Relationships between morphology and physiology of pyramid–pyramid single axon connections in rat neocortex *in vitro*

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1. Double intracellular recordings were made from 1163 pairs of pyramidal neurones in layer V–VI of the rat somatomotor cortex *in vitro* using sharp electrodes filled with biocytin. Monosynaptically connected pairs of cells were identified when an action potential in one could elicit a constant latency excitatory postsynaptic potential (EPSP) in the other and the cells were filled with biocytin. Labelled cells were subsequently identified histologically with avidin–horseradish peroxidase.

2. Thirty-four pairs of cells were found to be monosynaptically connected. Fifteen of these pairs were sufficiently stable for electrophysiological recordings and three of these were recovered sufficiently to permit full morphological reconstruction.

3. The EPSP recorded between the first pair of pyramids varied in amplitude between 0 and 3 mV (mean 1·33 ± 1·06 mV) and fluctuated considerably (coefficient of variation, 0·796). This was largely due to a high incidence of apparent failures of transmission. On reconstruction two boutons from the presynaptic pyramid axon were in close apposition to the proximal portions of basal dendrites of the postsynaptic cell.

4. In the second pair of pyramids the EPSP had a mean amplitude of 1·06 mV, and displayed a 10–90% rise time of 2·8 ms and a width at half-amplitude of 23 ms. This EPSP did not alter significantly with changes in membrane potential at the soma. The presynaptic axon closely apposed the distal apical dendrite of the postsynaptic cell in eight places.

5. In the third pair of pyramids, the EPSPs, recorded at a relatively depolarized membrane potential, were long lasting and could elicit slow dendritic spikes with long and variable latencies. These slow spikes suggested that the postsynaptic recording site was dendritic and on reconstruction a possible location was identified on the apical dendrite. A total of five presynaptic boutons closely apposed three separate, proximal branches of the postsynaptic apical dendrite.

6. These results provide the first illustration of a morphological basis for variations in functional properties of pyramid–pyramid connections in the neocortex.

The existence of local axon collaterals of pyramidal cells of the neocortex has been known for a considerable time (Ramón Y Cajal, 1899; Lorente de Nó, 1922; Sholl, 1955; Szentágothai, 1965, 1973; Scheibel & Scheibel, 1970; Lund, 1973). However, the full extent of the arborizations of local axon collaterals of pyramidal cells has only recently been revealed using intracellular injection of tracers such as horseradish peroxidase and biocytin (for discussion see Gilbert & Weisel, 1979, 1983; Feldman, 1984; Martin & Whitteridge, 1984; Noda & Yamamoto, 1984; Kisvárday, Martin, Freund, Maglóczky, Whitteridge & Somogyi, 1986; Gabbot, Martin & Whitteridge, 1987; McGuire, Gilbert, Rivlin & Weisel, 1991).

Initial indications that some of the pyramidal axon collaterals innervate other pyramidal neurones were obtained indirectly. Electron microscopic examination of pyramidal axon collaterals visualized by Golgi labelling or intracellular filling revealed that they gave rise to asymmetric synapses, a varying proportion of which were with dendritic spines (Feldman, 1984). Since pyramids are the most abundant spiny cells in the mammalian neocortex, many of these dendritic spines outside layer IV could be assumed to belong to other pyramidal cells (for discussions see Feldman, 1984; White, 1989; DeFelipe & Farinas, 1992).

In a few noteworthy studies the neurones postsynaptic to pyramidal cell axons have been conclusively identified

as other pyramids (Kisvárday *et al.* 1986; Gabbot *et al.* 1987; McGuire *et al.* 1991; Kisvárday & Eysel, 1992). By examining serial ultra-thin sections, Gabbot *et al.* (1987) studied the postsynaptic targets of two intracellularly filled pyramidal neurones in layer V of the cat visual cortex and suggested that they formed a maximum of four synapses with any one other pyramidal neurone. In an analogous study, McGuire *et al.* (1991) studied axons of pyramidal cells in the superficial layers of monkey area 17 and suggested that a maximum of two boutons were involved in contacts from one pyramid to another. Similarly, the synaptic targets of horseradish peroxidase (HRP)-filled layer III pyramidal cells in the cat striate cortex were identified as pyramidal dendrites by virtue of their morphology, and only one input was observed onto any one pyramidal dendrite (Kisvárday *et al.* 1986). However, the impracticalities associated with reconstruction of the entire unlabelled postsynaptic neurone restricted these studies to localized areas of the postsynaptic pyramids.

To our knowledge, to date only one study has fully identified both pre- and postsynaptic pyramids (Kisvárday & Eysel, 1992). Following extracellular injection of biocytin in layer III of cat visual cortex, labelled neurones were entirely filled and could be completely reconstructed. Although synaptic connections were not verified by electron microscopy, the number of apparent connections between pyramidal neurones in 'patches' 500 μm to 1 mm apart most often numbered one to four.

Electrophysiological techniques have also been used to study local circuit connections between neocortical pyramidal neurones, particularly in the rat (Mason, Nicoll & Stratford, 1991; Thomson & West, 1993; Thomson, Deuchars & West, 1993). In these studies, intracellular recordings were obtained from one cell identified as pyramidal by its electrophysiological characteristics, and from simultaneous intracellular recordings made sequentially from other pyramids in the same area. Synaptic connections between two pyramids were identified when an action potential in one pyramid elicited a constant latency excitatory postsynaptic potential (EPSP) in the other pyramid.

Analysis of EPSPs obtained with double intracellular recordings of connected pyramidal neurones in the deep layers of the rat somatomotor cortex has revealed some variations in their response properties. The average amplitudes of EPSPs evoked in one pyramid by action potentials in one other varied from $< 0{\cdot}1$ to 7 mV, which might indicate, for example, that some connections consisted of very few boutons, while others involved many contacts. The shape indices of these EPSPs also varied widely: the 10–90% rise time from 1 to 5 ms and the width at half-amplitude from 7·2 to 30 ms at somatic membrane potentials between −70 and −80 mV, perhaps suggesting that some connections involved proximal targets while others were more distally placed (Rall, Burke, Holmes, Jack, Redman & Segev, 1992). Another prediction arising from these electrophysiological studies was that all synaptic inputs contributing to a single pyramid–pyramid connection were at similar electrotonic distances from the postsynaptic soma (Thomson *et al.* 1993). Electrophysiological studies therefore suggest a wide diversity in the number and position of synapses comprising the connection between two pyramidal cells, but electrophysiology alone cannot confirm if this diversity is due to morphological differences.

In the present experiments we have studied the electrophysiology and anatomy of connections between pyramidal neurones in layer V of the rat motor cortex. Paired intracellular recordings have been made from pyramidal neurones *in vitro* and the recorded cells identified morphologically following intracellular injection of biocytin. A preliminary report has been published (Deuchars & Thomson, 1993).

METHODS

Male rats weighing 120–180 g were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (Sagatal; 60 mg kg^{-1}). They were perfused transcardially with 50–100 ml ice-cold artificial cerebrospinal fluid (ACSF) with added sodium pentobarbitone (6 mg ml^{-1}). The brains were removed and 400 μm coronal sections of the cortex were cut on a Vibroslice (Campden Instruments, Loughborough, UK). The slices were maintained at the interface between ACSF and warm, humidified 95% O_2–5% CO_2. The ACSF contained (mM): 124 NaCl, 25·5 $NaHCO_3$, 3·3 KCl, 1·2 KH_2PO_4, 1·0 $MgSO_4$, 2·5 $CaCl_2$ and 15 D-glucose at 34–35 °C.

Intracellular recordings were made with glass microelectrodes filled with 2 M potassium methyl sulphate plus 2% biocytin or 2% neurobiotin (resistance 80–160 MΩ). A third microelectrode of similar properties was connected to the bath headstage of the Axoprobe (Axon Instruments, Burlingame, CA, USA) positioned close to the two intracellular electrodes and adjusted to reduce capacitance coupling artifacts.

Search strategy

The first step was to obtain a stable intracellular recording from one pyramidal neurone, identified by its electrophysiological characteristics, in a region where no other cells had been previously penetrated. Subsequently, second intracellular recordings were made from other pyramidal neurones in the vicinity (see Fig. 1). Action potentials were evoked in one of the cells and then in the other, and any postsynaptic response elicited monitored. Only those pairs in which the postsynaptic response was visible above the baseline noise on at least some of approximately fifty trials were pursued. These second recordings were discarded as rapidly as possible if these trials were negative to reduce inadvertent dye filling of unconnected neurones. Positions of penetrated but rejected neurones were drawn on a representative section of the slice. Full details of the collection and analysis of responses are given in Thomson & West (1993).

The necessity for a search strategy and the first objective of these experiments, i.e. to label pairs of synaptically connected

neurones, compromised the quality of electrophysiological data that could be collected. This is particularly apparent in the baseline noise illustrated in Figs 4 and 6, which obscured EPSP amplitude fluctuations, in the capacitance coupling artifacts in Figs 2, 4 and 6 and in the relatively brief recording periods for the three illustrated pairs. A more complete picture of pyramid–pyramid connections can be found elsewhere (Thomson *et al.* 1993) and the present data should be viewed in this context.

Once sufficient data had been collected for analysis, biocytin was ejected from the electrode by 500 ms pulses of 0·5–1 nA of positive current at a frequency of 1 Hz for 5–15 min. The electrodes were left inside the cells for as long as stable recordings could be maintained. If the intracellular recordings were maintained for 30–60 min after the initial dye ejection, the same ejection parameters were applied for 2–5 min prior to withdrawing the electrode. The position of the electrodes in the slice was drawn on a representative section, and the electrodes slowly withdrawn from the cells. With the two recording electrodes in position just above the slice, the slice was trimmed around the location of the cells and one corner removed to aid its subsequent orientation. The trimmed portion was removed from the bath, sandwiched between two Millipore filters and immersed overnight at 4 °C in 2% glutaraldehyde–2% paraformaldehyde in 0·1 M phosphate buffer (PB).

Twelve to fourteen hours following fixation the slice was washed twice for 10 min each in 0·1 M PB. The slice was then washed in 0·1 M PB plus 10% sucrose for 10 min, followed by 0·1 M PB plus 20% sucrose for 30 min, placed in a plastic capsule and dipped in liquid nitrogen for approximately 20 s. After thawing for a few minutes the slice was removed from the plastic capsule and washed twice in 0·1 M PB. The slice was then flat embedded in gelatin and sectioned at 60–80 μm on a vibratome. The cut sections were then washed in phosphate-buffered saline (PBS) twice prior to incubating in avidin–biotinylated horseradish peroxidase complex (ABC, Vector Labs, Peterborough, UK) at 4 °C for 12–14 h on a shaker. Following incubation, the sections were washed twice in PBS and twice in Tris buffer. On a third wash in Tris, 3,3′-diaminobenzidine tetrahydrochloride (50 mg (100 ml)$^{-1}$) was added to the solution and the vials kept in the dark. After 10 min, 10 μl of 1% H_2O_2 was added to each millilitre of solution in the vials and the reaction allowed to proceed for 10 min in the dark before it was terminated by washing the sections in Tris buffer.

Following the reaction, the sections were washed twice in 0·1 M PB and placed in 1% osmium tetroxide (TAAB, Aldermaston, UK) made in 0·1 M PB for 1 h, following which they were washed once in 0·1 M PB and then in distilled water. Superfluous attached gelatin was removed with a scalpel blade. The sections were subsequently placed in 1% uranyl acetate in distilled water for 1 h and then washed in distilled water. The sections were then flattened between a glass slide and a coverslip and dehydrated in an ascending series of alcohols before being transferred to glass vials. Following two 15 min washes in propylene oxide (Merck, Poole, UK) the sections were embedded in Durcupan ACM resin (Fluka, Gillingham, UK). Approximately 12 h after embedding, the resin was heated and the sections removed and placed on glass slides. A coverslip was carefully placed on top of the sections and the slides cured in an oven at 60 °C for 48 h. Once cured, the slides were examined at the light microscopic level. Labelled cells were then reconstructed using a drawing tube and photographed to provide a permanent record.

RESULTS

Pyramidal neurones were identified by their established electrophysiological properties (McCormick, Connors, Lighthall & Prince, 1985). All cells which exhibited these electrophysiological characteristics and which were fully recovered were subsequently identified morphologically as pyramidal neurones.

In twenty-five experiments (100 slices) 1163 pairs of pyramids were tested for synaptic connections (2326 one-way tests). Thirty-four pairs of cells were found to be monosynaptically connected, as an action potential in one could elicit a constant-latency EPSP in the other. Of these connected pairs, fifteen were sufficiently stable to record, but following histological processing, only three of these pairs were recovered sufficiently to allow full reconstruction. In the other cases only one of the two neurones was clearly labelled, or both were labelled but the presynaptic axon was poorly filled, or too many neurones were labelled to allow unambiguous identification of the recorded pair. In the three pairs of neurones illustrated here, the entire presynaptic axon and the entire postsynaptic dendritic tree were drawn to ensure that all potential synaptic contacts were identified. For clarity, only the branch(es) of the presynaptic axon that made close appositions with the postsynaptic neurone are illustrated.

In the first of the recovered pairs both somata were located in layer V and the soma of the presynaptic pyramid was approximately 50 μm directly above, i.e. superficial to the postsynaptic neurone (Fig. 1). Two boutons from axon collaterals of the presynaptic pyramidal cell closely apposed separate basal dendrites of the postsynaptic pyramid (Fig. 1). Electrophysiological recordings (Fig. 2) revealed that a single presynaptic action potential evoked an EPSP at a latency of 2 ms which varied in amplitude between 0 mV (apparent failures of transmission) and 3 mV (mean 1·33 $\pm$ 1·06 mV; see Fig. 2*B*) at a postsynaptic membrane potential of -79 ± 2 mV. This EPSP fluctuated considerably as shown by the large standard deviation time course (SDTC; Fig. 2*A*) and its coefficient of variation of 0·796. The EPSP displayed a 10–90% rise time of 2·3 ms and a width at half-amplitude of 12 ms, although the rise time is at best an approximation due to capacitance coupling artifacts. When the presynaptic firing rate was increased from 0·33 to 1 Hz a larger proportion of presynaptic spikes apparently failed to elicit a postsynaptic response (Fig. 2*C*). After > 15 min, during which few presynaptic spikes were evoked, presynaptic firing at 0·33 Hz again elicited EPSPs (Fig. 2*C*).

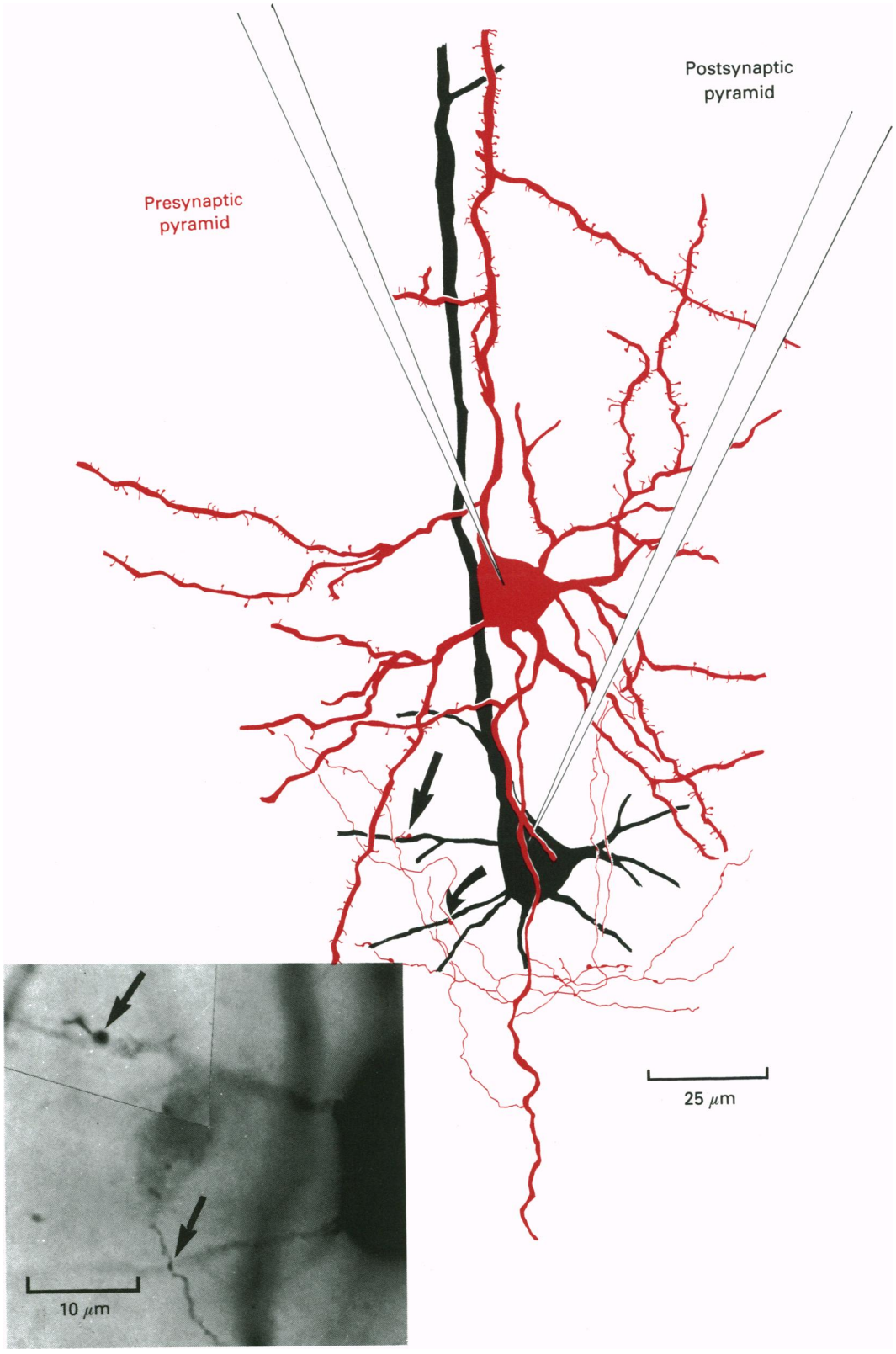

Figure 1. The axon of a layer V pyramidal neurone apposes the basal dendrites of another in two places

Reconstruction of a pair of connected pyramidal cells in layer V of the rat motor cortex. For clarity, only part of the axons and some of the dendrites are shown. The electrophysiological characteristics of this connection are illustrated in Fig. 2. The drawing also illustrates the procedure used in these

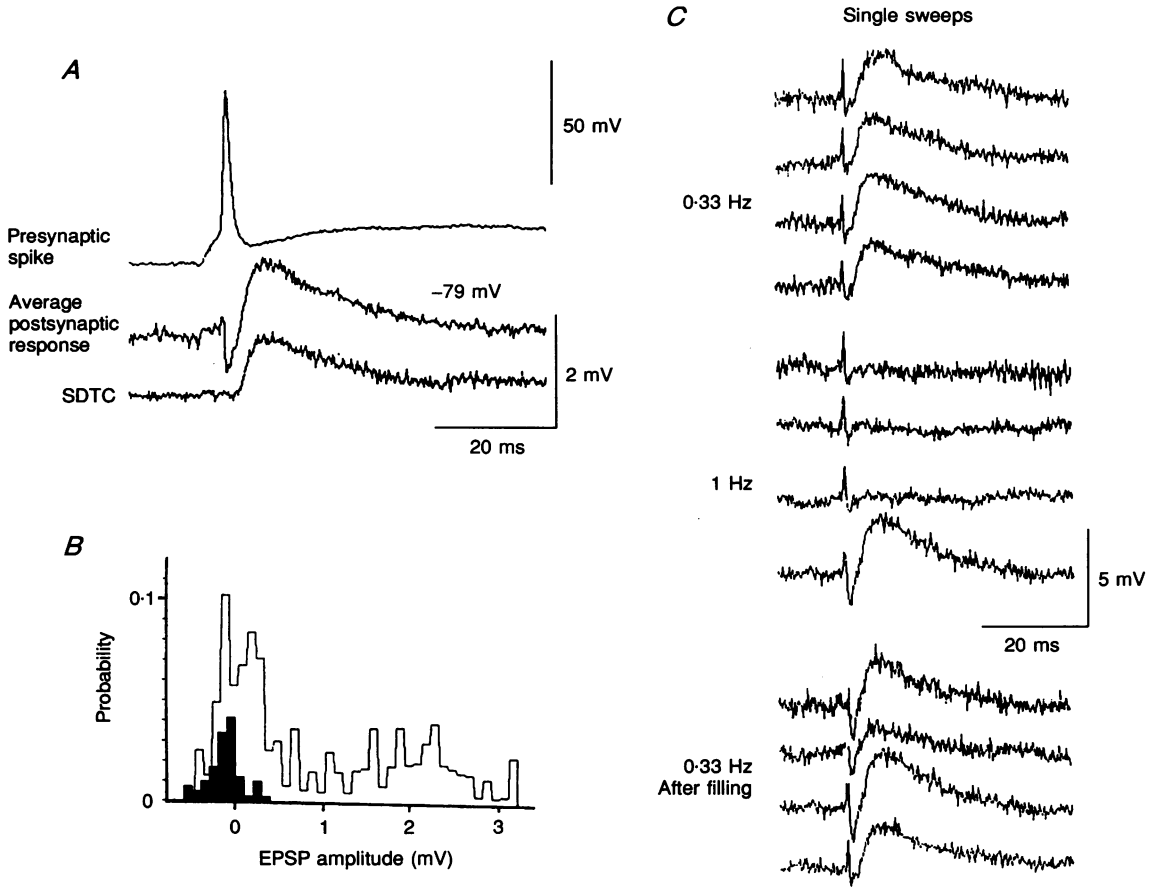

Figure 2. Postsynaptic responses associated with two close appositions on the postsynaptic basal dendrites display many apparent failures of transmission

Synaptic potentials elicited in the postsynaptic pyramid by action potentials in the presynaptic pyramid illustrated in Fig. 1. *A*, a single presynaptic spike, an average of the postsynaptic response (sweeps triggered from the rising phase of the presynaptic spike) and the standard deviation time course (SDTC). This EPSP fluctuated considerably in amplitude as evidenced by the large amplitude of the SDTC. *B*, the EPSP amplitude distribution. The filled bars indicate the distribution of the noise. *C*, single sweep records. The upper four sweeps were obtained early in the recording period with a presynaptic firing rate of 0·33 Hz. The middle four sweeps were obtained during a period of 1 Hz presynaptic firing. During this period many spikes apparently failed to elicit a postsynaptic response, indicating transmission failure. The two recorded neurones were then filled with biocytin for 15 min, during which time no presynaptic action potentials were elicited. The lower four records were obtained after filling, at a presynaptic firing rate of 0·33 Hz. The large SDTC, the peak around 0 mV in the amplitude distribution and the proportion of single sweeps in which no postsynaptic response was elicited might indicate either a very low probability of transmitter release, or that a small number of release sites contributed to this EPSP. The latter may correlate with the small number of close appositions, i.e. 2, observed between the presynaptic axon and postsynaptic dendrites (Fig. 1).

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experiments: intracellular recordings were made from a pair of pyramidal cells and when an action potential in the presynaptic pyramid (shown in red) evoked a short, constant-latency EPSP in the postsynaptic pyramid (drawn in black), the cells were considered monosynaptically connected. Following electrophysiological recordings, the neurones were filled with biocytin and processed for microscopy. The two neurones were located in layer V and the presynaptic pyramid was situated directly above the postsynaptic pyramid, suggesting that they were located in the same column. When reconstructed, two boutons from axon collaterals of the presynaptic pyramid closely apposed basal dendrites of the postsynaptic pyramid (arrows). The inset photomontage shows the labelled boutons of the presynaptic axon (arrows) in close proximity to the labelled dendrites of the postsynaptic pyramid.

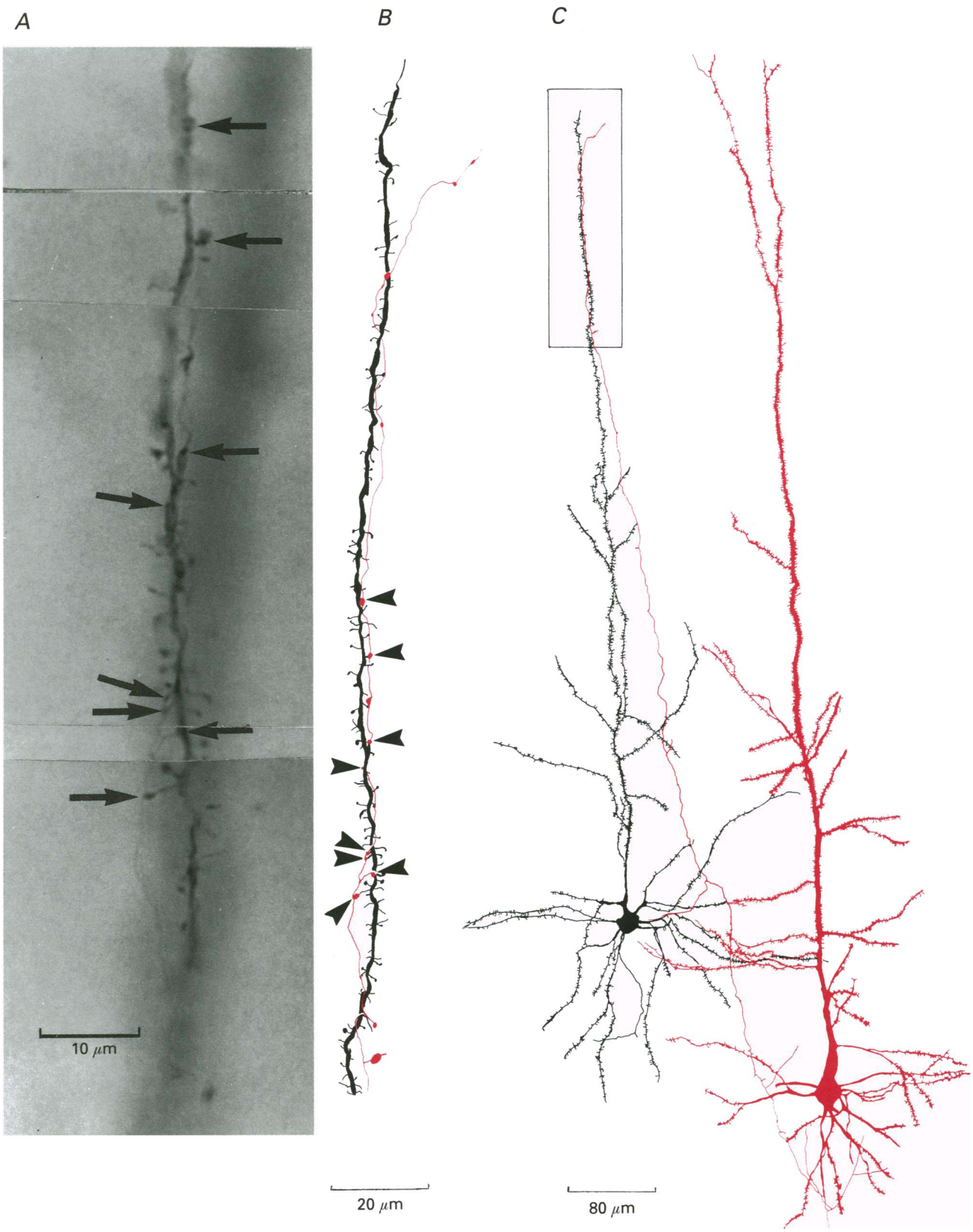

Figure 3. For legend see facing page.

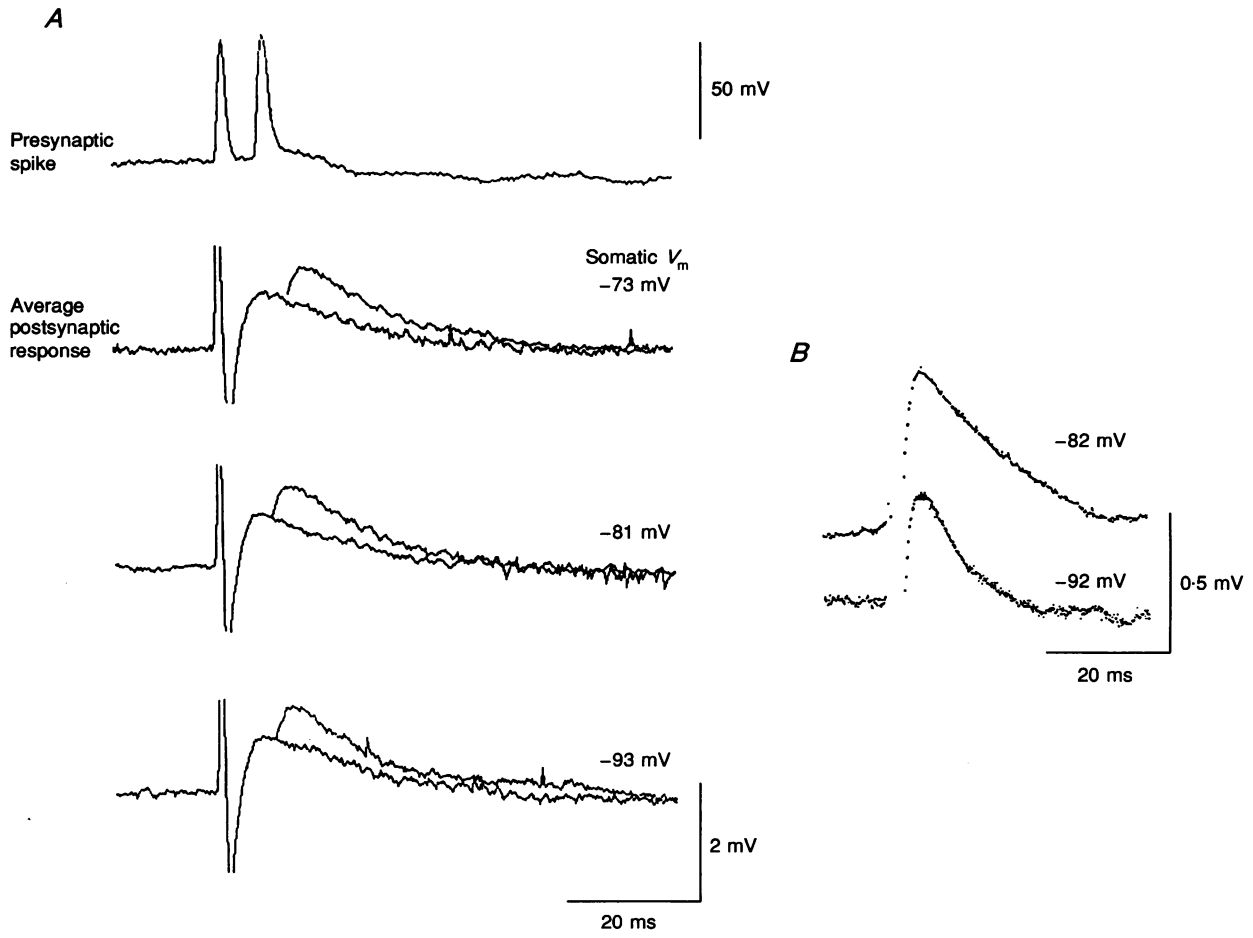

Figure 4. EPSPs associated with close appositions on the distal apical dendrite are insensitive to changing somatic membrane potential

A, synaptic potentials elicited in the postsynaptic pyramid by action potentials in the presynaptic pyramid illustrated in Fig. 3. The upper record shows a single presynaptic sweep, the lower records are averages of the postsynaptic response to single presynaptic spikes and to pairs of spikes. Superimposed averages were triggered from the rising phase of single presynaptic spikes and from the rising phase of the second of a pair of spikes. Each pair of averages was obtained from a period of recording in which the postsynaptic membrane potential was held within 2 mV of a pre-set value (−73 mV upper records, −81 mV and −93 mV lower records). This wide range of postsynaptic membrane potentials produced little change in either the amplitude or time course of this EPSP. *B*, averages of an EPSP recorded from a different pair of pyramidal neurones illustrated for comparison. This EPSP, which is more typical of pyramid–pyramid connections and whose time course was relatively rapid, increased significantly in amplitude and duration with 10 mV postsynaptic membrane depolarization. In *A* and *B* the postsynaptic recording was obtained from the soma of the postsynaptic neurone. Changes in postsynaptic membrane potential are therefore changes in somatic membrane potential (somatic V_m).

Figure 3. The axon of a layer V pyramidal neurone apposes the distal apical dendrite of another in eight places

Panel *C* shows a reconstruction of two connected pyramidal neurones in layer V of the rat motor cortex. For clarity, only the presynaptic axon apposing the postsynaptic cell is fully illustrated. The electrophysiological characteristics of this connection are illustrated in Fig. 4. Both the pre- and postsynaptic cell were located in layer V and were separated horizontally by approximately 200 μm. The presynaptic pyramid (in red) provided an axon collateral which travelled to layers II–III before nearing the apical dendrite of the postsynaptic pyramid (in black). *B*, an expansion of the boxed area of the apical dendrite of the postsynaptic pyramid shown in *C*. Some boutons of the presynaptic axon (in red) closely appose (arrowheads) the postsynaptic dendrite (black). *A*, photomontage illustrating that labelled boutons of the presynaptic axon closely appose (arrows) the distal apical dendrite of the postsynaptic pyramid in eight places.

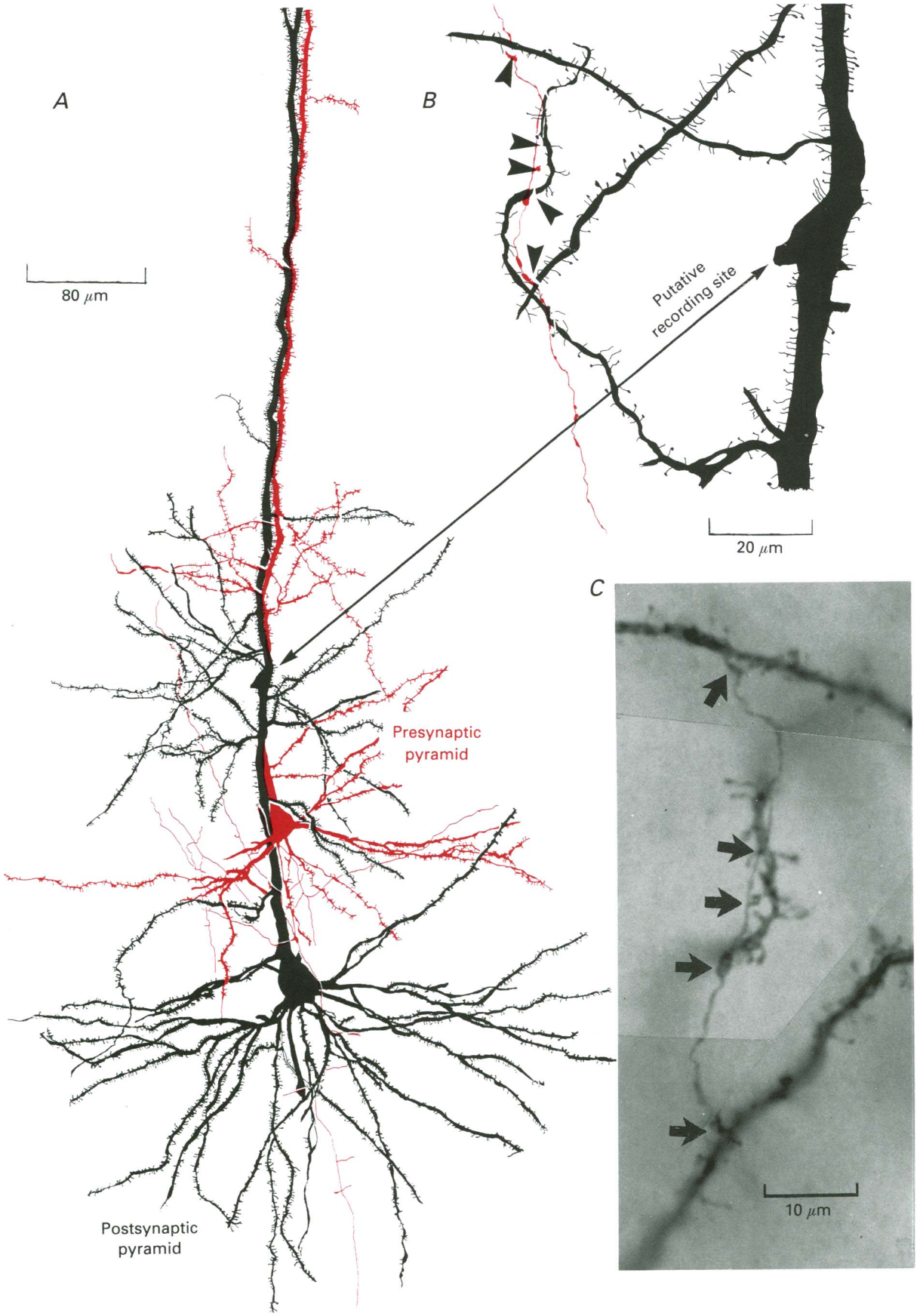

Figure 5. For legend see facing page.

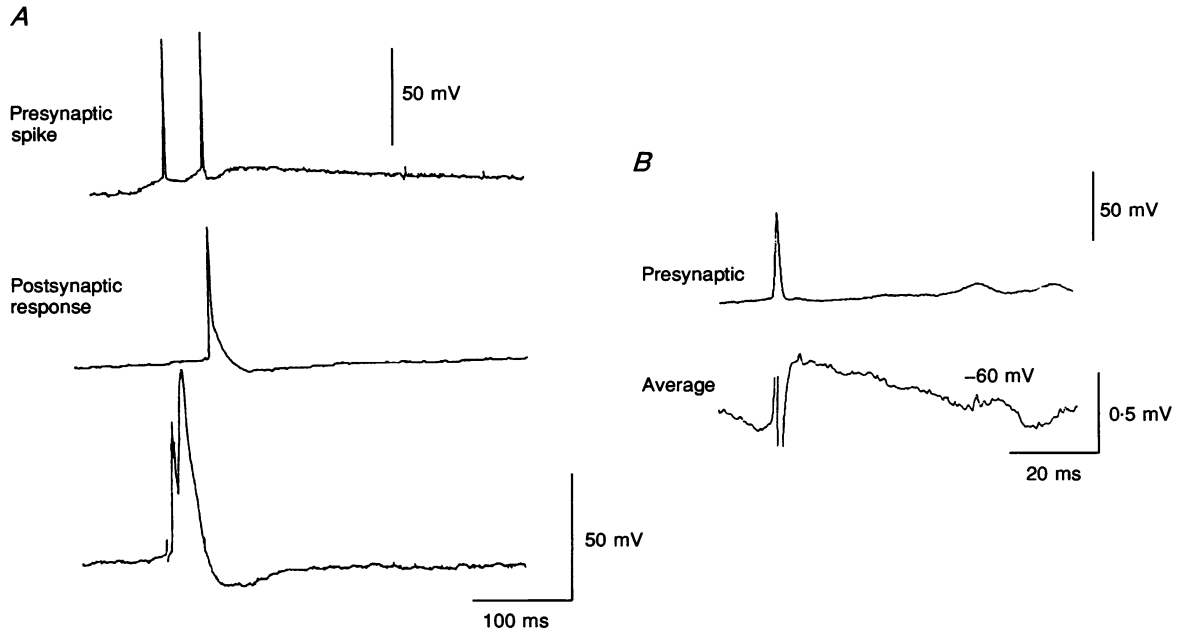

Figure 6. Postsynaptic responses recorded in the apical dendrite involve dendritic action potentials

A, single sweep postsynaptic responses (lower two records) elicited by pairs of action potentials in the presynaptic pyramid (top records) illustrated in Fig. 5 and recorded from the postsynaptic pyramidal dendrite (see arrow Fig. 5*B*). The postsynaptic membrane could not be held securely at potentials more negative than −60 mV and EPSPs elicited fast and slow spikes which exhibited varying latencies, shapes and durations. *B*, some of the smallest EPSPs elicited by single presynaptic action potentials that did not lead to slow spikes were averaged. The slow decay of the averaged EPSP may reflect the relatively depolarized postsynaptic membrane potential at which these responses were recorded.

The somata of the second pair of connected pyramidal neurones were located in layer V (Fig. 3*C*). The presynaptic soma was located approximately 240 μm below and 200 μm lateral to the postsynaptic soma (Fig. 3*C*). Despite axonal arborization in layer V (not illustrated, for clarity), an axon collateral of the presynaptic pyramid coursed to layer II–III before it neared the distal apical dendrite of the postsynaptic pyramid (Fig. 3*B* and 3*C*). At this level, boutons from the presynaptic axon collateral closely apposed the apical dendrite of the postsynaptic cell in eight places (Figs 3*A* and *B*). When action potentials in the presynaptic pyramid were elicited by current injection, short, constant-latency EPSPs were recorded in the postsynaptic pyramid (Fig. 4*A*). This EPSP had an average amplitude of 1·06 mV and single sweeps appeared to contain few failures of transmission. However, high noise levels precluded accurate fluctuation analysis. The EPSP

Figure 5. The axon of a layer V pyramidal neurone apposes three apical dendritic branches of another in five places

Illustration of connected pyramidal neurones in layer V of the rat motor cortex. *A*, reconstruction of the two pyramidal neurones. Drawings of the electrode positions made during recording indicated that the postsynaptic recording site was above the presynaptic recording site, but the soma of the postsynaptic neurone (in black) was below that of the presynaptic neurone (in red). These neurones were the first two penetrated in this area and there were no other labelled neurones present; therefore they must be the two recorded pyramids. Further support comes from the electrophysiological demonstration that the postsynaptic recording site was likely to be dendritic, as action potentials in the presynaptic pyramid elicited slow EPSPs and slow dendritic spikes in the postsynaptic neurone (see Fig. 6). *B*, an enlargement of the area in *A* indicated by the arrow. The possible postsynaptic recording site was identified anatomically by the expansion of the apical dendrite at one region (arrow). A labelled axon collateral from the presynaptic pyramid (in red) apposed (arrowheads) the dendrites of the postsynaptic neurone (in black). *C*, photomontage demonstrating that the labelled boutons of the presynaptic axon collateral closely appose dendrites of the postsynaptic neurone at five sites (arrows).

displayed an average 10–90% rise time of 2·8 ms and width at half-amplitude of approximately 23 ms. This EPSP did not vary significantly in amplitude or in time course with changes in membrane potential at the postsynaptic soma (Fig. 4*A*), suggesting that it was generated distally from the recording site. This lack of response of the EPSP to current injections at the postsynaptic soma was in contrast to observations in more typical pyramid–pyramid connections, one of which is illustrated for comparison in Fig. 4*B*.

The labelled somata of the third pyramidal pair were also located in layer V, and the presynaptic pyramid was located approximately 80 μm directly superficial to the soma of the postsynaptic pyramid (Fig. 5*A*). These neurones appeared to be connected through an axon collateral from the presynaptic pyramid which furnished three separate postsynaptic dendrites with a total of five closely apposing boutons (Fig. 5*B* and *C*). Action potentials in the presynaptic pyramid evoked slow, long lasting EPSPs (Fig. 6*B*) which could elicit slow dendritic spikes with variable latencies (Fig. 6*A*) in the postsynaptic pyramid. The postsynaptic membrane potential was close to −60 mV and could not be hyperpolarized further without introducing significant electrode noise. The slow postsynaptic spikes suggested that the postsynaptic recording site was dendritic. This was supported by reconstruction of the postsynaptic cell which disclosed an enlargement of the apical dendrite in the region where drawings of electrode positions during the experiment had placed the postsynaptic recording site (Fig. 5*A* and *B*).

DISCUSSION

These experiments illustrate that the variations in electrophysiological properties of connections between pyramidal neurones in layer V of the rat motor cortex are paralleled by diversity in morphological features of contacts between pyramidal cells.

Technical considerations

A major technical consideration of this study was the accurate morphological identification of the two neurones that had been electrophysiologically identified as connected. This difficulty arose because cells that were recorded and rejected were sometimes filled inadvertently with biocytin. Consequently, we developed a procedure that allowed us to identify those two connected cells reliably. Firstly, drawing the positions of electrodes on maps representative of the slices accurately predicted the location of labelled neurones on subsequent histological examination of sections. All cells which were penetrated and recorded but rejected were also drawn on these maps. Secondly, cutting a corner from the slices allowed sections subsequently obtained from them to be oriented correctly, enabling the positioning of the filled cells to be correlated with the drawing. It is possible that some real connections between pairs were not evident electrophysiologically as their EPSPs were too small to distinguish from noise levels, but we have yet to observe a connection morphologically where none was apparent physiologically, even when several neurones were filled in a restricted area.

Morphological considerations

The finding that pyramids innervate other pyramids with as many as eight possible contacts (Fig. 3) is contrary to previous suggestions. Szentágothai (1975, 1979) predicted pyramidal neurones to be interconnected by one or very few contacts by extrapolating from observations on Golgi-impregnated material. Similar numbers of contacts were proposed by others who combined intracellular filling of a single axon with electron microscopy (Kisvárday *et al.* 1986; Gabbott *et al.* 1987; McGuire *et al.* 1991). The apparent discrepancy between the number of contacts observed in these previous studies and the present study may be explained by the procedures used to reveal connections. In most previous investigations, the postsynaptic targets of intracellularly labelled pyramids have not been labelled in any way and have been identified by serial reconstruction at the ultrastructural level. As it is not feasible to reconstruct whole neurones in this manner, it is impossible to be certain that all the potential contacts from one pyramid to another have been identified.

In our experiments, it is likely that the entire pre- and postsynaptic neurones have been visualized. The necessity for labelling the entire postsynaptic neurone is illustrated by the examples in Figs 1 and 5, where the presynaptic axon closely apposed separate dendrites of the postsynaptic neurone, similar to observations of Kisvárday & Eysel (1992; see their Fig. 4*A* and *D*). Although we did not verify synaptic contacts with electron microscopy, monosynaptic connections between pyramids were demonstrated electrophysiologically and some of the close appositions between the presynaptic axon and postsynaptic dendrites illustrated in Figs 1, 3 and 5 are likely to be the areas of contact. We can also exclude the possibility that connections with other parts of the dendritic tree contributed to recorded events.

Relationships between physiology and morphology

In a previous study, we demonstrated that local circuit pyramid–pyramid connections in the deep layers of the rat motor cortex display a wide range of physiological properties (see Thomson *et al.* 1993). The present study indicates that these variations might be accounted for by heterogeneity in the anatomical aspects of pyramid–pyramid connections. Closer examination of the pairs described here illustrates how these anatomical and physiological aspects may be related.

In the first pyramid–pyramid pair shown (Figs 1 and 2), boutons from the presynaptic axon were in close apposition to basal dendrites of the postsynaptic cell (Fig. 1). The characteristics of the EPSP recorded in the postsynaptic cell in response to action potentials in the presynaptic cell were in accordance with the anatomy (Fig. 2): the modest average amplitude of the EPSP (1·33 mV) which was in the middle of the range observed previously (< 0·5 mV to > 5 mV; Thomson *et al.* 1993) and the relatively brief half-width 12 ms (cf. a range of 6–23 ms for a larger population in Thomson *et al.* 1993) suggested that a small number of boutons were involved which were close to the recording site. A small number of boutons was also indicated by the high incidence of apparent failures of transmission and the considerable degree of sweep-to-sweep fluctuation in this EPSP as indicated by the large standard deviation time course (SDTC) and the c.v. of 0·796 (c.v. range reported previously, < 0·1 to 0·8; Thomson *et al.* 1993). The shape of the SDTC paralleled that of the average EPSP, signifying that there was little sweep-to-sweep variation in EPSP shape and implying that these EPSPs were generated at the same electrotonic distance from the recording site (in this case the soma). Although the two apparent contacts are with separate dendrites, they are indeed at similar distances from the soma.

In the second pair of pyramids, the observation of up to eight presynaptic boutons apposing the distal apical dendrite of the postsynaptic pyramid can also be correlated with the electrophysiological characteristics of the connection (Figs 3 and 4). The average EPSP amplitude was again relatively small (1·06 mV). The shape indices of this EPSP are relatively slow and did not change significantly with current injection at the soma (Fig. 4). In a previous study the time course of EPSPs was found to correlate with their sensitivity to current injected at the soma (Thomson *et al.* 1993). Fast EPSPs with rise times < 2·3 ms and half-widths < 15 ms increased by between 2·5 and 10% in amplitude with each millivolt depolarization of the soma. Slower EPSPs like the one illustrated in Fig. 4 changed much less. The lack of responsiveness of this EPSP to changes in somatic membrane potential indicates that the EPSP was generated at a point distant to the somatic recording site from whence synaptic currents will be strongly attenuated in their passage along the dendrite (Iansek & Redman, 1973). Since the average amplitude was similar to that observed in the connection illustrated in Figs 1 and 2 which had fewer, more proximal connections, the larger number of contributory synapses apparent on morphological examination (Fig. 3) might also be predicted from the electrophysiology of this connection. If the data from the first (Figs 1 and 2) and second (Figs 3 and 4) pairs are compared a reasonable conclusion is that each of the two boutons in Fig. 1 contributes a larger somatic postsynaptic response than each of the eight illustrated in Fig. 3. The failure, to date, to find a clear correlation between EPSP amplitude and time course (see Thomson *et al.* 1993) may reflect the wide variation in numbers of contributory boutons amongst pyramid–pyramid connections. It should, perhaps, be noted that some connections between deep layer pyramids display very much larger EPSPs than those reported here (see Thomson *et al.* 1993): up to 7 mV in average amplitude. We have not, so far, been able to reconstruct the morphology of such a connection, but might predict from the data presented here that such large EPSPs could involve as many as twenty boutons.

It was concluded that the postsynaptic recording site in the third pyramidal pair was dendritic since the slow spikes recorded in response to action potentials in the presynaptic neurone (Fig. 6) were similar to those observed in the rat motor cortex *in vivo* (Pockberger, 1991) or apical dendrites of pyramids in the neocortex *in vitro* (Amitai, Friedman, Connors & Gutnick, 1982). Variations in the shape, duration and amplitude of these slow spikes may reflect activation of a population of fast (Na^+) and slow (Ca^{2+}) spike generating zones, possibly indicating that the inputs onto three separate dendrites recruit these zones separately. Whether or not a dendritic spike is elicited may depend on whether an EPSP is elicited close to its generation zone. The slow time course of the smaller EPSPs that did not give rise to action potentials was probably due to the relatively depolarized postsynaptic membrane potential during this recording (−60 mV). At this potential the NMDA component of pyramid–pyramid connections prolongs the time course of EPSPs (Nowak, Bregestovski, Ascher, Herbert & Prochiantz, 1984; Thomson *et al.* 1993).

Despite a wide range of average EPSP amplitudes and time courses for pyramid–pyramid connections reported previously (Thomson *et al.* 1993), sweep-to-sweep fluctuations in amplitude were not accompanied by large changes in EPSP shape. This may indicate that each connection between a pair of pyramids consists of synapses that are at a similar electrotonic distance from the soma. The data presented here are from a small sample, but are consistent with this suggestion, one connection involving only basal dendrites, one only the distal portion of the apical dendrite and one three closely neighbouring branches of the proximal apical dendrite.

Possible morphological differences between pyramid–pyramid connections in layer II–III and layer V–VI

Comparison of the electrophysiological attributes of connections between pyramidal neurones in layer II–III with those in layer V–VI may indicate differences in the number and position of presynaptic boutons in these connections. In the somatomotor cortex of the rat, average amplitudes of pyramid–pyramid EPSPs were smaller and exhibited a narrower range of amplitudes in layer II–III (Thomson & West, 1993) than in layer V–VI (Thomson *et*

al. 1993), perhaps indicating that some pyramids in layer V–VI may be connected by a greater number or by more proximally placed boutons than those in the superficial layers. This theory has anatomical correlates in other studies as well as our own. The maximum number of contacts reported between pyramids in the superficial layers in the cat visual cortex was four, and these contacts were located distally on the basal or apical dendrites (Kisvárday & Eysel, 1992). Another study suggested that a maximum of two contacts were made by pyramids in layer II–III of the cat visual cortex onto the apical dendrites of other layer II–III pyramids (McGuire *et al.* 1991). Conversely, in layer V of the cat visual cortex a pyramidal cell was found that made at least four contacts with another pyramid, and these contacts were onto the proximal apical dendrite (Gabbot *et al.* 1987). The range of shape indices of EPSPs observed in layer V–VI connections was greater than that in layer II–III pyramid–pyramid connections (compare those in Thomson & West, 1993, and Thomson *et al.* 1993), perhaps reflecting the fact that the apical dendrites of layer V–VI pyramids are generally longer than those in layer II–III, and that at least some connections between pyramids in both regions are made through their distal apical dendrites (see Fig. 3 and McGuire *et al.* 1991). Interestingly, the range of time courses observed in pyramid–pyramid EPSPs in layer II–III of the rat visual cortex (Mason *et al.* 1991) was greater than that seen in comparable layers in the somatomotor cortex when similar techniques were applied (Thomson & West, 1993). This might indicate a sampling bias or a greater number of pyramid–pyramid contacts onto distal apical dendrites in visual than in somato-motor cortex.

The connections of only a few pyramids have been studied to date and further studies are required to unravel the wiring of local circuits in the cortex. However, the present study provides the first indications of a morphological correlate of functional properties of pyramid–pyramid connections in the cortex.

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