Presubicular and parasubicular cortical neurons of the rat: functional separation of deep and superficial neurons *in vitro*

Makoto Funahashi and Mark Stewart

Department of Physiology, State University of New York Health Science Centre at Brooklyn, 450 Clarkson Avenue, Brooklyn, New York 11203, USA

1. The presubiculum and parasubiculum are retrohippocampal structures bordered by the subiculum and medial entorhinal cortex. Deep layer (IV–VI) neurons from this region exhibit stable synaptically triggered burst behaviour which distinguishes them from superficial layer (I–III) cells. This functional separation was examined with intracellular and field potential recordings from horizontal slices of rat brain. Neurobiotin labelling and rapid Golgi techniques were used to obtain anatomical evidence of axonal trajectories.

2. Extracellular stimulation of the subiculum, deep medial entorhinal cortex or superficial pre- or parasubiculum caused, in deep layer cells only, a short latency burst discharge which could be followed by one or more after-discharges. Bursts appeared after repetitive stimulation and were stable for the life of the slice. Each event was supported by giant excitatory postsynaptic potentials (EPSPs). Events were similar whether they were evoked in horizontal slices or slices cut perpendicular to the horizontal plane.

3. Bath application of the NMDA receptor antagonist 3-[2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP; 5 μM) elevated the threshold for evoking the giant EPSP. Higher concentrations (10–15 μM) reduced the amplitude and duration of the giant EPSP. Bath application of the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5 μM) eliminated the evoked EPSP.

4. In intact slices, superficial layer neurons of pre- and parasubiculum could exhibit EPSPs coincident with bursts recorded in the deep layers. However, in isolated subsections of horizontal slices or in 'vertical slices', both of which contained only pre- and/or parasubiculum, evoked or picrotoxin-induced bursts occurred only in deep layer cells. Superficial layer cells in these subsections showed no response to deep layer events.

5. Antidromic population spikes confirmed projections from superficial cell layers of pre- and parasubiculum down to their deep cell layers. Reciprocal antidromic responses were absent.

6. Axons of superficial layer stellate and pyramidal cells had horizontal collaterals and at least one ascending and one descending collateral. Branches of the descending collaterals were given off in layer V and some axons were found to reach the angular bundle. Axons of deep layer stellate and pyramidal cells also had horizontal collaterals and descending collaterals which could be traced to the angular bundle. One ascending axon collateral was found among the thirty-one deep layer cells examined morphologically.

7. We conclude that the deep layer cells of the presubiculum and parasubiculum are richly interconnected with excitatory synapses. These interconnections can generate giant excitatory synaptic potentials that support the bursting behaviour exhibited by these neurons. Any of the excitatory inputs to deep layer cells can trigger the population bursts and specific inputs from entorhinal cortex produce the after-discharges. Further, connections between superficial and deep layer cells appear to be almost exclusively in the direction of superficial to deep. The absence of significant ascending input can account for the functional separation of superficial and deep layer neurons of presubiculum and parasubiculum.

The hippocampal formation consists of six-layered periallocortical regions (the entorhinal cortex, parasubiculum, presubiculum and postsubiculum) and three-layered allocortical regions (the subiculum, Ammon's horn and dentate gyrus). The connections among these regions have been studied anatomically and electrophysiologically. One of the best known circuits within the hippocampal formation is the trisynaptic pathway. Axons of superficial layer entorhinal neurons perforate the white matter bundle that separates the deepest entorhinal layer and the deepest subicular layer. These axons continue through the subiculum itself to reach the apical dendrites of hippocampal neurons (reviewed e.g. in Witter, Groenewegen, Lopes da Silva & Lohman, 1989; see also Jones, 1993). The perforant projection from layer II of entorhinal cortex to the apical dendrites of the dentate granule cells is the first synapse in the so-called trisynaptic pathway. A complete loop of excitatory connections is made with the additional synapes made by CA1 pyramidal cells onto subicular pyramids and a projection from the subiculum to the entorhinal cortex. The projection from the subiculum back to the entorhinal cortex can again perforate the white matter to reach deep layer neurons of the entorhinal cortex directly. This circuit, which includes the entorhinal cortex and the hippocampal subfields, has been extensively studied.

The presubiculum and parasubiculum are situated between the subiculum and the medial entorhinal cortex. In the circuit just described, however, these structures are completely bypassed by the perforant projections in and out of the entorhinal cortex. Anatomically, interconnections of presubicular and parasubicular neurons with entorhinal and subicular neurons have been described (e.g. Kohler, 1985; van Groen & Wyss, 1990). Functionally, these connections have received little attention.

Extracellular single-cell recordings taken from behaving animals have been used effectively to identify neurons as participants in particular neural circuits based on their firing correlates. Such single-unit studies of retrohippocampal neurons have shown that presubicular, parasubicular and postsubicular neurons have sharply defined behavioural correlates, which suggests that they are involved in navigational circuitry along with other hippocampal formation neurons. Head-direction cells have been found in deep layers of the presubiculum and postsubiculum (Ranck, 1985; Taube, Muller & Ranck, 1990). Place cells have been recorded in the parasubiculum (Taube, 1995).

Deep layer neurons of the presubicular, parasubicular and entorhinal cortices have also been shown to participate in population burst (sharp wave) generation *in vivo* (Chrobak & Buzsáki, 1994, 1996). Superficial layer cells of the same region, on the other hand, have shown different firing correlates. Many superficial layer retrohippocampal cells fired in strict relation to the EEG theta rhythm (e.g. Alonso & García-Austt, 1987; Stewart, Quirk, Barry & Fox, 1992; Chrobak & Buzsáki, 1994, 1996).

We found that stellate and pyramidal cells of the deep layers of the presubiculum and parasubiculum exhibited burst responses to subicular or deep entorhinal stimulation *in vitro*, but not to direct current injection (Funahashi & Stewart, 1997). While about 70% of intracellularly recorded subicular cells exhibit burst firing (e.g. Stewart & Wong, 1993), the burst responses by deep layer presubicular and parasubicular neurons were much longer in duration (often exceeding 300 ms), far outlasting the multiphasic EPSPs that were sometimes isolated and considered to be monosynaptic (Funahashi & Stewart, 1997). This striking pattern of activity was seen in every sampled deep layer cell, yet superficial layer cells exhibited no such behaviour. The *in vitro* data mirrored the patterns of cell involvement in the sharp waves recorded *in vivo* (Chrobak & Buzsáki, 1994) and offered support for the notion of a functional dissociation of superficial and deep layer neurons in some retrohippocampal areas. In addition, the size and synaptic nature of the bursting behaviour could be a powerful tool to explore the connectivity of presubicular and parasubicular neurons electrophysiologically. A large, reliably generated burst event should be easy to detect in potential target cells, especially if it reflects synchronous activity in a large group of afferent neurons.

In this paper, we describe the use of intracellular and field potential recordings to explore: (1) the cortical afferents which can be used to evoke the bursting behaviour exhibited by deep layer presubicular and parasubicular neurons; (2) the synaptic basis of the burst behaviour by deep layer neurons; and (3) the basis for the apparent functional separation of superficial layer and deep layer pre- and parasubicular neurons. This last point is supported by anatomical data for axonal domains obtained from single-filled neurons and neurons visualized by rapid Golgi methods. The results offer a basis for the functional subdivisions within the presubiculum and parasubiculum which appears to distinguish these areas from other six-layered cortices, including the entorhinal cortex.

METHODS

Slice preparation and maintenance

Male Sprague–Dawley albino rats (150–250 g) were anaesthetized with halothane and decapitated. Each brain was removed from the skull, bisected and placed briefly in ice-cold artificial cerebrospinal fluid (ACSF). Thick slices of tissue (about 2–4 mm thick), which included portions of the hippocampus and retrohippocampal areas, were cut horizontally from the intact hemispheres from the region of the hippocampal genu. Thin slices (300–450 μm) were cut from such blocks using a Vibroslice sectioning system and transferred to a holding chamber. From the holding chamber, single slices were placed on a nylon mesh support in the recording chamber. The lower surface of the slice contacted the perfusing solution (mM: 124 NaCl, 5 KCl, 2 $CaCl_2$, 1·6 $MgCl_2$, 26 $NaHCO_3$ and 10 glucose; pH 7·4 when gassed with 95% O_2–5% CO_2). The upper surfaces were exposed to a warmed, moistened atmosphere of 5% CO_2 in O_2. The temperature of the chamber was maintained at 35–36 °C.

For comparison with the typical horizontal slices, recordings were taken from some cells in 'vertical' slices. These slices were cut from the thick (1–2 mm) horizontal slices described above. A subsection of the thick slice was taken by making two cuts, both parallel to the radial axis of the parasubiculum, presubiculum or medial entorhinal cortex. The angle of the cuts was between the coronal and sagittal planes. Cuts were closer to the coronal plane for presubicular or parasubicular slices and closer to the sagittal plane for entorhinal slices. This piece of tissue was laid on the cutting surface such that one of the newly cut faces contacted the holding block. Thin slices (400–450 μm) were cut as above. The resulting plane of the slice was orthogonal to the plane of the horizontal slices, but preserved the radial architecture of the cortex.

Recording techniques

Extracellular recording electrodes were stainless steel (acute conical tips; Roboz Microprobe, Rockville, MD, USA) with tip impedances (at 1 kHz) of 0·9–1·1 MΩ. Signals, referred to the bath, were amplified (Model 1800; A-M Systems, Inc., Everett, WA, USA), filtered (0·1 Hz to 10 kHz, −6 dB octave^{-1}), and digitized (Digidata 1200; Axon Instruments) for off-line analysis. Intracellular recording electrodes were pulled from 1 mm diameter filament-containing glass capillary tubes and filled with 3 M potassium acetate (tip resistances, 75–100 MΩ) or a solution containing 2% Neurobiotin in either 2 M potassium methylsulphate or 2 M potassium acetate (tip resistances, 80–180 MΩ; Kita & Armstrong, 1991). Some electrodes were filled with 3 M potassium acetate solution that contained *N*-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide (QX-314; Research Biochemicals International) in concentrations from 30 to 100 mM. The lowest concentrations were used to prolong the time before action potentials were suppressed in the recorded cell. Intracellularly recorded signals were amplified by a high-input impedance amplifier with facilities for current injection using a bridge circuit and capacitance compensation (IR-283, Neurodata, New York, USA). Extracellular stimulating electrodes were parallel bipolar (150 μm diameter stainless steel, 0·5 mm tip exposure, 0·2 mm tip separation; FHC, Brunswick, ME, USA). Stimulating pulses were put through constant-current isolation units (Isolator-10; Axon Instruments) at 1 per 2 s.

All electrodes were placed under direct visual guidance with the dissecting microscope. During experiments, the locations for all recording and stimulating electrode placements were drawn by hand using the dissecting microscope. Cell locations of unlabelled cells were based on measurements from observable landmarks (e.g. pial surface, angular bundle, alveus). Cell locations of labelled neurons were obtained by locating cells directly in Nissl-stained sections. In stained sections, changes in the appearance of layer II helped to identify the transitions between the presubiculum (very compact layer II), parasubiculum (broad layer II) and medial entorhinal cortex (more compact again; e.g. Köhler, 1985; van Groen & Wyss, 1990). Regional specification of deep layer cells was based on radial extension of superficial layer boundaries down through the deep layers. Lastly, there was an electrophysiological marker (identified in Funahashi & Stewart, 1997). Most neurons of the subiculum (Stewart & Wong, 1993) and at least a few neurons of the deep layers of the medial entorhinal area (Jones & Heinemann, 1988) were found to burst in response to depolarizing current injection. No cells of the presubiculum or parasubiculum were ever found to burst in response to current injection (Funahashi & Stewart, 1997).

Previously, we showed that stellate and pyramidal cell types in the superficial or deep layers of the presubiculum and parasubiculum were nearly indistinguishable on the basis of passive properties, firing properties or simple evoked responses (Funahashi & Stewart, 1997). In fact, superficial layer cells of one morphology more closely resembled (electrophysiologically) other superficial layer cells with different shapes than they resembled deep layer cells with the same shape. Likewise, deep layer cells were an electrophysiologically uniform group. Whether in superficial or deep cell layers, pyramidal cells represented approximately 70% of Neurobiotin-labelled cells (Funahashi & Stewart, 1997). As such, we expect that the cells reported herein include stellate and pyramidal cell shapes. In some recordings, cell morphology was directly determined by Neurobiotin labelling of the recorded cell.

Pharmacological and microsurgical manipulations

In some experiments 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) and 3-[2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP; 10 μM) were added to the bathing medium to antagonize, respectively, non-NMDA- and NMDA-mediated glutamatergic transmission. Both compounds were obtained from Tocris Cookson (St Louis, MO, USA). In other experiments, ACSF calcium levels were raised to 4–10 mM by addition of calcium chloride solution (1 M) to the continuously bubbled perfusate. While there was no evidence of precipitate in the ACSF reservoir, even at 10 mM, we obviously cannot rule out precipitation elsewhere in the setup. We, therefore, refer to these solutions simply as high-calcium ACSF. Picrotoxin (10–100 μM) was used to antagonize $GABA_A$-mediated inhibitory transmission. All other chemicals were obtained from Sigma.

An acute wedge-shaped microsurgical knife was used to make cuts in slices by plunging it vertically downward with a micromanipulator. Long cuts were made with multiple stabs. Field electrodes were left in place in some instances where cuts were remote. Cuts made very close to field electrodes or, more commonly, between field electrodes, required removal of at least one electrode from the slice to make the cut. Electrodes were withdrawn vertically to allow accurate replacement. Cuts were confirmed to be complete by: (1) plunging the knife through the slice; (2) visually inspecting the space left after the knife was removed; and (3), in some instances, removing the unneeded tissue by suction.

Neurobiotin labelling of single cells

For cells recorded with Neurobiotin-containing electrodes, after electrophysiological recording, Neurobiotin was injected into these cells by using 2–4 nA depolarizing rectangular current pulses (150 ms duration at 3·3 Hz for 20–30 min). Post-injection survival times ranged from 10 to 60 min. Slices with Neurobiotin-injected cells were fixed in 4% paraformaldehyde and 0·2% picric acid in 0·1 M phosphate buffer (pH 7·4) from overnight to 10 days. Frozen sections (40–80 μm thick) were cut from the fixed tissue and kept in phosphate buffered saline (PBS; pH 7·4). After rinses with PBS, these sections were treated with 0·1% H_2O_2 for 20 min and Triton X-100 (0·4–0·5% in PBS) for 2–3 h. They were rinsed in PBS and then incubated in the Vectastain ABC Reagent (Vector Labs, Burlingame, CA; USA) in PBS for 2–3 h. After rinses with PBS, sections were reacted with diaminobenzidine (DAB) and H_2O_2 (0·003%) in PBS (DAB substrate kit for peroxidase; Vector Labs) to visualize the injected cells. The sections, which included the successfully stained cells, were counterstained by Nissl staining. It must be noted that the size of the dendritic tree or axonal domain may be limited because tracer failed to reach the most distal processes and/or because dendrites or axons left the plane of the slice.

Rapid Golgi methods

Rats, deeply anaesthetized with halothane, were perfused through the heart with saline, followed by 1·5% glutaraldehyde in water. Brains were removed and horizontal or coronal slabs (about 2 mm thick) were cut and transferred into a solution of 0·25% osmium tetroxide in 3% potassium dichromate for 2 days. Tissue pieces were rinsed in water and transferred to a solution of 0·75% silver nitrate for up to 5 days. Tissue pieces were then dehydrated in ethanol and equilibrated in a 1 : 1 solution of absolute ethanol : absolute ether. Tissue pieces were embedded in parlodion by first soaking pieces in 6% parlodion in alcohol : ether and transferring them to a solution of 12% parlodion in alcohol : ether. The parlodion hardened with slow evaporation of the alcohol : ether over several days. Parlodion blocks were stored in 80% ethanol. Sections (~80 μM) were cut from the parlodion blocks on a Vibratome and collected into 95% ethanol. Sections were dehydrated by changes in 100% terpineol, cleared in xylene and mounted.

Expression of results

Results are given as means ± S.E.M.

RESULTS

Intracellular recordings were taken from ninety-six neurons of the presubicular and parasubicular cortices in 105 horizontal and fourteen vertical slices. Cells had stable resting membrane potentials in the range of −58 to −72 mV, membrane input resistances in the range of 54–88 MΩ and overshooting action potentials. Multiple field potential recordings or field potential and intracellular recordings were taken from each slice. Intracellular recordings were stable for from 5 min to 1 h. Bursting responses (seen with intracellular recordings or field potential recordings) were stable for the life of the recording, or until manipulations were made to reduce or enhance activity.

The burst response by deep layer presubicular and parasubicular neurons is described first: its appearance, how it is triggered, and evidence that it is synchronous and supported by glutamatergic synapses. It is the scope and clarity of the event that makes it such a valuable tool for exploring the outputs of the deep layer cells. The behaviour of the superficial layer cells is described next. Connections suggested by differences in firing patterns are tested electrophysiologically (using antidromic field potential studies) and morphologically (using Neurobiotin injection and Golgi impregnation to reveal axon paths). Finally, a specific role is suggested for entorhinal afferents in the generation of after-discharges in the presubiculum and parasubiculum.

Deep layer cell bursts

Single extracellular stimulus pulses (50–100 μs, 0·1–1·0 mA, 0·5 Hz) delivered to the subiculum evoked a large burst response, at short-latency (stimulus to EPSP onset,

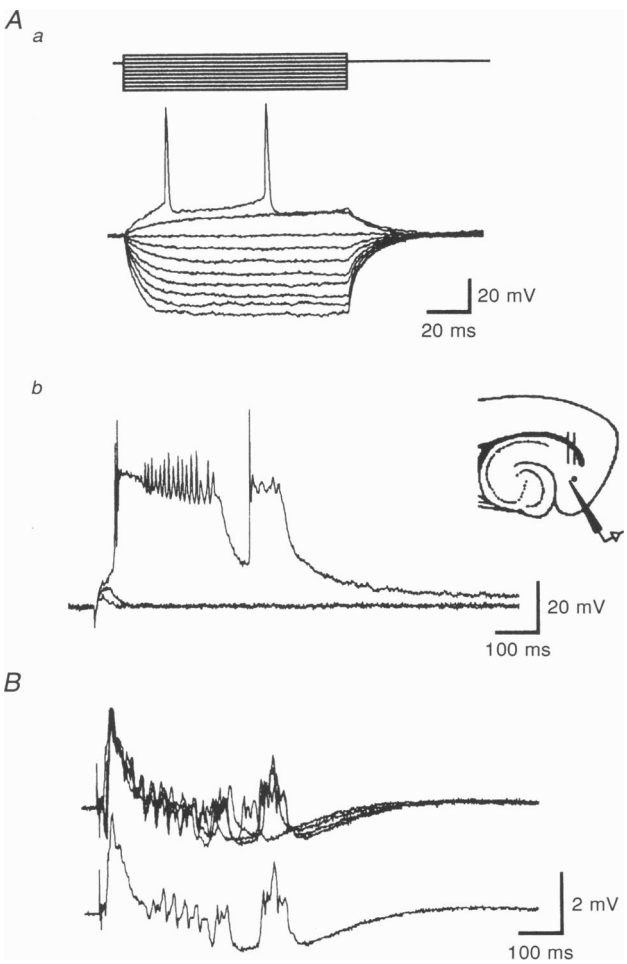

Figure 1. Deep layer cells burst and the bursts are synchronous

Responses of a deep layer presubicular neuron to direct current injection and extracellular stimulation of subiculum. Intracellular responses are shown in *A*, field potential recordings in *B*. *Aa*, direct current pulses ranged in amplitude from −1·05 to 0·3 nA, in 0·15 nA increments. Response to depolarizing current injection was repetitive single spiking. Resting potential, −68 mV. *Ab*, responses to three extracellular stimulus intensities (0·2, 0·35 and 0·55 mA, 70 μs duration). A primary burst with a single after-discharge is shown. Note within the primary burst, the initial brief period of firing (< 10 ms duration), the period of no firing (~60 ms duration), and finally, the period of oscillation (~140 ms duration). Note the absence of a late oscillation period in the after-discharges. *B*, superimposed field potential responses (top) and single response (below) illustrate components of the primary burst as seen by extracellular electrodes. Inset: drawing of horizontal slice showing electrode location. ●, field potential electrode, in this and subsequent figures.

4·9 ± 1·2 ms) in individual deep layer neurons and in field potential recordings taken from the deep layers (most commonly, layer V) of the presubiculum and parasubiculum (Fig. 1). Such responses were seen in sixty of sixty-seven horizontal slices. Intracellular events (referred to as the 'primary burst') ranged in duration from 100 to 400 ms (218 ± 86 ms, $n = 14$). An intracellularly recorded primary burst was characterized by an initial burst of action potentials (lasting 30 ± 19 ms; range, 10–80 ms) which was typically followed by a period of no firing, but sustained depolarization (lasting 105 ± 39 ms; range, 50–180 ms), and finally a return to firing for the remainder of the burst response (Figs 1 and 2). Consistent with the period of no firing that followed the initial burst of action potentials was the finding that individual deep layer neurons exhibited pronounced spike frequency adaptation when tested with long-duration depolarizing current pulses (Funahashi & Stewart, 1997). The return to firing was variable in duration, but was most commonly characterized by an interspike interval of about 10–25 ms (40–100 Hz). In fifty-one out of sixty slices, primary bursts were followed by one or more after-discharges (mean number, 1·6 ± 1·0 in 60 slices).

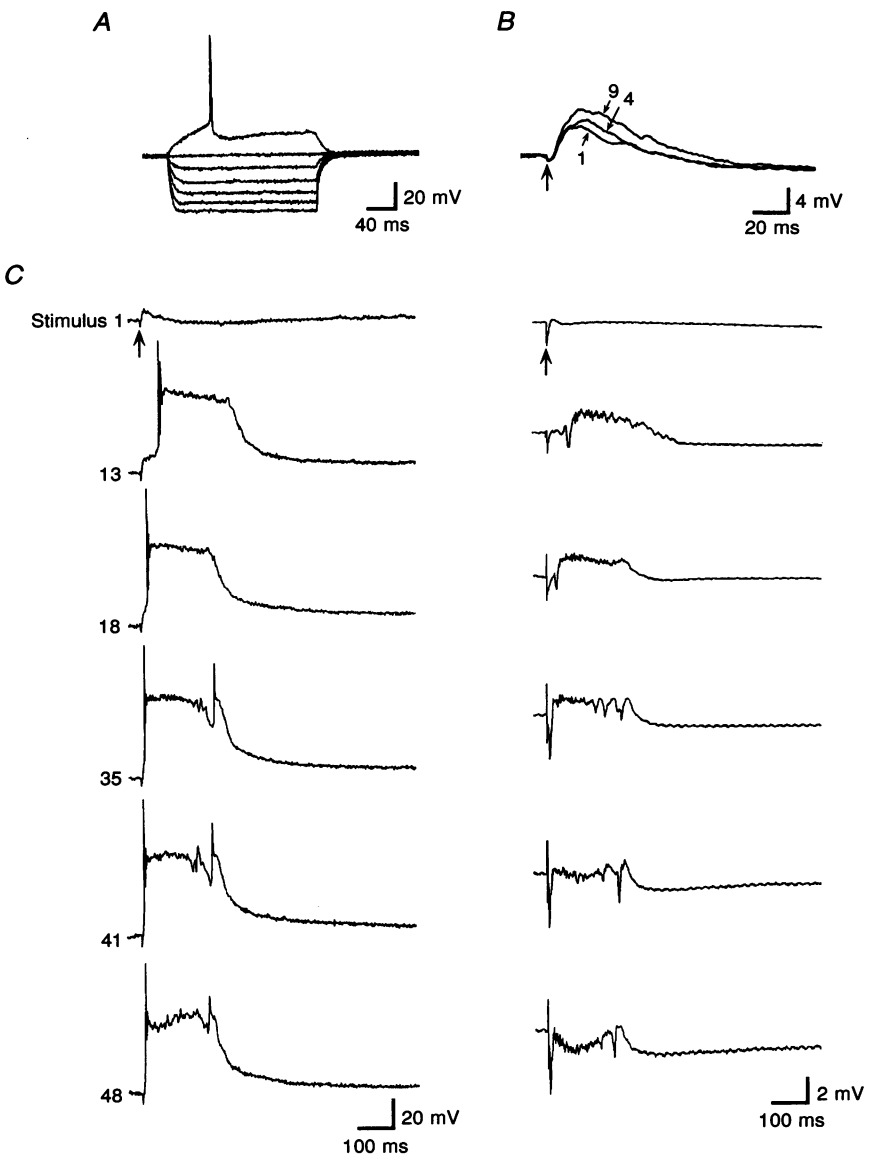

Figure 2. Development of deep layer cell bursts

Recordings taken from the first impaled cell in a slice that was not previously stimulated. *A*, intracellular responses to current injection. Repetitive extracellular stimuli (0·12 mA, 70 μs, 0·05 Hz) were used to trigger burst discharges. *B*, growth of intracellularly recorded EPSP illustrated with responses to pulses 1, 4 and 9. *C*, intracellular (left column) and simultaneous field potential (right column) recordings for 6 different pulses between 1 and 48. First response is shown again. Note the first burst response (pulse 13) occurred long after the start of the EPSP. In response to subsequent pulses, the latency decreased. Stimulus number given to left of intracellular traces. Arrows mark the time of occurrence of stimulus pulse.

Individual cells did not burst in response to direct current injection (Fig. 1). All cells responded to direct current injection with regular spiking. However, all deep layer cells could respond to synaptic input with the primary burst response. It must be noted that these events were not the only responses to extracellular stimulation that could be exhibited by deep layer neurons. The development of the deep layer cell burst discharge is described below.

Deep layer cell bursts are synchronous

Burst responses were synchronous among deep layer cells as judged by the presence of population responses in field potential recordings. In field potential–intracellular recording pairs, the field response was found to consist of an early negative spike that correlated with the initial burst of action potentials by individual cells, a large positive component that correlated with the period of sustained depolarization without firing, and finally, variable amounts of activity that correlated with the late firing seen in the burst response. This late firing by individual cells was also synchronous, as judged by its appearance in field potential records (see below, e.g. Fig. 1). After-discharges were seen, as indicated above, in many of the recordings.

Development of bursting by deep layer neurons

In deep layer cells, which were the first ones recorded from a particular slice, initial suprathreshold stimuli elicted simple EPSPs with single action potentials. Successive stimuli caused larger EPSPs, until a burst response occurred (Fig 2). Once triggered, burst responses were stable in amplitude and duration for the life of a given cell recorded intracellularly or for the life of the slice, based on field potential recordings. The majority of cells were initially impaled after burst responses had been seen in other cells of the same slice. In these cells, burst responses were their threshold response to orthodromic activation (cf. Fig. 3).

Ten to sixty pulses were sufficient to trigger the first burst response at our usual stimulation rate of 0·5 Hz. Jones (1993) showed a similar process in deep layer entorhinal neurons *in vitro* and suggested that a delicate balance of excitation and inhibition was altered to favour excitation. When we used stimuli which were well below threshold for

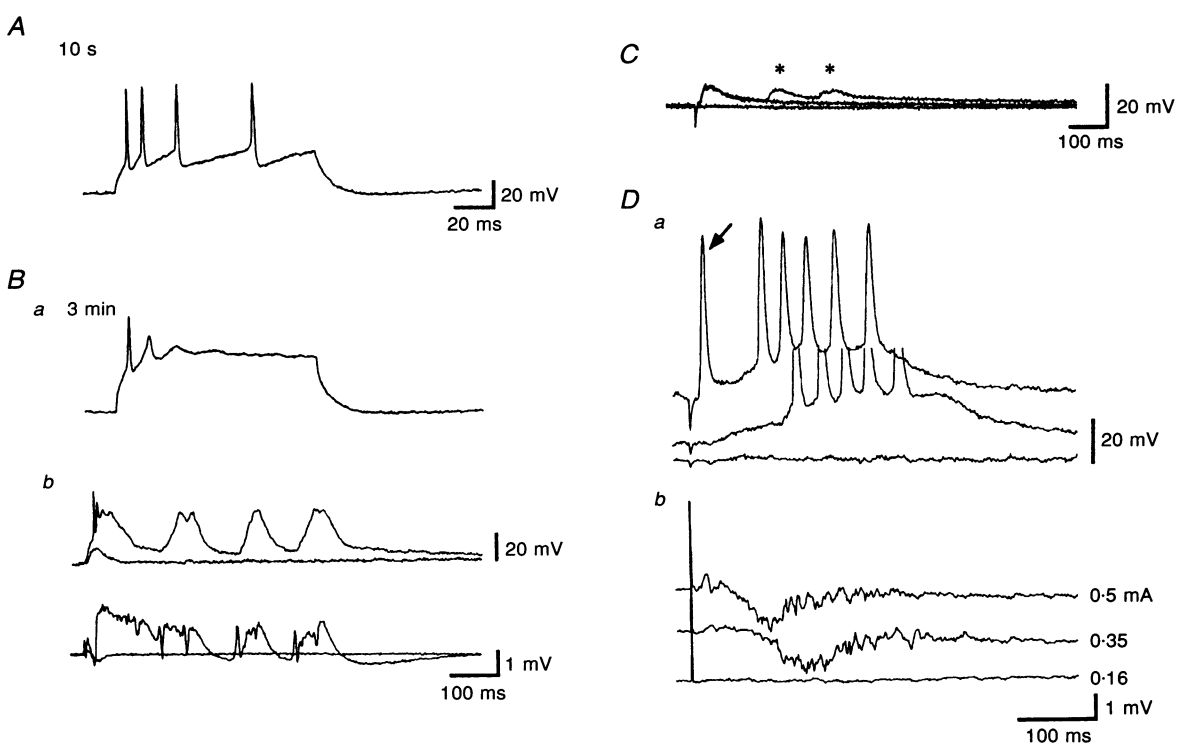

Figure 3. Bursts are synaptically triggered and supported

A, effects of intracellular QX-314 application on responses to direct current injection and extracellular stimulation. Intracellular response immediately after impalement (*A*) and 3 min after impalement (*Ba*) with a QX-314 pipette. *Bb*, intracellular responses (above) and simultaneous field responses (below) confirm synaptic potentials underneath burst responses. *C*, this cell responded only with EPSPs to subicular activation. Note that EPSPs corresponding to after-discharges (indicated by asterisks) only occur if the primary burst occurs. Resting potential, −67 mV, stimulus intensity, 0·75 mA. *D*, intracellular (*Da*) and field (*Db*) responses to three extracellular stimulus intensities (0·16, 0·35 and 0·5 mA). At the highest intensity, this cell was antidromically activated by the subicular stimulus. Resting potential, −67 mV. Arrow marks the antidromic spike at the beginning of the sweep.

firing cells, we detected no appreciable growth in the EPSP intracellularly or in field potential recordings (0·5 Hz stimulation rates, $n = 2$ slices). When stimulus intensities were suprathreshold or near threshold, EPSP growth (as judged from intracellular and/or extracellular recordings) and, ultimately, burst firing were seen with stimulation rates of 1 Hz ($n = 3$ slices), 0·5 Hz ($n = 63$ slices) and 0·05 Hz ($n = 3$ slices; Fig. 2).

As the number of times the preparation was stimulated increased, the latency of the population burst response decreased until it had the same latency as the EPSP itself. If the stimulating electrodes were relocated to another afferent system, burst responses were triggered with the first or one of the first few pulses, suggesting that at least some of the change was stored at synapses other than the particular afferent synapse.

Deep layer cell bursts are synaptically triggered and supported

Stimuli applied to the subiculum ($n = 57$ slices), superficial parasubiculum ($n = 4$ slices), superficial presubiculum ($n = 4$ slices), or deep layers of medial entorhinal cortex ($n = 15$ slices) could each trigger the primary burst discharge. The only apparent difference among the afferent systems was the presence of a very early small multiphasic field component when the subiculum was stimulated.

Burst responses were synaptically supported as judged by intracellular recordings where: (1) EPSPs were below threshold for firing the neuron (Fig. 3*C*); (2) membrane hyperpolarization could suppress firing and leave behind a depolarizing potential (not shown); or (3) QX-314 (30–100 mM) in recording pipettes suppressed firing and revealed a depolarizing potential (Fig. 3). Burst responses had lower thresholds than antidromic responses (Fig. 3*D*).

When evoked responses were tested during depolarizing current injection, clear IPSPs were present and confirmed when depolarizing current injection was used to elicit a continuous train of action potentials that was arrested by the evoked response (not shown).

Burst responses were confirmed pharmacologically to be supported by non-NMDA- and NMDA-mediated components. Bath application of a low concentration of CPP (5 μM) to antagonize the NMDA-mediated component of the synaptic response had the effect of raising the threshold for evoking the primary burst response with no effect on its size or

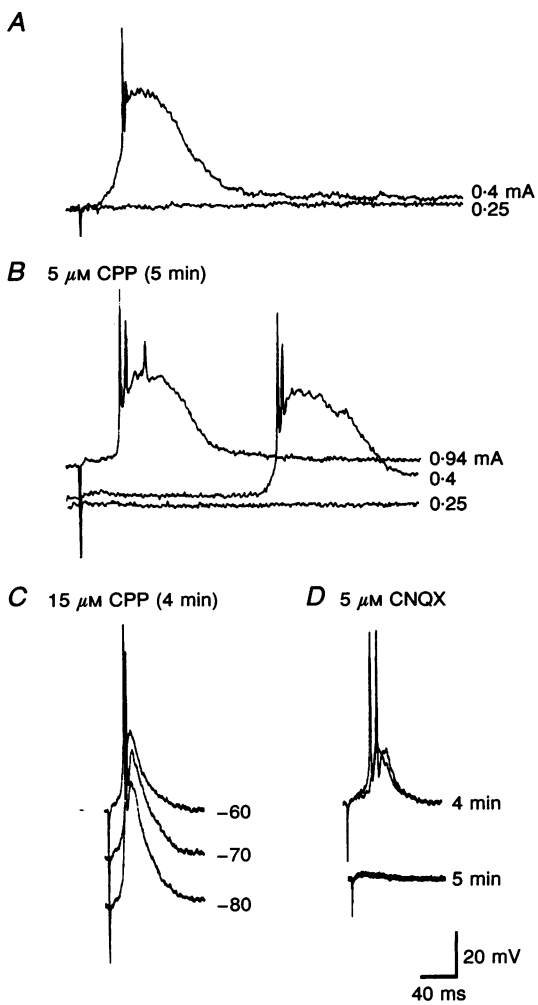

Figure 4. Glutamatergic components of deep layer cell bursts
Primary burst and the effects of CPP to antagonize NMDA-mediated responses and CNQX to antagonize non-NMDA-mediated responses. Resting potential, −59 mV. *A*, before application of glutamate receptor antagonists. *B*, after low concentration of CPP (5 μM), only the threshold for evoking primary burst was visibly changed. *C*, higher concentrations (15 μM) reduced the amplitude and duration of responses. *D*, CNQX (5 μM) abolished the evoked event. Stimulus intensity, 0·9 mA.

duration (Fig. 4*B*). Higher concentrations (10–15 μM) produced clear reductions in the amplitude and duration of the primary burst response (Fig. 4*C*). Bath application of CNQX in concentrations as low as 5 μM completely eliminated the evoked response (Fig. 4*D*).

Deep layer cell activity depends on the amount of deep layer tissue

Burst responses appeared to depend on the number of functionally connected deep layer cells present in the slice. During recordings taken from the presubiculum, near the border with the subiculum, the primary burst response was progressively reduced in duration and amplitude by successive radially oriented cuts through the parasubiculum and presubiculum ($n = 9$ slices). The response decreased as more and more of the deep layer tissue was cut away (Fig. 5). In three slices, enough deep layer tissue was removed to reduce the response to subicular stimulation to what appeared to be a monosynaptic event. In these recordings, intracellularly recorded cells responded with single action potentials to subicular stimuli which had previously triggered the bursting behaviour (Fig. 5).

As an alternative method for altering the network of deep layer neurons which might generate the population bursts, the firing thresholds of cells in the slice were raised by increasing the extracellular calcium concentration ($n = 4$ slices) (Miles, Traub & Wong, 1988). Nominal concentrations between 4 and 10 mM eliminated after-discharges (see below) and reduced the size and duration of the primary burst response (Fig. 5).

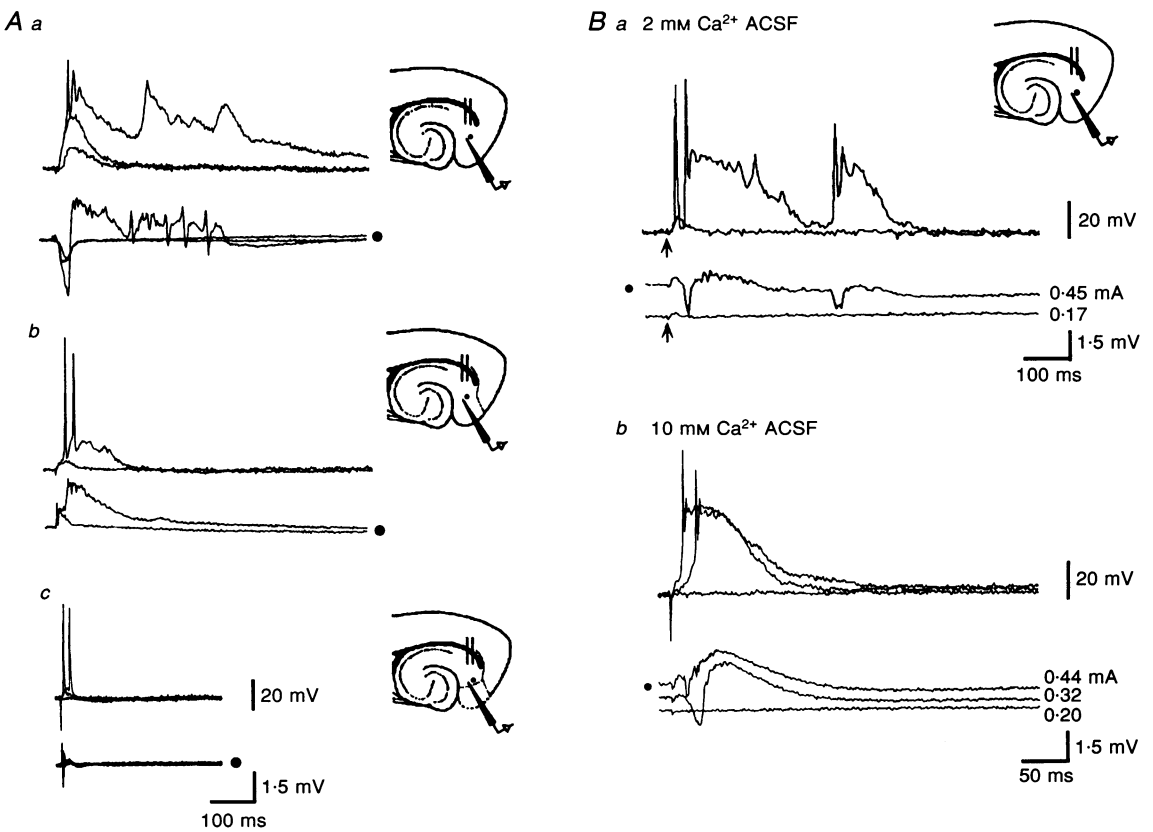

Figure 5. Bursts and after-discharges are reduced by knife cuts or elevated extracellular calcium

A, effects of progressive cuts to remove retrohippocampal tissue. *Aa*, control response in intact horizontal slice. Intracellular responses (above) and simultaneous field responses (below). Resting potential, −65 mV. Stimulus intensities, 0·13, 0·21 and 0·27 mA. *Ab*, responses after a knife cut which separated the medial entorhinal cortex. Note the loss of after-discharges and the relative lack of effect on the primary burst response. Stimulus intensities, 0·53 and 0·66 mA. Cut indicated by dashed line on the drawing (inset). *Ac*, reduction of primary burst response to a presumed monosynaptic event by removal of much of the presubiculum and parasubiculum. Stimulus intensity, 0·9 mA. *B*, effects of elevated extracellular calcium on population responses. *Ba*, in ACSF containing 2 mM calcium ('normal' solution), intracellular (above) and field potential (below) responses are shown which consist of a primary burst and a single after-discharge. Resting potential, −64 mV. *Bb*, after exposure to ACSF containing high-calcium (nominal 10 mM; recording taken 1 h after change), response consists only of the primary burst. Note that the late activity seen in the primary burst is also eliminated.

Superficial layer cell responses to afferent stimulation

In contrast to the prominent bursting behaviour exhibited by every recorded deep layer neuron, orthodromic responses by presubicular and parasubicular cells of layers II and III consisted of simple excitatory postsynaptic potentials which could be sufficient to elicit single action potentials. In only two superficial layer cells was it possible to see EPSPs coincident with the field potential burst in the deep cell layers (Fig. 6). These cells also showed EPSPs in response to stimulation of superficial cell layers of entorhinal cortex. Superficial layer cells of entorhinal cortex themselves fired in response to the evoked burst (Fig. 6) and may have been the actual source of the long-latency EPSP seen in superficial layer presubicular and parasubicular cells.

Superficial layer cell responses to spontaneous deep layer cell bursts

Attempts to orthodromically activate superficial layer cells by extracellular stimulation in the deep cell layers may be contaminated by concurrent activation of afferent fibres passing through the deep layers to reach their targets in the superficial layers. Subicular afferents, for example, may take this path. To eliminate this problem as well as similar problems of activating superficial layer cell axons directly, spontaneous deep layer cell bursts were caused by picrotoxin application to isolated pieces of presubiculum and parasubiculum. The picrotoxin treatment has other advantages. First, it is possible that the superficial layer cell responses were small or apparently absent because of differences in the inhibitory inputs to the cells. If this were true, picrotoxin treatment may reveal excitatory inputs that were actively suppressed in undrugged slices. By eliminating any 'resting level' of inhibition, picrotoxin treatment should also enhance excitatory responses, even if there were no active feed-forward inhibitory mechanisms.

To eliminate long-loop connections of deep layer pre- and parasubicular cells through entorhinal cortex or subiculum, subsections which contained only pre- and parasubiculum were cut from full horizontal slices by making radially oriented knife cuts from the angular bundle to the pia at the presubiculum–subiculum border and at the parasubiculum–medial entorhinal cortex border. Subsections were bathed in picrotoxin (50 or 100 μm; $n = 13$ isolated pre-/parasubicular pieces). It must be emphasized that, while we preferred to use spontaneous activity for the reasons given above, deep layer burst responses were evoked in undrugged subsections in the same way as in intact slices ($n = 6$ cells).

Spontaneous population burst responses were seen in the deep layers (single cells, $n = 7$ cells; and field potentials, $n = 13$ pre-/parasubicular pieces), but superficial layer cells ($n = 6$) showed no synaptic events related to the deep layer cell bursts (Fig. 7). Only when connections with the subiculum or entorhinal cortex remained intact could

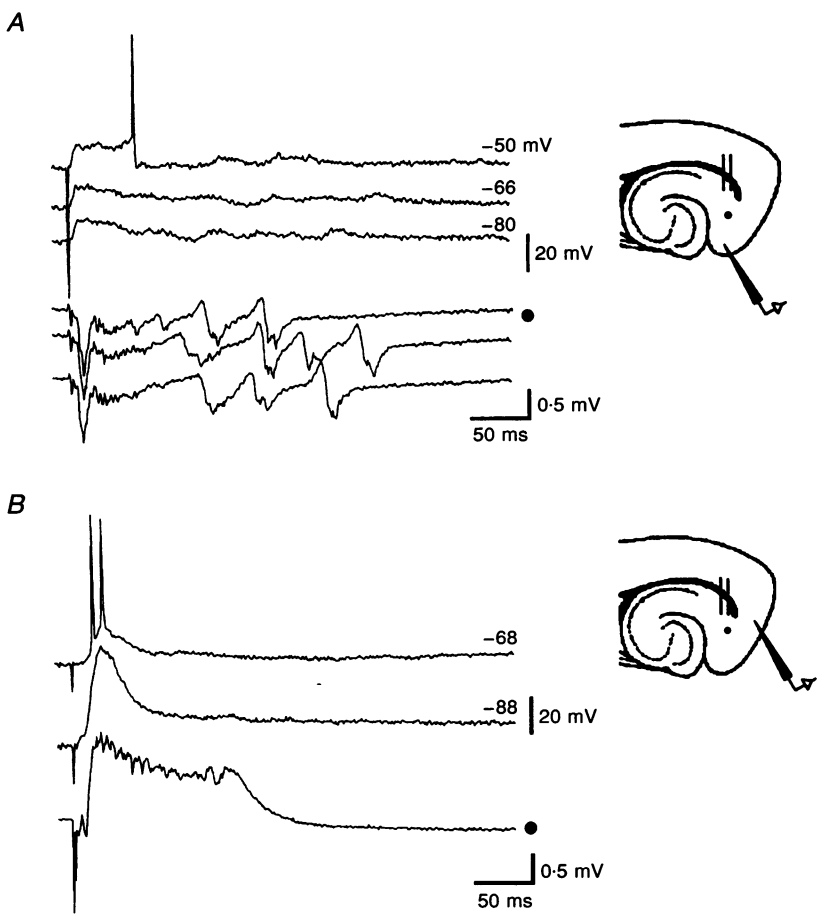

Figure 6. Comparison of evoked superficial layer cell responses from presubiculum and medial entorhinal cortex

Intracellular responses of superficial layer cells of presubiculum (*A*) and medial entorhinal cortex (*B*) during evoked population bursts. *A*, when presubicular cell was polarized to −80 mV, low amplitude EPSPs corresponding to peaks in the field response became visible. Three intracellular (above) and corresponding field potential (below) sweeps shown. Extracellular electrode marked with a filled circle in drawings. Stimulus intensity, 0·78 mA. *B*, superficial layer medial entorhinal neuron exhibits a clear evoked response. Stimulus intensity, 0·49 mA.

superficial pre-/parasubicular cells show EPSPs and firing in relation to the deep layer bursts in slices bathed in picrotoxin ($n = 8$ cells in 9 slices).

Similar slices of entorhinal cortex showed clear burst responses by superficial layer cells. Note that these were always smaller in amplitude and shorter in duration than burst responses by deep layer neurons.

Responses in 'vertical' slices

In the previous section, the possibility of powerful inhibitory circuits suppressing deep to superficial projections was addressed. Another possible explanation for the lack of response by superficial layer cells is that the slicing procedure itself severed the axons which would have reached the superficial layer cells. We were comfortable with the horizontal slice for several reasons: (1) the radial extent of the dendritic tree of deep layer and superficial layer pyramidal cells is preserved in horizontal slices (Funahashi & Stewart, 1997); (2) electrophysiologically, connections from superficial layers to deep layers can be demonstrated (this paper); (3) deep to superficial projections can be shown for entorhinal cortex (this paper; Lorente de Nó, 1933; Jones, 1990; Jones & Lambert, 1990); and (4) long-range connections of deep layer cells with subicular and deep layer entorhinal neurons are present within the slices. However, to address the possibility that the ascending axons of deep layer presubicular and parasubicular cells might travel in a plane other than the horizontal, we examined superficial

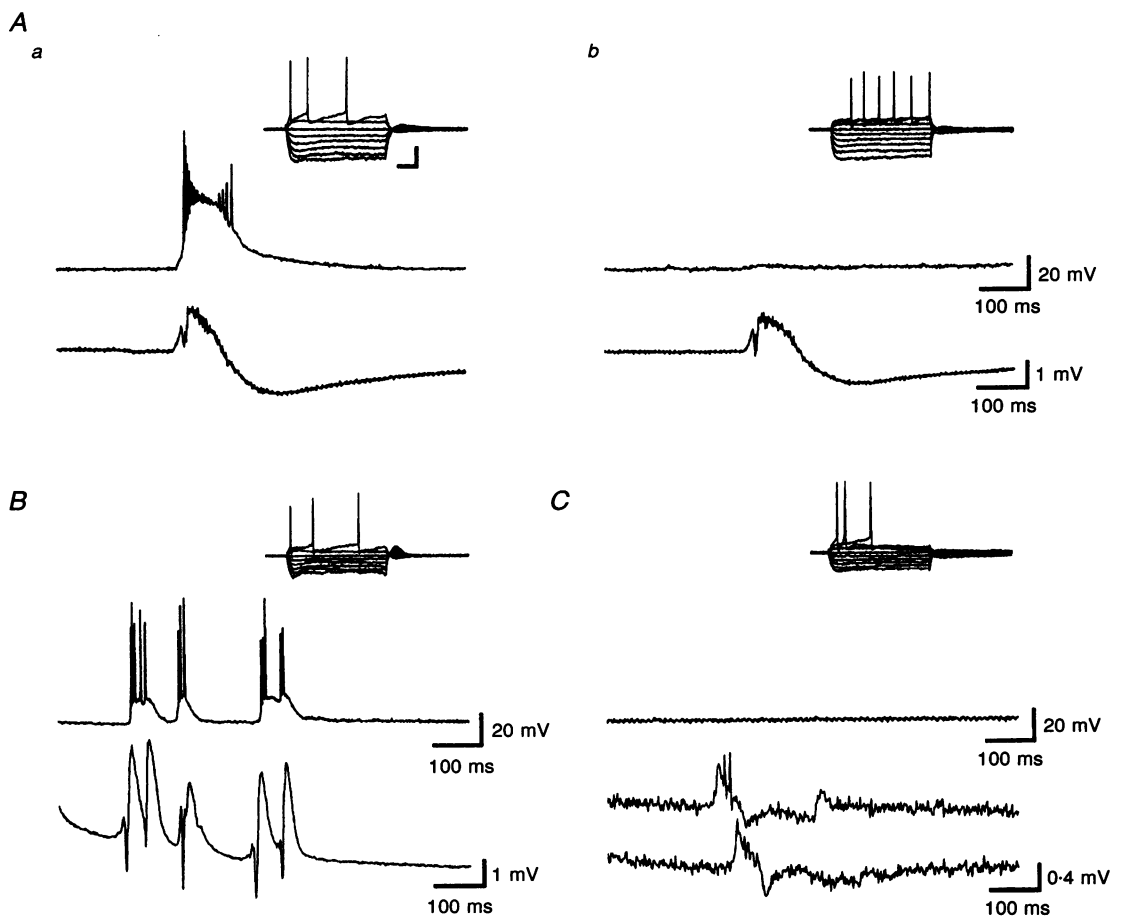

Figure 7. Superficial layer cell responses to spontaneous deep layer events

Subsections, which preserved the radial architecture of presubiculum and/or parasubiculum, were bathed with 100 μM picrotoxin to induce spontaneous burst firing by deep layer neurons. Two different preparations were used with identical results: (1) 'vertical slices' (described in Methods); or (2) subsection of horizontal slice containing only presubiculum and parasubiculum. Recordings in *A* and *B* were taken from vertical slices. Recordings in *C* were taken from a subsection of a horizontal slice. *Aa*, intracellular (above) and simultaneous field (below) responses show deep layer cell (layer V) spontaneous burst. Response to current injection shown in inset. *Ab*, intracellular and field responses from a layer II neuron of the same vertical slice. *B*, intracellular and field responses from a vertical entorhinal slice. Layer II entorhinal neuron exhibits large synaptic potentials and firing in response to spontaneous burst and after-discharges recorded from the deep entorhinal layers. *C*, responses by a layer III presubicular neuron to spontaneous deep layer events. Calibration for all insets: 20 mV, 40 ms.

layer cells in vertical slices which also preserved the radial dimension, but in a plane perpendicular to that of the horizontal slice.

A total of twenty cells were recorded in fourteen vertical slices. Six cells were recorded from layer V of the pre- or parasubiculum, nine cells from layers II and III of the pre- and parasubiculum, and five cells from layers II and III of the medial entorhinal cortex. Results obtained in these slices were identical to those from horizontal slices. Superficial layer presubicular and parasubicular cells exhibited no activity in relation to evoked or spontaneous deep layer cell bursts, even in the presence of picrotoxin (Fig. 7). Again, by comparison, superficial layer medial entorhinal neurons exhibited large EPSPs with multiple spiking in association with bursts by deep layer entorhinal neurons (Fig. 7).

After-discharges were only seen in slices or subsections which contained deep layers of medial entorhinal cortex.

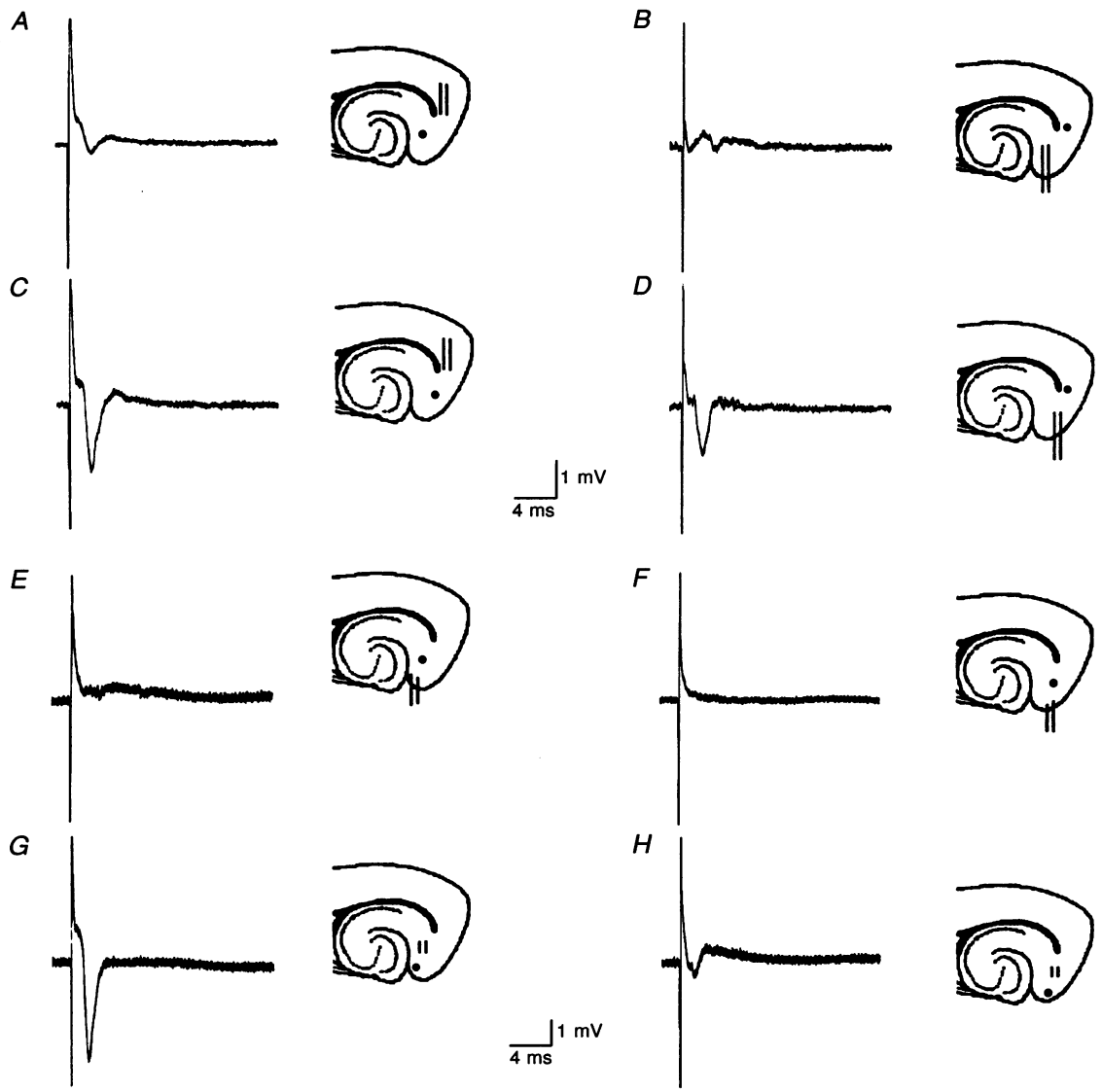

Figure 8. Antidromic population responses

Antidromic population spikes recorded in slices bathed in 10 μM CNQX and 10 μM CPP to block excitatory neurotransmission. *A*, deep presubicular response to deep entorhinal stimulation (0·4 mA). *B*, deep entorhinal response to deep presubicular stimulation (0·4 mA). *C*, deep parasubicular response to deep entorhinal stimulation (0·4 mA). *D*, deep entorhinal response to deep parasubicular stimulation (0·3 mA). *E*, deep presubicular response to superficial presubicular stimulation (0·3 mA). *F*, deep parasubicular response to superficial presubicular stimulation (0·3 mA). Note that while a single antidromic spike can be recorded in every other configuration, there is no antidromic response in deep layer presubicular or parasubicular recordings to superficial layer stimulation. *G*, superficial presubicular response to deep presubicular stimulation (0·3 mA). *H*, superficial parasubicular response to deep parasubicular stimulation (0·3 mA). These last two panels are additional evidence of a pronounced descending projection.

Antidromic field potential evidence for projections

Attempts to antidromically activate cells in slices bathed in 10 μM CNQX–10 μM CPP failed to reveal antidromic spikes in the deep cell layers when superficial cell layers were stimulated ($n = 8$ slices), but did show antidromic superficial layer population spikes when deep cell layers were stimulated ($n = 8$ slices). These results (shown in Fig. 8) are consistent with the superficial to deep projections which could trigger deep layer cell bursts, and the apparent absence of reciprocal connections as judged by the lack of a burst response by superficial layer cells.

Reciprocal connections (based on antidromic population spikes) of the presubiculum and parasubiculum with deep layers of medial entorhinal cortex were also seen ($n = 3$ slices) (Fig. 8). Whereas we can clearly say that the presubicular and parasubicular recordings depended on entorhinal cortex for after-discharges we cannot quantify any contribution of entorhinal deep layer cell projections to the amplitude or duration of the primary burst in the presubiculum or parasubiculum.

Morphological evidence for projections

In some recorded cells, Neurobiotin was used to label the cell. We were interested in the axonal trajectories and arborizations of identified cells for the purpose of supporting or refuting the notion, derived from the electrophysiological studies, that there was a unidirectional system of connections from superficial to deep layers in the presubiculum and parasubiculum. Axon collaterals were followed in some cells over distances up to 800 μm. A total of twenty-one pre- and parasubicular cells with axons longer than 200 μm were taken from a population of forty-nine stained cells. The distribution was as follows: two layer II stellate cells, eight layer III pyramidal cells, eight layer V pyramidal cells, and three layer V stellate cells. Five entorhinal cells were labelled: two layer II stellate cells, two layer V pyramidal cells, and one layer V stellate cell.

In addition, the brains of three rats were prepared with rapid Golgi techniques and sections cut in the horizontal ($n = 2$ brains) or coronal ($n = 1$ brain) planes. It is important to note that these data were intended to supplement the

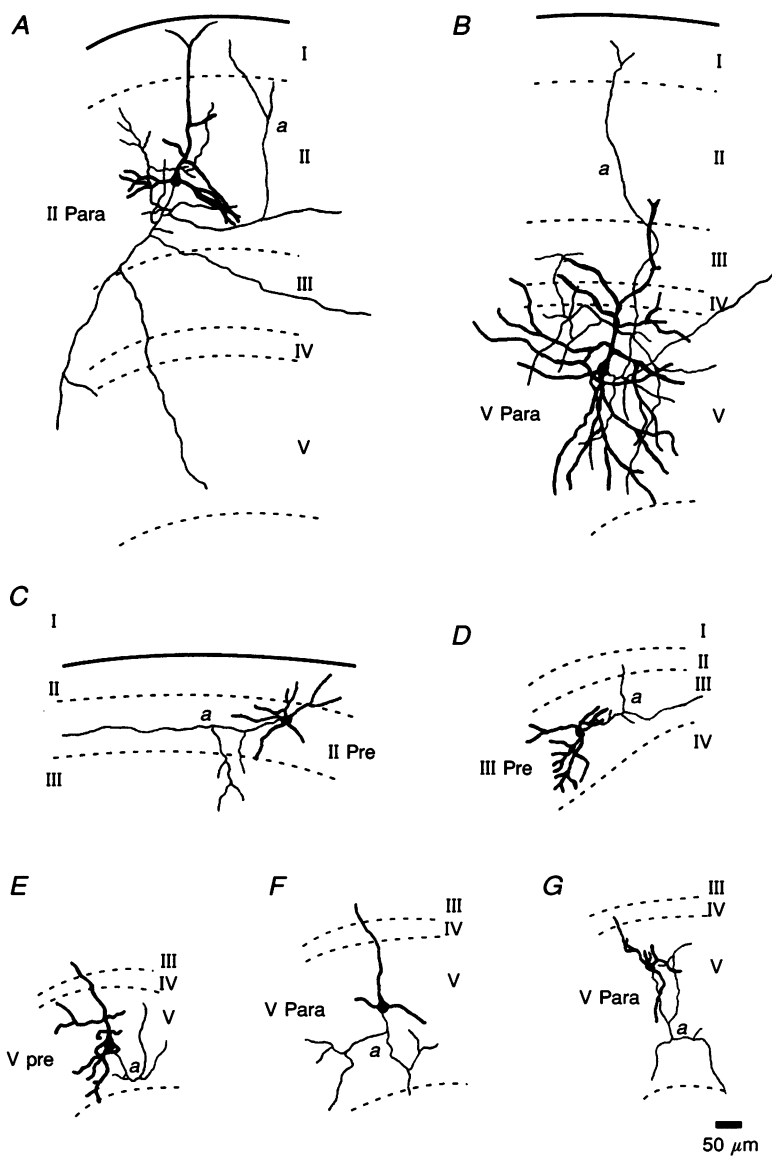

Figure 9. Axonal trajectories of morphologically identified presubicular and parasubicular cells
Drawings of individual presubicular and parasubicular neurons. Neurobiotin-labelled cells (*A* and *B*) were reconstructed from serial 40 μm sections. Golgi-impregnated cells were captured in 80–100 μm sections. Dendrites are shown as thick processes and axons as thin processes to permit distinction in the illustration. *A*, pyramidal cell from layer II of parasubiculum. *B*, pyramidal cell from layer V of parasubiculum. This was the only deep layer cell found which had even a single collateral reach the superficial cell layers. *C*, spiny stellate cell from layer II of presubiculum. The axon of this cell reached into subiculum. Descending collaterals were traced into layer III. *D*, spiny stellate cell in layer III of presubiculum. *E*, pyramidal cell in layer V of presubiculum. *F*, pyramidal cell in layer V of parasubiculum. *G*, aspiny stellate cell in layer V of parasubiculum.

data from Neurobiotin-labelled cells, not to represent a complete Golgi study of the presubiculum and parasubiculum. A total of thirty pre- and parasubicular cells were examined with the following distribution: two layer II–III stellate cells, eight layer III pyramidal cells, four layer IV pyramidal cells, eleven layer V pyramidal cells, and five layer V stellate cells.

The axonal domains of Golgi-impregnated cells were similar to those seen in Neurobiotin-labelled cells, so they are described together. In some instances, where many cells were visible in the Golgi material, clear identification of the cell of origin of particular axon collaterals was difficult. The cells described were all well isolated. In some cases they were one of only a few cells impregnated in a section. Examples are drawn in Fig. 9.

Axons of superficial layer pyramidal cells were found to give off numerous collaterals in layers II and III (cell in Fig. 9*A*). Each cell could have one or more ascending collaterals. These typically branched repeatedly after climbing past the level of the cell body. One or more descending fibres were present and could be traced into layer V where they gave off several additional branches. In two cases, fibres were traced to the angular bundle.

Axons of superficial layer stellate cells were also found to branch within the layer of the cell body (layer II). Examples are cells in Fig. 9*C* and *D*. Collaterals were found to descend into layer III, and rarely into layer V. The axon of one cell was traced over 500 μm horizontally into the apical dendrites of the subiculum (cell in Fig. 9*C*). This axon branched repeatedly within layer II of the presubiculum and gave off several collaterals which descended into layer III of the presubiculum. Stellate cell axons, from superficial or deep cell layers, were more delicate than the axons of pyramidal cells.

Axons of deep layer pyramidal cells (layer V only in Neurobiotin material, layer IV and V in Golgi material) were found to give off numerous horizontal collaterals within layer V (cells in Fig. 9*B*, *E* and *F*). Boutons were present along the entire length of each collateral. One or more descending collaterals were present in every cell, and were followed in some to the angular bundle (cells in Fig. 9*B* and *F*). One cell (cell in Fig. 9*B*) was found to have a single non-branching axon collateral that reached the border of layers I and II. There, the collateral divided. This is in contrast to deep layer entorhinal neurons where ascending collaterals are common. The axons of entorhinal cells were also found to branch in layers III and II (see also Lorente de Nó, 1933). No other deep layer presubicular or parasubicular cells were found to have ascending collaterals that extended beyond layer IV. Those that did extend to layer IV appeared to end there with a tuft of short branches.

Axons of deep layer stellate cells were found to branch within layer V. The dendrites of deep layer stellate cells

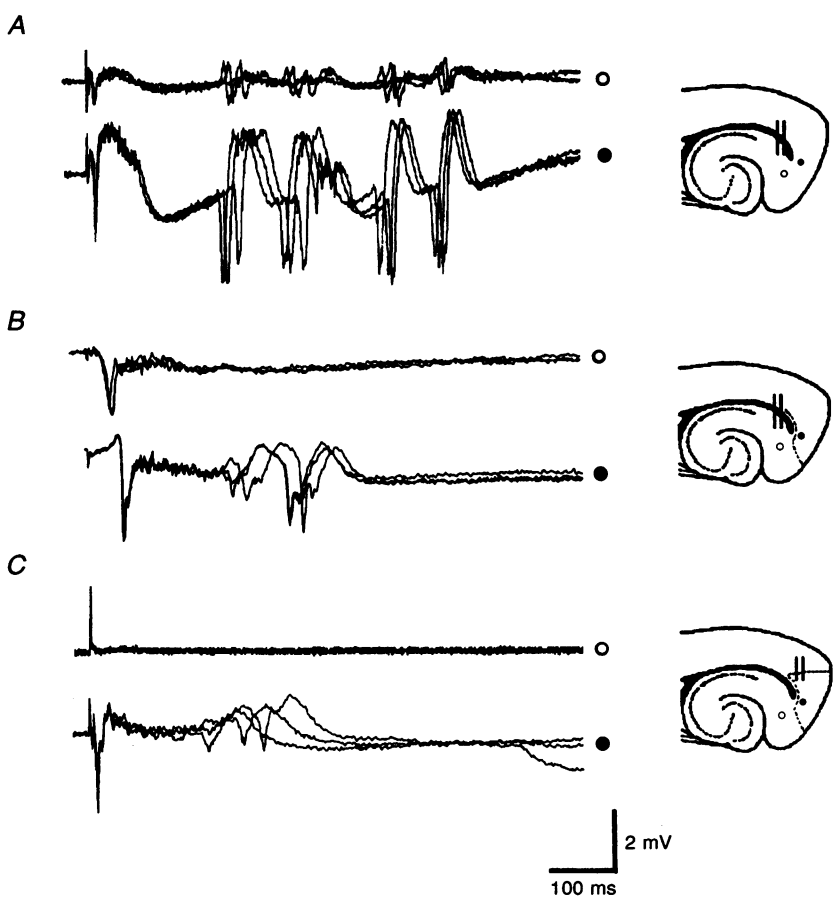

Figure 10. Entorhinal origin for after-discharges
A, Presubicular (○) and medial entorhinal (●) field potential recordings of evoked primary burst and after-discharges. After-discharges in entorhinal cortex uniformly lead presubicular events by about 18 ms. Stimulus intensity, 0·76 mA. *B*, after separating medial entorhinal cortex with a knife cut (dotted line). Presubiculum still shows primary burst, but no after-discharges. *C*, stimulation and recording within the isolated medial entorhinal area shows primary burst and after-discharge.

were distinctly less spiny than superficial layer stellate cells or any pyramidal cells. Axons branched within layer V and a few could be traced to the angular bundle (cell in Fig. 9*G*). None were found to ascend to beyond layer IV.

Entorhinal origin for after-discharges

Regardless of the site stimulated to trigger the primary burst in deep layers of the presubiculum or parasubiculum, after-discharges uniformly lagged simultaneously recorded events in deep layers of medial entorhinal cortex (Fig. 10). Latencies between the onset of the first or later after-discharges recorded from one location and the onset of corresponding after-discharges at another location ranged from 8 to 15 ms and depended upon the separation between the recording electrodes. For given recording configurations that compared after-discharges in deep layers of the entorhinal cortex with presubicular, parasubicular, or superficial entorhinal after-discharges, these latency values were constant.

Following the primary burst discharge, there could be one or more after-discharges. It was possible to eliminate the after-discharges selectively, with no effect on the primary burst response, by making a knife cut in the slice that disconnected medial entorhinal cortex from the presubiculum and parasubiculum (Fig. 10; see also Fig. 5). Such knife cuts, while they abolished after-discharges in presubicular and parasubicular recordings, did not eliminate after-discharges from recordings taken from deep layers of medial entorhinal cortex (Fig. 10). Lastly, these data illustrate the range of connectivity present within the horizontal slice.

DISCUSSION

There is a sharp functional separation of superficial layer and deep layer neurons of the presubiculum and parasubiculum of the rat. Electrophysiological and anatomical data are offered in support of a structural basis for the separation: ascending projections from deep layer neurons to superficial layer neurons are virtually absent. This finding distinguishes the presubiculum and parasubiculum from other six-layered cortices, including the entorhinal cortex, where a columnar organization is present.

The difference in function can be striking. All deep layer neurons exhibited stable synaptically supported bursting behaviour. Superficial layer neurons never did, unless there were intact connections with the subiculum or entorhinal cortex.

Local excitatory interconnections appeared to be more potent in the deep cell layers. The bursting behaviour of deep layer neurons was triggered by afferent stimulation or occured spontaneously after cells were disinhibited. The amplitude and duration of burst events were reduced by reducing the amount of deep layer tissue. These findings suggest that there are local excitatory interconnections among deep layer cells and that these contribute to the generation of the burst discharges. The absence of similar behaviour in superficial layer neurons of normal or disinhibited slices was taken by us as evidence of weak or absent excitatory interconnections among superficial layer cells.

Whereas superficial layer cells were found to project to the deep cell layers, a reciprocal projection appeared to be absent. Extracellular stimulation of superficial cell layers of the presubiculum or parasubiculum could evoke the deep layer bursts. Superficial layer cells were antidromically activated by deep layer stimulation. Axons of superficial layer neurons were traced into the deep cell layers. These findings suggest that at least some superficial layer neurons project into the deep layers where they may excite deep layer neurons. In contrast, even though all deep layer cells appeared to participate in the population bursts, excitatory synaptic potentials correlated with the bursts were never seen in superficial layer cells unless there were intact paths through the subiculum or medial entorhinal cortex. Attempts to antidromically activate deep layer neurons by superficial layer stimulation failed. In morphological studies, a single pyramidal cell axon collateral, from a group of thirty-one examined cells, was found to reach the superficial cell layers (layer I–II). Every superficial layer entorhinal neuron recorded showed EPSPs and firing correlated with deep layer entorhinal bursts. Deep layer medial entorhinal neurons with axon collaterals which reached the superficial cell layers were commonly observed.

Functional subdivisions in other cortical areas

In isocortical areas, a columnar organization of superficial and deep layer neurons is well established electrophysiologically and anatomically. For example, synchronized oscillations which originated in layer V were shown to occur synchronously in all cell layers (Silva, Amital & Connors, 1991). In slices exposed to 4-aminopyridine, deep layer neurons exhibited epileptiform responses to extracellular stimulation while superficial layer cells responded with small EPSPs and single action potentials (Barkai, Friedman, Grossman & Gutnick, 1995). This functional difference was attributed to differences in the patterns of inhibition in superficial *versus* deep layer cells (Kang, Kaneko, Ohishi, Endo & Araki, 1994; Barkai *et al.* 1995; van Brederode & Spain, 1995; Salin & Prince, 1996).

In the entorhinal cortex, interconnections between superficial layer and deep layer neurons have been demonstrated electrophysiologically and anatomically (Lorente de Nó, 1933; Köhler, 1986; Jones, 1990; Jones & Lambert, 1990). Projections from deep layer neurons to superficial layer neurons appear capable of relaying population burst activity of deep layer cells to the superficial layers (Jones & Lambert, 1990; this paper). As in the isocortical studies of similar activity, superficial layer cell responses were more limited than responses seen in deep layer neurons. Strong inhibition has been described in superficial layer entorhinal cells (Jones, 1993; Wouterlood, Härtig, Brückner & Witter, 1995), but detailed comparisons of inhibition in superficial *versus* deep layer cells have not been done.

Data on inhibitory circuits of the presubiculum and parasubiculum are also lacking. At least part of the functional separation of superficial and deep layers in these areas can, however, be attributed to an entirely different mechanism. There do not appear to be any significant ascending connections. Since the activity of deep layer neurons can reach the superficial layers via paths which include subicular neurons or entorhinal cortical neurons, there may be inhibitory control over these inputs in the same manner as in isocortical areas or the entorhinal cortex.

Clearly, it is difficult to prove the *absence* of a deep to superficial projection, especially in view of the fact that similar connections are present in other six-layered cortices. And, we were able to discover one axon collateral from a layer V pyramidal cell which ascended to layer I. This collateral did not appear to branch until it was through layer II. It is possible that this collateral, and others like it, did not synapse on superficial layer stellate or pyramidal cells such as those we recorded. This could explain the lack of orthodromic responses by superficial layer cells to deep layer cell activity. Even so, these collaterals would be activated with extracellular stimulation in the superficial cell layers when we were searching for antidromic responses in deep layer cells. Such responses were never found, suggesting that similar ascending collaterals may be rare.

Retrohippocampal circuit organization

Anatomically, projections from presubicular and parasubicular neurons of layers II and III have been shown to reach the entorhinal area: entorhinal layers I and III were labelled when anterograde tracer was injected into the presubiculum, and entorhinal layer II was labelled when tracer was injected into the parasubiculum (Köhler, 1985; van Groen & Wyss, 1990). In return, however, there was little in the way of reciprocal connections from superficial layer entorhinal neurons to superficial layer cells of the presubiculum or parasubiculum. Instead, deep layer (V) entorhinal neurons were shown to project to all layers of the presubiculum and parasubiculum (Köhler, 1986; van Groen & Wyss, 1990).

The most potent intrinsic connections (all excitatory), based on our electrophysiological data, appear to be: (1) connections among the deep layer neurons of the presubiculum and parasubiculum; (2) reciprocal connections between deep layer cells of the presubiculum–parasubiculum and medial entorhinal cortex; (3) reciprocal connections between deep layer cells of the presubiculum–parasubiculum and cells of the subiculum; and (4) non-reciprocal connections from superficial layer presubicular–parasubicular neurons to deep layer neurons. Weak synaptic responses by some superficial layer presubicular–parasubicular cells can be the result of any of the following: (1) the sparse projection from superficial cell layers of the entorhinal cortex (Köhler, 1986); (2) the projections of entorhinal layer V neurons to all layers of the presubiculum and parasubiculum (Köhler, 1986); (3) a relay in the subiculum; or (4) weak reciprocal connections from deep layer cells of the presubiculum and parasubiculum. We cannot, at this time, specify the extent to which each of these is present. Based on the data of this paper, we feel the last option has been eliminated.

With respect to participating neurons, every deep layer presubicular and parasubicular cell recorded in this study exhibited the long-duration burst response to afferent input, and as such is considered to participate in the excitatory network of deep layer neurons. In a previous study (Funahashi & Stewart, 1997), we showed deep layer cells to exhibit various morphologies, broadly classified as stellate and pyramidal, and that these cell classes were electrophysiologically similar.

Population bursts by deep layer neurons: comparison with entorhinal cortex

Jones and others (Jones, 1987; Jones & Heinemann, 1988; Jones & Lambert, 1990) have characterized the population burst discharges of entorhinal cortex. In magnesium-free medium or after application of convulsant to the bath, deep layer medial entorhinal neurons displayed large burst discharges that could be followed by after-discharges. Here we confirm this finding and identify the participation by presubicular and parasubicular neurons. There can be little doubt that our population events are identical to those originally described by Jones in entorhinal cortex. We have used this activity as a powerful tool to explore the connectivity of the presubicular and parasubicular cortices. While we have shown the the deep layer cells of the presubiculum and parasubiculum can generate a population event similar to that described for deep layer entorhinal neurons, there are differences in the deep layer events, and the behaviour of presubicular and parasubicular superficial layer cells does not parallel the behaviour of superficial layer entorhinal neurons.

Primary events could be triggered in deep layer neurons of the entorhinal cortex or presubiculum–parasubiculum by any of their excitatory inputs. In fact, we used this finding, together with antidromic responses by superficial layer neurons to deep layer stimulation, to argue for substantial superfical layer to deep layer connectivity within the presubiculum and parasubiculum. Our morphological studies indicate that superficial layer pyramidal cells project into the deep layers. Descending collaterals of some superficial layer stellate cells may also reach the deep layers.

One notable difference between primary burst responses by deep layer entorhinal neurons, as compared with deep layer presubicular–parasubicular cells, was the relative prominence of very-high-frequency components (~200 Hz) especially in the beginning of events seen in field potential recordings taken from entorhinal cortex. It is interesting to speculate that this represents the frequency of firing within intrinsic burst events exhibited by deep layer entorhinal neurons. Deep layer presubicular and parasubicular neurons were never found to burst in response to direct current injection (this paper; Funahashi & Stewart, 1997).

Perhaps the biggest difference between the entorhinal cortex and the presubiculum–parasubiculum is the absence of involvement by superfical layer presubicular and parasubicular neurons in these population events. Responses by superficial layer entorhinal neurons are much more limited than responses by deep layer entorhinal neurons (Jones & Lambert, 1990), but they are much more robust than the responses by superficial layer neurons of the presubiculum and parasubiculum (this paper). Many of our recorded superficial layer cells showed no apparent response at all, in spite of the fact that the deep layer population bursts appear to involve every cell in the deep layers. The breadth of the deep layer cell response is what makes the event such a powerful tool for searching for deep layer to superficial layer connectivity. The weak synaptic responses in superficial layer presubicular and parasubicular cells that were occasionally seen appeared to be entirely due to interconnections with subicular or superficial layer entorhinal neurons.

Is the primary burst an *in vitro* sharp wave?

In recordings from single neurons in freely behaving rats, individual deep layer presubicular and parasubicular neurons exhibited high-frequency firing that was clearly correlated with sharp waves recorded in the hippocampus (Chrobak & Buzsáki, 1994) or locally recorded in the presubiculum or parasubiculum (Chrobak & Buzsáki, 1996). Superficial layer cells in these animals did not fire in relation to the sharp wave. It is remarkable that the *in vitro* demonstration of patterns of cellular involvement corresponds completely with the *in vivo* sharp wave data.

The second point of similarity is the composition of the primary burst event itself. As described above, the initial evoked event consisted of a brief period of firing by individual cells, followed by a period of sustained depolarization without firing, and ending with a variable period of synchronous activity at 40–100 Hz. These three major components can be seen in intracellular recordings as well as the field potential recordings. Precisely the same pattern of activity is found *in vivo*: an early spike associated with firing by individual extracellularly recorded neurons, a slow wave, and what has been referred to as the 'gamma-frequency tail' (e.g. Traub, Whittington, Colling, Buzsáki & Jefferys, 1996). We must make a cautionary note that while the pattern of the primary burst *in vitro* matches the pattern of the sharp wave *in vivo*, we cannot claim that the underlying mechanisms are identical.

The third point of similarity is that the *in vivo* sharp wave appears to be triggered in presubicular and parasubicular neurons by events which are relayed in Ammon's horn and the subiculum (Chrobak & Buzsáki, 1996). We found that any of the inputs to the deep layer cells could trigger the population burst.

Two findings that our *in vitro* preparation has already provided, which would have been difficult to obtain *in vivo*, are: (1) every deep layer cell (stellate and pyramidal) appears capable of participation in these population events; and (2) no deep layer cell exhibits a simple intrinsic burst response – all cells appear to be regular spiking cells. An *in vitro* model of the sharp wave will permit experimental access to a complex population event which has been implicated in normal memory formation (e.g. Buzsáki, 1989) and which may reveal more of the complexities of hippocampal formation circuitry.

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Author's email address

M. Funahashi: makoto@theta.hippo.hscbklyn.edu

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