarin and Dudek, 1996). This result is generally consistent with the view that functional glutamate receptors only exist at neuronal somata and dendrites but not axons of hippocampal neurons.

RESULTS

To examine the hypothetical CA1 inputs from subiculum, we recorded CA1 pyramidal cells that were relatively close to the subiculum (i.e., 200-500 µm from the distal end of CA1) and aimed flash stimulations in the subiculum at sites that were 150-600 µm from the distal end of CA1 (350–1100 µm from the recorded neurons; Fig. 3A). Of 24 CA1 pyramidal cells (from 21 slices), 25% of them (6 neurons, from 6 slices) responded with repetitive EPSCs to flash stimulation in the subiculum (Fig. 3B). Three to
four sites in the subiculum were stimulated at a distance of 150–200 µm from each other. In 5 of the 6 responding neurons, two or three subicular sites were effective at evoking EPSCs. The furthest subicular stimulation site that evoked EPSCs was approximately 600 µm from the distal end of CA1 and 1,100 µm from the recorded CA1 pyramidal cell. In all 6 CA1 pyramidal cells that responded with EPSCs to subicular flash stimulation, the responses were more robust to stimulation at sites relatively far from than closer to the distal end of CA1. The most robust and consistent responses occurred when stimulation was directed at subicular sites about 400-450 µm from the distal end of CA1. Figure 3 shows an example of the responses of a CA1 pyramidal cell to flash stimulation at a subicular site approximately 400 µm from the CA1 distal end and 900 µm away from the recorded neuron. Repeated flash stimulations consistently evoked repetitive EPSCs lasting for a few hundred milliseconds (Fig. 3B), which was consistent with the duration of glutamate excitation of presynaptic neurons under these experimental conditions (Figs. 1 and 2). The EPSCs were not present when the shutter of the light was closed (sham flash, Fig. 3C), which further confirmed that the EPSCs were evoked by photostimulation of subicular neurons.

Because only postsynaptic neurons (i.e., CA1 pyramidal cells) were recorded in this study, it is impossible to measure the actual latency from the activation of subicular neurons (i.e., presynaptic neurons) to the appearance of EPSCs in CA1 pyramidal cells, which would be useful to differentiate monosynaptic vs. polysynaptic responses (Miles and Wong, 1986; 1987). However, we have indirect evidence based on analyzing the latency of the first action potential and the latency of the first EPSC in response to the focal uncaging of glutamate. The duration from the beginning of the flash stimulation to
the onset of the first action potential in CA1 pyramidal cells ranged from 8 to 30 ms (mean=15.2 ± 1.5 ms, n=17). The latency from flash stimulation in subicular sites to the beginning of the first EPSC in CA1 pyramidal cells ranged from 10 to 33 ms (mean=18.2 ± 2 ms, n=13 effective subicular sites from 6 slices). The difference between the two mean latencies is ~ 3 ms, which is similar to the latency of monosynaptic CA3-CA1 synapses (3.4 ± 1.2 ms, Sayer et al, 1990). Thus, the relatively short latency difference between the first action potential and the first EPSC in response to flash stimulations strongly suggests that these EPSCs were not mediated by polysynaptic intervening connections between the subiculum and CA1 (i.e., subiculum→ EC→ hippocampus, see Discussion). Rather, it favors a direct connection from the subiculum to CA1.

DISCUSSION

These experiments showed that CA1 pyramidal cells were depolarized when glutamate was uncaged at the dendritic and somatic regions, but axonal stimulation did not excite the neurons. Focal glutamate stimulation of subicular neurons led to EPSCs of CA1 pyramidal cells. These data provide new and more direct functional evidence demonstrating that CA1 pyramidal cells receive excitatory synaptic inputs from its output (i.e., the subiculum), thus forming a reciprocal circuit between the hippocampal CA1 area and the subiculum.
Focal flash photolysis of caged glutamate to detect synaptic circuits: technical considerations

The technique of focal flash photolysis of caged glutamate was chosen to test the hypothesis of a subiculum-to-CA1 synaptic circuit because of the critical concern regarding inadvertent activation of fibers-of-passage. An important feature of this approach over the traditional method of electrical stimulation is that the glutamate released during focal flash stimulation does not activate axons-of-passage, whereas electrical stimulation unavoidably does stimulate nearby axons (Fig. 2, also see Christian and Dudek, 1988a,b; Wuarin and Dudek, 1996, 2001). Thus, this experimental approach excluded the possibility of excitation of axons that pass through the subiculum en route to CA1. Therefore, the EPSCs evoked in CA1 pyramidal cells by subicular photostimulation are most likely due to excitation of those subicular neurons that project excitatory synaptic outputs to CA1 pyramidal cells. The most direct approach to demonstrate the presence of such a projection would be to perform dual whole-cell recordings from subicular and CA1 neurons. However, previous studies have shown that the probability of detecting neuronal connectivity is very low, even in well established intrahippocampal projections such as the CA3-to-CA1 Schaffer collateral projection (Bolshakov and Siegelbaum, 1995; Sayer et al., 1990) or the local CA3-to-CA3 recurrent excitatory circuits (MacVicar and Dudek, 1980; Miles and Wong, 1986; 1987). For a particular level of synaptic connectivity, the approach of using focal flash photolysis of caged glutamate would be expected to yield a higher detection rate, because each flash stimulus with caged glutamate excites a population of neurons, but one that is spatially
circumscribed. In the present study, 25% of the recorded CA1 pyramidal cells responded with EPSCs to focal photostimulation in the subiculum, which strongly supports the hypothesis that CA1 pyramidal cells receive excitatory synaptic input from subicular neurons. However, the quantitative properties of the subicular-CA1 synaptic circuits are difficult to determine, because each flash would excite an unknown number of subicular neurons and several subicular sites were stimulated per recorded CA1 pyramidal cell, so tens to hundreds of neurons might be excited in each experiment. Therefore, although the approach of using flash photolysis of caged glutamate has the advantage of a high experimental efficiency for detection of synaptic circuits while remaining a comparatively direct test, the proportion of neuronal pairs with synaptic connections can only be estimated and comparisons across brain regions are necessarily only relative.

*Monosynaptic vs. polysynaptic connections*

The present study demonstrates that EPSCs were evoked in CA1 pyramidal cells by activating a small group of subicular neurons using focal flash photolysis of caged glutamate. The most parsimonious interpretation of these data is that the subiculum makes direct excitatory connections with CA1 pyramidal cells, but other explanations are possible. For instance, previous studies have reported anatomical evidence that EC projects to hippocampal CA3 and CA1 via the perforant path (Naber et al., 2001; Tamamaki and Nojyo, 1995). Therefore, excitation of subicular neurons might propagate through long loops via EC to eventually excite CA1 pyramidal cells. Possible examples of such circuits include: (1) subiculum→EC→perforant path→CA1; (2) subiculum→EC→perforant path→CA3→CA1; or (3) subiculum→EC→perforant
path → DG → CA3 → CA1. However, the probability of the presence of such a polysynaptic connection in slice experiments is extremely low because these long-loop circuits were most likely cut during slice preparation (particularly in slices of 300 µm thickness with CA1/subiculum isolated from CA3 (see Fig. 3A)). Moreover, a relatively short latency difference between the first action potential and the first EPSC in response to flash stimulations in this study favors a monosynaptic versus polysynaptic mechanism. A more likely source for polysynaptic EPSCs would be local recurrent connections within the subiculum or CA1, such as: (1) stimulated subicular neurons → other subicular neurons → recorded CA1 pyramidal cell; (2) stimulated subicular neurons → other CA1 pyramidal cells → recorded CA1 pyramidal cell; or (3) stimulated subicular neurons → other subicular neurons → other CA1 pyramidal cells → recorded CA1 pyramidal cell. None of these scenarios, however, would change the basic conclusion that the subiculum makes direct excitatory connections with CA1.

Functional implications of reciprocal CA1-subiculum circuits

While the EC-hippocampus-EC loop has generally been regarded as unidirectional (i.e., from EC to the dentate gyrus to Ammon’s horn, to subiculum and back to EC; Amaral and Witter, 1989; Amaral et al, 1991), anatomical evidence for bidirectional connections between the EC and subiculum/CA1 (Naber et al., 2001; Tamamaki and Nojyo, 1995; Witter et al., 1989), and between the subiculum and pre- and parasubiculum (Kohler, 1985) has been observed. When combined with previous research (Berger et al., 1980; Commins et al., 2002; Harris and Stewart, 2001; Kohler,
1985; Seress et al., 2002), the present study has provided new electrophysiological
evidence that subicular neurons synaptically excite CA1 pyramidal cells, thus supporting
the hypothesis that a bidirectional circuit between CA1 and subiculum is present. A
question remains, however, concerning the functional significance of these circuits. The
subiculum-CA1 excitatory synaptic circuits may serve as a positive feedback mechanism
to set the gain of the hippocampal output. In addition, given the presence of many
bursting pacemaker neurons in the subiculum (Staff et al., 2000; Stewart and Wong,
1993; Taube, 1993), which are probably interconnected with other subicular neurons
(Harris et al., 2001; Harris and Stewart., 2001), the subiculum has the capacity of
generating rhythmic epileptiform activity. Thus, the subicular-CA1 circuit may serve to
synchronize the activity between the two regions (Harris and Stewart, 2001). Further
experiments are needed to explore this issue.
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Figure legends

Fig. 1. Effectiveness of focal flash photolysis of caged glutamate. A: lack of response of a CA1 pyramidal cell to photostimulation in medium without caged glutamate. Flash stimulation (50-100 mJ, 0.5 ms) alone failed to evoke any response. B: when caged glutamate (250 µM) was added to the medium, the same photostimulus evoked a burst of action potentials in the recorded neuron. C: when the shutter was closed to block the light stimulus, no response was evoked.

Fig. 2 Anatomic specificity of the excitation caused by focal flash photolysis of caged glutamate. Left panel shows the responses of a CA1 pyramidal cell to flash stimulation at the corresponding regions (arrows) of the neuron indicated in the right (1-4). A burst of action potentials was evoked when flash stimulation was directed at the basal dendritic (stratum oriens, 1), somatic (2) or apical dendritic region (stratum radiatum, 3) of this neuron. However, the same photostimulation evoked no response when presented to the region of the presynaptic axons (stratum radiatum, ~ 400 µm to the side of the apical dendrites, 4). Parallel lines in 4 diagrammatically represent presynaptic axons.

Fig. 3. Evidence that CA1 pyramidal cells receive excitatory synaptic input from the subiculum. A: diagram showing the experimental approach in a hippocampal slice containing the subiculum, which was isolated from the CA3/CA2 region by a knife cut (dash line). Whole-cell recordings were performed in CA1 pyramidal cells (triangles) and focal flash stimulations were aimed in different sites in the subiculum (solid circles). B: in a CA1 pyramidal cell, repetitive EPSCs were consistently evoked in response to
repeated flash stimulations (arrow) at a spot in the subiculum approximately 400 μm from the distal end of CA1 layer and 900 μm from the recorded CA1 pyramidal cell. The responses to five consecutive flash stimulations are shown in B. C: the responses were blocked by the closure of the shutter to prevent the transmission of the light stimulus (i.e., sham flash).
Figure 1.
Figure 2.
Figure 3.