Cerebral Cortex October 2005;15:1485–1496
doi:10.1093/cercor/bhi027
Advance Access publication January 12, 2005

Excitatory Connections Made by Presynaptic Cortico-Cortical Pyramidal Cells in Layer 6 of the Neocortex

Audrey Mercer, David C. West, Oliver T. Morris, Sarah Kirchhecker, Jane E. Kerkhoff and Alex M. Thomson

Department of Pharmacology, The School of Pharmacy, University of London, 29–39 Brunswick Square, London WC1N 1AX, UK

Paired intracellular recordings with biocytin labelling were made in slices of adult rat somatosensory and visual cortex and in cat visual cortex to examine the properties of synaptic connections made by layer 6 pyramidal cells, to determine whether cortico-cortical pyramids more commonly provide input to other layer 6 pyramids than cortico-thalamic cells, and whether these connections exhibit paired pulse and brief train depression. Pyramidal cells with cortico-cortical like morphology were 2–4 times more likely to innervate other pyramidal cells than were cortico-thalamic like cells, but less likely to innervate inhibitory interneurons. The excitatory postsynaptic potentials elicited by presynaptic, phasically firing cortico-cortical pyramids in all classes of postsynaptic infragranular layer pyramidal cells exhibited strong, presynaptically mediated paired pulse and brief train depression. Those with larger paired pulse ratios also exhibited post-tetanic potentiation, but this was accompanied by stronger paired pulse and brief train depression. Both the firing characteristics and the outputs of cortico-cortical pyramidal cells display pronounced phasic characteristics, indicating that these cells respond most effectively to and preferentially pass on information related to novelty.

Keywords: cortico-cortical, EPSP (excitatory postsynaptic potential), layer 6 pyramidal cell, microcircuitry, paired pulse depression, synapse

Introduction

Layer 6 of the neocortex has some features that make it unique amongst cortical layers. In primary sensory regions it is both a thalamo-recipient layer, receiving primary sensory input from specific thalamic nuclei, and an output layer, providing inputs to specific and non-specific thalamic regions and other subcortical structures. Layer 6 is also a source of long range, ipsilateral cortico-cortical axons. The pyramidal cells that provide cortico-thalamic and cortico-cortical outputs are born on different embryonic days (Arimatsu and Ishida, 2002) and have distinctive morphologies.

In a study of the morphology of layer 6 neurons in adult rat barrel cortex, Zhang and Deschenes (1998) filled neurons *in vivo* by juxtacellular labelling with biocytin. They described three broad categories of layer 6 pyramidal cells. Cortico-thalamic (CT) cells that projected to the nucleus reticularis thalami (nRT) and/or the ventroposteriomedial (VPm) nuclei were short, upright pyramidal cells with a well-developed apical dendritic tuft and terminal axonal arbour in layer 4, the latter extending in some cases into layer 3. CT cells that projected to both VPm and non-specific thalamic regions such as Po (posterior thalamic group) were more commonly found in deep layer 6. They were also short, upright pyramids but their apical dendrites typically terminated in upper layer 5 where their ascending axons also terminated. Neither subgroup of CT cells had long horizontal axon collaterals in the infragranular layers; rather the majority of axonal branches turned upwards towards the pial surface near their origin. In contrast, cortico-cortical (CC) cells generated long horizontal axonal arbours that were confined to the infragranular layers. These cells displayed a variety of dendritic morphologies including short upright pyramidal cells whose apical dendrites rarely extended beyond upper layer 5, modified and inverted pyramids and spiny bipolar cells.

Additional complexity is indicated by studies in higher mammals. Subclasses of these broad groupings have been identified in primate primary visual cortex (Lund, 1987; Wiser and Callaway, 1996) where the subdivisions of layer 4 are morphologically and functionally distinct. Simplistically, in each CT subclass, dendritic branches and axonal ramifications are restricted to specific sublayers of layer 4. In a combined study of the physiology and morphology of layer 6 cells in adult cat visual cortex (Hirsch *et al.*, 1998a,b) the axonal arbours of pyramidal cells with simple cell characteristics were found to target layer 6 and/or layer 4, layers that are rich in simple cells. In contrast, layer 6 complex cells targeted layers 2/3 and layer 5, layers rich in complex cells. A further distinction may involve classification of these cells as either first- or second-order complex cells, i.e. whether, like simple cells, they receive direct thalamocortical input. Those described by Hirsch *et al.* (1998a) had response characteristics of second-order complex cells, while the layer 6 complex cells described by McGuire *et al.* (1984) had dendritic branches in both layers 4 and 6 and may therefore have been first-order, thalamo-recipient cells.

A simple overview would be that the CT cells that target specific thalamic regions [e.g. VPm and lateral geniculate nucleus (LGN)] and the nRT, receive direct input from these specific thalamic regions and have both dendritic branches and terminal axonal fields in layer 4. In visual cortex these first-order, thalamo-recipient cells may include both simple and complex cells. The CT cells that also target non-specific thalamic regions (but not the nRT) would be upright pyramids whose axons ramify primarily in the infragranular layers in rat and also in the supragranular layers in cat. CC cells with a wide range of dendritic morphologies have less complex, but broader, horizontally directed axonal arbours. Typically, the latter two categories would not be thalamo-recipient cells and in visual cortex might be predicted to display complex receptive fields.

To investigate the properties of the synaptic connections received by layer 6 pyramidal cells, Beierlein and Connors (2002) stimulated the thalamus electrically and recorded in layer 6 of rat somatosensory cortex in thalamo-cortical slices. The shorter latency excitatory postsynaptic potentials (EPSPs)

elicited, which were assumed to be due to activation of thalamic relay cells, displayed paired pulse depression similar to that previously described for thalamic inputs to layer 4 (Gil *et al.*, 1999). The less frequently observed, longer latency EPSPs were taken to be due to antidromic activation of CT cells and in contrast to thalamo-cortical inputs, displayed paired pulse facilitation. However, when pairs of synaptically connected pyramidal cells were recorded in layer 6, the EPSPs elicited by local circuit connections displayed paired pulse depression. This finding suggests two possible explanations, either the extracellular stimulus used to elicit EPSPs from the thalamus recruited additional CT fibres in paired pulse and train protocols, resulting in an apparent facilitation, or a different group of non-CT presynaptic neurons was involved in the paired recording data. No anatomical data were provided that might indicate whether the local circuit connections studied involved CC rather than CT cells. The possibility arises that the majority of intra-laminar layer 6 pyramid–pyramid connections results from connections made by CC, rather than by CT cell axons and that these connections may display paired pulse depression. Previous studies of layer 5 had indicated this kind of bias, the small, short, regular-spiking pyramidal cells being much more commonly presynaptic to other layer 5 cells than the large, intrinsically burst firing cells (Thomson and Bannister, 1998; Thomson and Morris, 2002).

To explore these questions further, paired intracellular recordings with biocytin-filling were made in layer 6 of rat and cat neocortex. The characteristics of EPSPs elicited by presynaptic cells whose morphology resembles that of CC cells described previously are reported.

Materials and Methods

Dual intracellular recordings were made from synaptically connected neurons in rat somatosensory and visual cortex and in cat visual cortex as described previously (Thomson and West, 2003).

Male Sprague–Dawley ($n = 20$) or Wistar ($n = 8$) rats (120–200 g) were anaesthetized with inhaled Fluothane and intraperitoneal pentobarbitone sodium (Sagatal, 60 mg/kg). Male cats ($n = 7$, 2.5–3.4 kg) were anaesthetized intravenously with a mixture of α-chloralose (70 mg/kg) and pentobarbitone sodium (6 mg/kg) for a different series of experiments (procedures similar to Wang and Ramage, 2001). Rats were perfused transcardially and cats (following an overdose of barbiturate) via the carotid arteries, with ice-cold modified artificial cerebrospinal fluid (ACSF) with added pentobarbitone (60 mg/l) in which 248 mM sucrose replaced NaCl. Rats were decapitated and the brain removed. Visual cortex was removed from cats via a hole in the skull. Slices of neocortex, 450–500 μm thick, were cut (Vibroslice, Camden Instruments, UK) and transferred to an interface recording chamber where they were maintained for 1 h in sucrose-containing medium, before switching to standard ACSF containing (in mM) 124 NaCl, 25.5 $NaHCO_3$, 3.3 KCl, 1.2 KH_2PO_4, 1.0 $MgSO_4$, 2.5 $CaCl_2$, 15 D-glucose equilibrated with 95% O_2/5% CO_2 at 35–36°C. All procedures complied with UK Home Office regulations for animal use.

Paired intracellular recordings were made with conventional sharp micro-electrodes, containing 2 M $KMeSO_4$ and 2% w/v biocytin, tip resistance 90–150 MΩ. Presynaptic neurons were depolarized with combinations of square-wave and ramped currents, typically delivered at 1 pulse per 3 s to elicit trains of action potentials (APs) at different frequencies and postsynaptic responses were recorded (Spike-2, Cambridge Electronic Designs). Cells were filled with biocytin and slices fixed and processed histologically for identification of recorded neurons, as described previously (Hughes *et al.*, 2000; Thomson *et al.*, 2002).

During off-line analysis (in-house software), data sets in which the first EPSP shape and amplitude and the postsynaptic membrane potential were stable were selected. Single sweeps were checked by hand to ensure that every presynaptic AP was recognized by the software and that the trigger points used for subsequent analysis were accurately aligned with the rising phase of each AP. Sweeps including artefacts or large spontaneous events were excluded from averaged records. All sweeps in which the second AP followed the first AP within a given time window were then selected. The second EPSPs within each window were then averaged, using the rising phase of the second AP as the trigger. This second EPSP average was then superimposed on an average of all responses to single APs. The amplitude of the averaged second EPSP was then measured from its peak to the appropriate point on the falling phase of the averaged first EPSP. Averaged responses to later APs in trains were analysed similarly. These points were then plotted against the interval between the 1st and 2nd (3rd, 4th,. . ., 7th) spike. Where postsynaptic responses exhibited an adequate signal-to-noise ratio, single sweep events were also measured (by hand with cursors) using an average of all single spike EPSPs, scaled to match the amplitude of the first EPSP in each sweep, to measure second EPSPs.

D-2-Amino-5-phosphonopentanoic acid (D-AP5, Tocris, UK) 60 μM was added to the bathing medium in one experiment.

Results

Electrophysiological Characteristics of Layer 6 Pyramidal Cells

Paired intracellular recordings were made in 28 experiments in rat and seven in cat neocortex. During experiments, cells were categorized according to their firing characteristics and pyramidal cells separated into two broad classes, tonic and phasic cells (Fig. 1), each constituting about half the cells recorded. Subsequent morphological analysis demonstrated that the tonic cells (13:14, 93% in rat) exhibited CT-like characteristics with both an apical dendritic tuft and axonal ramification in layer 4. The cat layer 6 pyramidal cells with tonic characteristics that were recovered histologically either had morphology reminiscent of claustrum projecting cells with a long, slender apical dendrite, sometimes reaching layer 1, but without a well-developed dendritic tuft in layer 4 and an axonal arbour restricted to the infragranular layers (Katz, 1987, see the presynaptic cell in Fig. 6) or were CT-like cells (e.g. postsynaptic neuron in Fig. 6). In contrast, phasic cells more typically (23:29, 79% in rat) exhibited CC-like morphology, with long horizontal axonal arbours restricted to the infragranular layers and a range of dendritic morphologies that included short upright pyramids (e.g. Fig. 1 and presynaptic cell in Fig. 8), inverted pyramids (see Fig. 5 for two examples in cat) and spiny bipolar cells (Fig. 4). Similar differences in the electrophysiological properties of layer 6 cells antidromically activated from the white matter (putative CT cells), and those that were not, have been reported in mouse (Brumberg *et al.*, 2003).

Probabilities of Synaptic Connections Between Layer 6 Pyramidal Cells

Some 1500 pairs of layer 6 pyramidal cells were tested, yielding 56 excitatory connections (an average probability of around 1:27). To remove bias that might have been introduced by tests involving cells with severely pruned axons and to provide a comparison between the two major classes of pyramidal cells, connectivity probabilities were calculated for those cells that were held while several (6–10) potential pre- or postsynaptic partners were tested, and including only those cells that were subsequently identified morphologically and found to have significant axonal arbours within the slice. This group included five CT-like and four CC-like cells in cat and 19 CT-like and 16 CC-like cells in rat. For these morphologically identified cells,

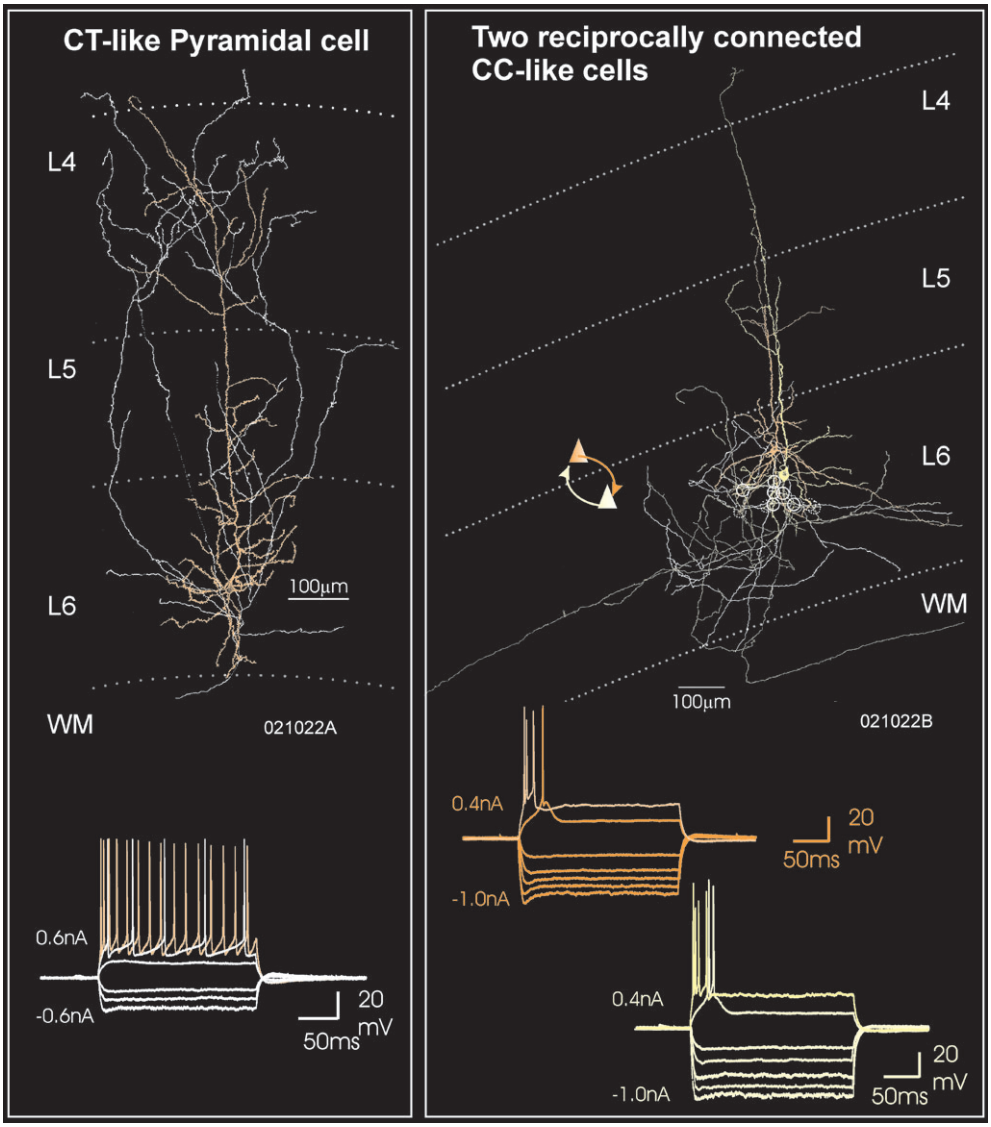

Figure 1. The left panel shows the reconstruction of a rat cortico-thalamic (CT)-like layer 6 pyramidal cell (soma/dendrites orange, axon white). These cells have an apical dendritic tuft and axonal arbour in layer 4. Responses of this cell to injected current pulses (−0.6, −0.4, −0.2, 0.2, 0.4, 0.6 nA) are shown below. Although there is some spike frequency adaptation, these neurons continue to fire tonically throughout a suprathreshold square wave current pulse. Two reciprocally connected rat layer 6 cortico-cortical (CC)-like pyramidal cells are reconstructed on the right (soma/dendrites of the lower cell pale yellow, axon white, soma/dendrites of the upper cell orange, axon grey). Neither CC-like cell has a well-developed dendritic tuft or ramifying axonal arbour in layer 4. The responses of these cells to current injection are shown below (−1.0, −0.8, −0.6, −0.4, −0.2, 0.2, 0.4 nA). The discharge pattern typical of these neurons is extremely phasic with a single spike, or brief burst followed by no further firing despite continued strong depolarization. Scale bars here and throughout indicate 100 µm.

the probability that a CC-like cell would be presynaptic to another pyramidal cell was 1:30 in rat and 1:6 in cat. The probability that an identified CT-like cell would be presynaptic to another pyramid was lower, 1:75 in rat, while none of the five identified CT-like cells in cat was found to be presynaptic to another layer 6 pyramidal cell in 44 tests.

These data suggest that CC-like cell axons make contact with other layer 6 pyramidal cells more than twice as frequently as CT-like cell axons. This bias was also indicated when only the presynaptic neurons in demonstrably connected cell pairs were analysed. In rat, only five of 27 pyramid–pyramid pairs involved a presynaptic CT-like cell, while 11 involved a presynaptic CC-like cell. The remaining 11 presynaptic cells were not sufficiently well stained for unambiguous identification, but nine exhibited the phasic firing patterns typical of CC-like cells and only one the tonic pattern more typical of CT-like cells. This suggests that CC cells are four times more likely to provide input to other layer 6 pyramidal cells than CT cells. In cat a similar pattern emerged. Three presynaptic cells were inverted CC-like pyramids, three had morphology reminiscent of claustrum projecting cells and none was CT-like. Of the two presynaptic pyramids recorded in cat that were not recovered histologically, one fired phasically.

Correlation Between Pyramid–pyramid Connectivity and Presynaptic Axonal Arbour Density

To determine whether the preponderance of presynaptic CC-like cells in this layer correlates with relative local axonal arbour densities for the two classes of pyramidal cells, the total length of axon within 150 µm of the parent soma in three adjacent 60 µm sections was measured and the number of axonal swellings

(putative synaptic boutons) counted for six CT-like and nine CC-like pyramidal cells in rat. This area was selected as one that would include the majority of paired recordings in this series. Although CC-like cells have much broader axonal arbours within the infragranular layers and therefore more axon and boutons in total within layer 6 than CT cells (Fig. 2), this axon does not ramify densely close to the soma. On average, therefore, while CC-like cells did have more axon length within this volume (2100 ± 900 μm, mean ± SD) than CT-like cells (1750 ± 550 μm) and a larger number of putative boutons (480 ± 230 versus 350 ± 160) the differences did not reach significance and were not large enough to account for the 1:2 to 1:4 connectivity ratio observed.

There was, however, no apparent bias in the types of postsynaptic neurons involved in layer 6 pyramid–pyramid connections. In cat two were CC-like, four CT-like and one claustrum projecting (one was not recovered). In rat, 11 were CC-like and nine CT-like (seven were not recovered histologically).

Time Course and Amplitudes of EPSPs Resulting from Pyramid–Pyramid Connections Involving Presynaptic Cortico-cortical Cells

Seven rat layer 6 pairs in which the postsynaptic neuron was another CC-like cell and four in which it was a CT-like cell were compared. Many properties were similar (see Table 1) Inputs to CC-like cells were, however, smaller than those recorded in postsynaptic CT-like cells. This difference correlated with a higher CV (coefficient of variation) for EPSPs recorded in CC-like cells than in CT-like cells and a higher proportion of total apparent failures of transmission in response to the first AP of a train (see Fig. 3).

Two cat CC- to CT-like and two CC- to CC-like cell EPSPs were compared with the rat data. All parameters were similar except that widths at half amplitude in both cat groups and rise times in CT cells were longer than in rat. This difference did not, however, reach significance.

Voltage-dependent Components of Pyramid–Pyramid EPSPs

At membrane potentials more negative than –65 mV, short interval second EPSPs that summed with first EPSPs were of a similar shape, i.e. summation appeared to be linear (also indicated by the results of plotting changes in CV^{-2} against changes in M) and unaffected by voltage-dependent events that might have been associated with the first EPSP. At less negative potentials, however, the shape of the EPSP indicated that it was truncated by a hyperpolarizing event (Figs 4*B* and 5*C*). In these pairs, EPSPs, like the majority of pyramid–pyramid EPSPs in

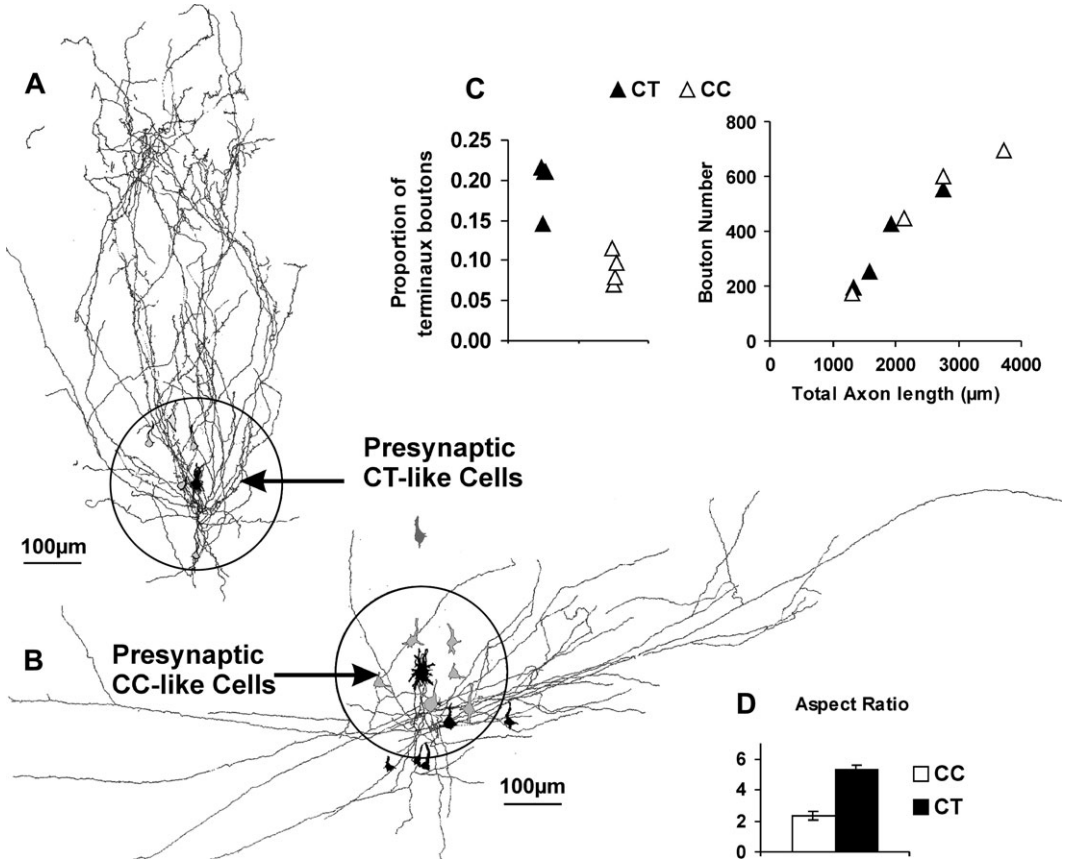

Figure 2. (*A*) Axons of four rat cortico-thalamic (CT)-like cells are reconstructed and superimposed. The superimposed somata of these cells can be seen at the centre of the circle. This circle indicates the region analysed in C (radius 150 μm, depth of analysed volume equivalent to three consecutive 60 μm sections). The somata of the postsynaptic targets of these four presynaptic neurons are also indicated (grey). In *B* a similar superimposition of four cortico-cortical (CC)-like cells is shown. Postsynaptic CT-like cells (and one layer 5 postsynaptic target) are indicated in grey and postsynaptic CC-like cells in black. (*C*) A larger proportion of putative axon boutons (swellings visible at the light level) are terminal boutons (bouton terminaux versus bouton en passage) in the axons of CT-like pyramids than in the axons of CC-like cells. To the right, the total putative bouton count is plotted against the total length of the axon contained within the slice for these eight neurons. In *D* the maximum vertical extent of the axonal arbour within the cortical grey matter in the slice divided by its maximum horizontal extent (aspect ratio) is plotted for 17 CT-like and 12 CC-like pyramidal cells (mean ± SEM, $P < 0.01$, unpaired *t*-test).

Table 1
Properties of EPSPs elicited in layer 5 and layer 6 pyramidal cells by presynaptic layer 6 CC-like pyramids

	CC to CC rat (n = 7)	CC to CT rat (n = 4)	CC to L5 rat (n = 1)	CC to CC cat (n = 2)	CC to CT cat (n = 2)	CC to L5 cat (n = 1)
Postsynaptic MP (mV)	−73 ± 3	69 ± 7	−65	−68, −70	−67, −69	−66
Amplitude (mV)	0.9 ± 0.7*	1.7 ± 0.4*	1.8	0.75, 0.67	1.6, 0.4	0.97
Latency (ms)	2.1 ± 0.8	2.0 ± 0.4	2.2	1.8, 2.6	2.9, 2.2	2
10–90% RT (ms)	1.6 ± 0.7	1.7 ± 1.0	2.5	0.8, 2.4	3.4, 2.8	2
HW (ms)	12 ± 3	13 ± 18	19	12, 20	21, 23	24
CV	0.6 ± 0.5	0.2 ± 0.03	0.16	0.47, 0.25	0.2, 0.5	0.2
Proportion of failures (%)	20 ± 20	2 ± 2	0	2, 1	0, 0	0

Postsynaptic membrane potential and EPSP amplitude, latency, 10–90% rise time (RT), width at half amplitude (HW), coefficient of variation (CV) and the proportion of apparent failures of transmission are given for connections in which both pre- and post-synaptic neurons were identified morphologically. Means and standard deviations are given where $n > 3$, individual measurements are given elsewhere. The asterisks indicate the comparison between groups that yielded a significant difference ($P < 0.05$, Student's unpaired t-test).

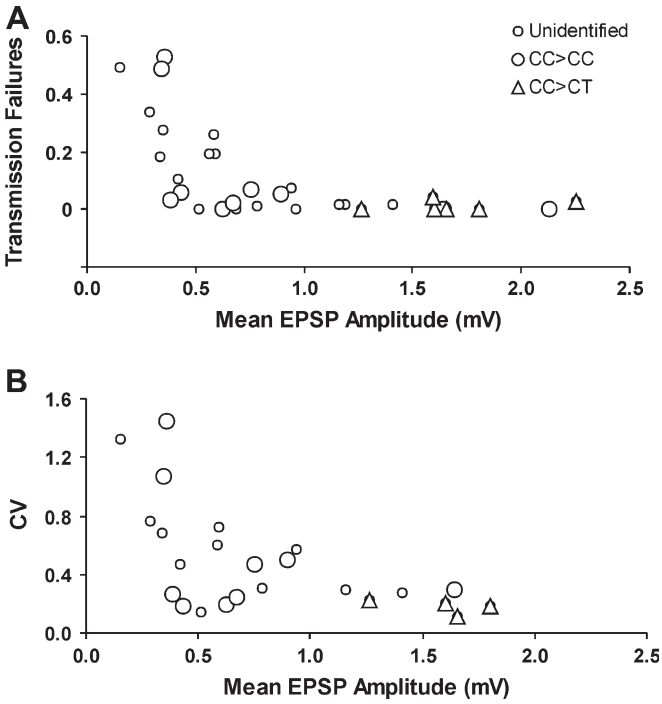

Figure 3. (*A*) Proportion of apparent failures of transmission plotted against mean EPSP amplitude for 36 pairs of layer 6 pyramidal neurons. (*B*) Coefficient of variation (CV) of the EPSP amplitude plotted against mean EPSP amplitude. Both the proportion of failures and the CV decrease with increasing mean EPSP amplitude. The larger amplitudes typical of EPSPs recorded in CT-like cells (triangles) are apparent.

neocortex, increased in amplitude and duration as the postsynaptic membrane was depolarized (Figs 4*B* and 5). To determine whether the EPSP was partially mediated by *N*-methyl-D-aspartate (NMDA) receptors and whether the hyperpolarizing event was dependent on activation of this component, D-AP5 (60 μM) was added to the bathing medium. Partial blockade of the EPSP (19% of average peak amplitude at –58 mV) demonstrated the involvement of NMDA receptors, but the hyperpolarization that curtailed the second EPSP was still apparent (Fig. 5*A*,*B*).

Experiments with partial blockade by D-AP5 address another question; whether the second EPSP involves the same postsynaptic receptor population as the first EPSP. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are more rapidly activated and inactivated by glutamate than NMDA receptors. A second, short interval EPSP utilizing the same receptors would therefore be expected to involve a relatively smaller AMPA receptor-mediated component and a relatively larger NMDA receptor-mediated component, i.e. the EPSP recorded in the presence of D-AP5 would demonstrate a smaller paired pulse ratio. There was, however, no significant difference between control and D-AP5 blocked EPSPs in the paired pulse ratios obtained for a range of interspike intervals (15–100 ms). This indicated that while other, less powerfully expressed mechanisms cannot be discounted, release site refractoriness had indeed prevented a second release from the release sites that had contributed to the first EPSP.

Paired Pulse Depression at Pyramid–Pyramid Connections Made by Cortico-cortical Cell Axons

The paired recordings reported by Beierlein and Connors (2002) indicated that some pyramid–pyramid connections in layer 6, like those reported previously in other layers, display paired pulse and brief train depression. To determine whether connections made by CC-like axons did indeed exhibit synaptic depression, postsynaptic responses to pairs and trains of presynaptic action potentials (APs) were analysed in 17 pairs in rat (seven CC to CC, four CC to CT, one CC to unidentified and five in which a phasic cell was presynaptic) and six pairs in cat (two CC to CC, one CC to CT and three pairs in which the presynaptic cell was a claustrum projection-like cell).

All 23 pyramid–pyramid connections involving a presynaptic CC-like cell, a phasic cell or a claustrum projection-like cell exhibited paired pulse depression at all interspike intervals between 10 and 100 ms (Figs 4*A*,*C*, 5, 6 and 7*A*,*B*). Averaged second EPSPs were 69 ± 22% (mean ± SD) of average first EPSP amplitudes at intervals of 18–22 ms (n = 16). Whether this depression was mediated by a presynaptic mechanism was investigated in two ways. In those pairs (n = 6) in which a significant number of apparent failures of transmission were recorded, the proportion of failures was found to be greater for second EPSPs than for first EPSPs, indicating a presynaptic locus (see Figs 4*C* and 6*B*). For eight pairs (four CC to CC, four CC to CT), the normalized inverse square of the coefficient of variation (CV^{-2}) was plotted against the normalised average EPSP amplitude (M). For a binomial distribution, CV^{-2} is equal to $[np/(1 - p)]$ and therefore independent of q, while M is equal to $[npq]$ (where q is the quantal amplitude, p is the release probability, n is the number of release sites and M is the mean EPSP amplitude; for a review, see Thomson, 2003). A slope of zero indicates a change only in q (a postsynaptic change), a slope of 1 indicates a change in n (or both p and q) and a slope >1 a change in p. All points for seven of the eight pairs (including points for several interspike intervals in some pairs)

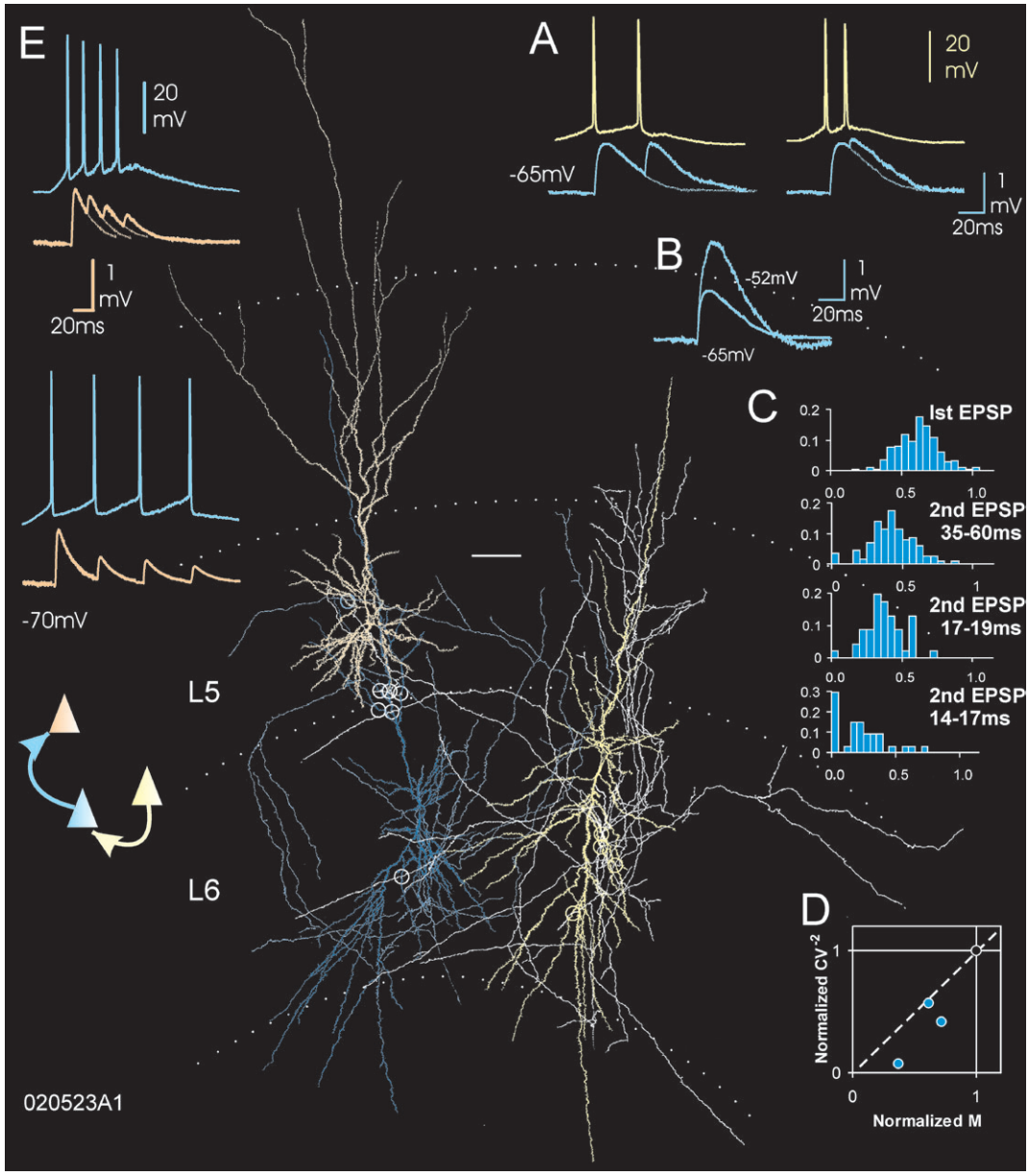

Figure 4. Three cat pyramidal cells: a bipolar layer 6 CC-like pyramidal cell (soma/dendrites yellow, axon white) that was presynaptic to another bipolar CC-like pyramid layer 6 (soma/dendrites blue, axon pale blue), which was presynaptic to a small layer 5 pyramid (soma/dendrites sand) are reconstructed in the centre. Positions of putative synaptic contacts (and autapses, see yellow dendrites) are indicated by the circles, which are colour-coded for the presynaptic axon involved (similar in other figures). (*A*) The EPSP elicited in the postsynaptic layer 6 neuron exhibited paired pulse depression which was more pronounced at short interspike intervals. These and all EPSPs illustrated in this paper are averages of between 10 and 200 sweeps. (*B*) This EPSP increased in amplitude with postsynaptic depolarization and the afterhyperpolarization that followed the EPSP became more pronounced. (*C*) EPSP amplitude distributions for first EPSPs and second EPSPs at different interspike intervals show that paired pulse depression was associated with a higher proportion of apparent failures of transmission at short interspike intervals and was probably of presynaptic origin since in the plot of normalized CV^{-2} against normalized mean EPSP amplitude (*M*) all points fell on a slope >1 (*D*). (*E*) The EPSP elicited in the layer 5 pyramid also exhibited paired pulse and brief train depression that were more pronounced at higher frequencies (see Fig. 7 for further analysis of this pair). In these phasically firing presynaptic neurons, more tonic, maintained firing was achieved by injection of combinations of square wave and ramped current pulses.

fell on a line with slope >1 (Figs 4*D* and 7*F*), indicating that the depression was mediated at least in part by a decrease in release probability, as described previously for pyramid–pyramid connections in other layers. One point fell on the unity line.

Single exponentials fit to the recovery from paired pulse depression, over a range of interspike intervals from 10 to 100 ms, yielded time constants for decay between 3.5 and 61 ms (29 ± 18 ms, n = 10 pairs). However, as reported previously (Thomson and West, 2003), extrapolation of this decay predicted a residual depression at infinity (second EPSP 82 ± 10% of averaged first EPSP amplitude, n = 10).

Brief Train Depression

Previous studies of neocortical pyramid–pyramid connections have demonstrated more profound depression of third and subsequent EPSPs in brief trains than of second EPSPs. This was also typical of many layer 6 CC-like pyramid–pyramid connections (Figs 4*E* and 7*A,B* and in Figs 4*E* and 7*H,I* for layer 6 to layer

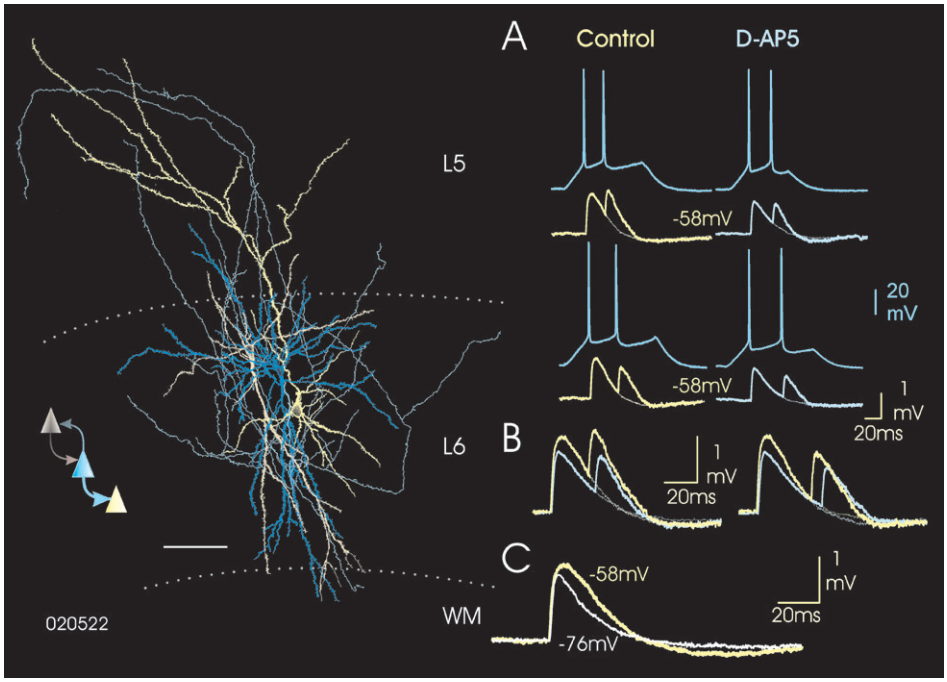

Figure 5. An inverted CC-like cat layer 6 pyramidal cell (soma/dendrites blue, axon pale blue) which was presynaptic to a short, upright CC-like cell (yellow soma/dendrites, EPSPs from this pair illustrated in *A–C*) and reciprocally connected with another inverted pyramid (soma/dendrites sand, EPSPs not illustrated). (*A*) Averaged EPSPs elicited by pairs of pyramidal spikes in control (yellow traces) and in D-AP5 (pale blue traces) are shown with control and D-AP5 recordings superimposed at higher gain in *B*. (*C*) Averaged EPSPs recorded under control conditions at two different membrane potentials, −58 and −76 mV, are superimposed. EPSPs recorded at −58 mV are followed by an afterhyperpolarization that is less evident at −76 mV.

5 connections). That this additional depression is also mediated presynaptically is indicated by an increasing proportion of failures with successive EPSPs in brief trains (Fig. 7*A*) and a slope >1 in the plots of CV^{-2} against *M* for third and fourth EPSPs compared with first EPSPs (Fig. 7*F*).

Pyramid–Pyramid Connections Between Layer 6 and Layer 5

CC-like pyramidal cells innervate both layer 6 and layer 5, although they rarely send axon collaterals beyond upper layer 5. To compare their inputs to layer 5 cells, with inputs to layer 6 cells, two pairs (one in rat, one in cat) in which a layer 6 CC-like cell was presynaptic to a layer 5 pyramidal cell were recorded (Figs 4*E*, 7*C–I* and 8, and Table 1). Paired pulse depression was apparent at both connections with average second EPSP amplitudes 31% (cat) and 52% (rat) of average first EPSP amplitudes at interspike intervals of 10 ms. Third and fourth EPSPs were further depressed. These connections exhibited too low a proportion of transmission failures following the second AP to allow the pre- or post-synaptic locus for depression to be assessed by this method, but the proportion of failures increased between the second and fourth EPSP in brief trains (Fig. 7*H*). In plots of normalized CV^{-2} against *M* all points for both connections fell on a slope >1, again indicating a presynaptic locus for paired pulse and brief train depression at these connections (Fig. 7*F*).

In addition, two pairs in which the presynaptic cell was a layer 5 pyramid and the postsynaptic an inverted CC-like pyramidal cell in layer 6 were recorded. In one, the presynaptic was a small regular spiking cell and in the other, a large tufted, intrinsically burst firing pyramidal cell. Both EPSPs exhibited paired pulse depression. In a third briefly recorded pair, a large, tufted layer 5 pyramid was presynaptic to an unidentified layer 6 pyramid. Although the numbers of pairs sampled involving a layer 5 and a layer 6 pyramid were too low to provide connectivity probabilities, connections between the layers appeared to be as common as those within layer 6.

Post-tetanic Potentiation

Post-tetanic potentiation (PTP) was assessed by comparing EPSPs elicited 3 s after a single preceding spike with EPSPs that followed trains of two or more spikes. In four pairs [one phasically firing to CT, one CC to CC (Fig. 7*B*), and one CC to layer 5 pyramid (Fig. 8*C*) and one CC to CC in cat] three spikes in the preceding train resulted in an increase in mean EPSP amplitude of between 10 and 25% (14 ± 7%). In two pairs, four preceding spikes resulted in increases of 26 and 20%, while a preceding train of six spikes produced a 40% increase in average first EPSP amplitude in one pair tested (Fig. 7*B*). This increase in the first EPSP amplitude was, however, accompanied by a decrease in the average amplitude of second and subsequent EPSPs. Second EPSPs following trains of three spikes were decreased by 22, 20 and 6% in three pairs and unchanged in one, while a 38% decrease in average third EPSP amplitude followed trains of six spikes. The overall effect of PTP, by increasing first EPSPs and depressing subsequent EPSPs in trains, was therefore to enhance the phasic characteristics of the connections, as might be expected following an increase in low-frequency release probability (Markram *et al.*, 1997). To determine, therefore, whether PTP is more readily elicited at connections that display a low probability of release, the paired pulse ratios for those connections that displayed PTP and for

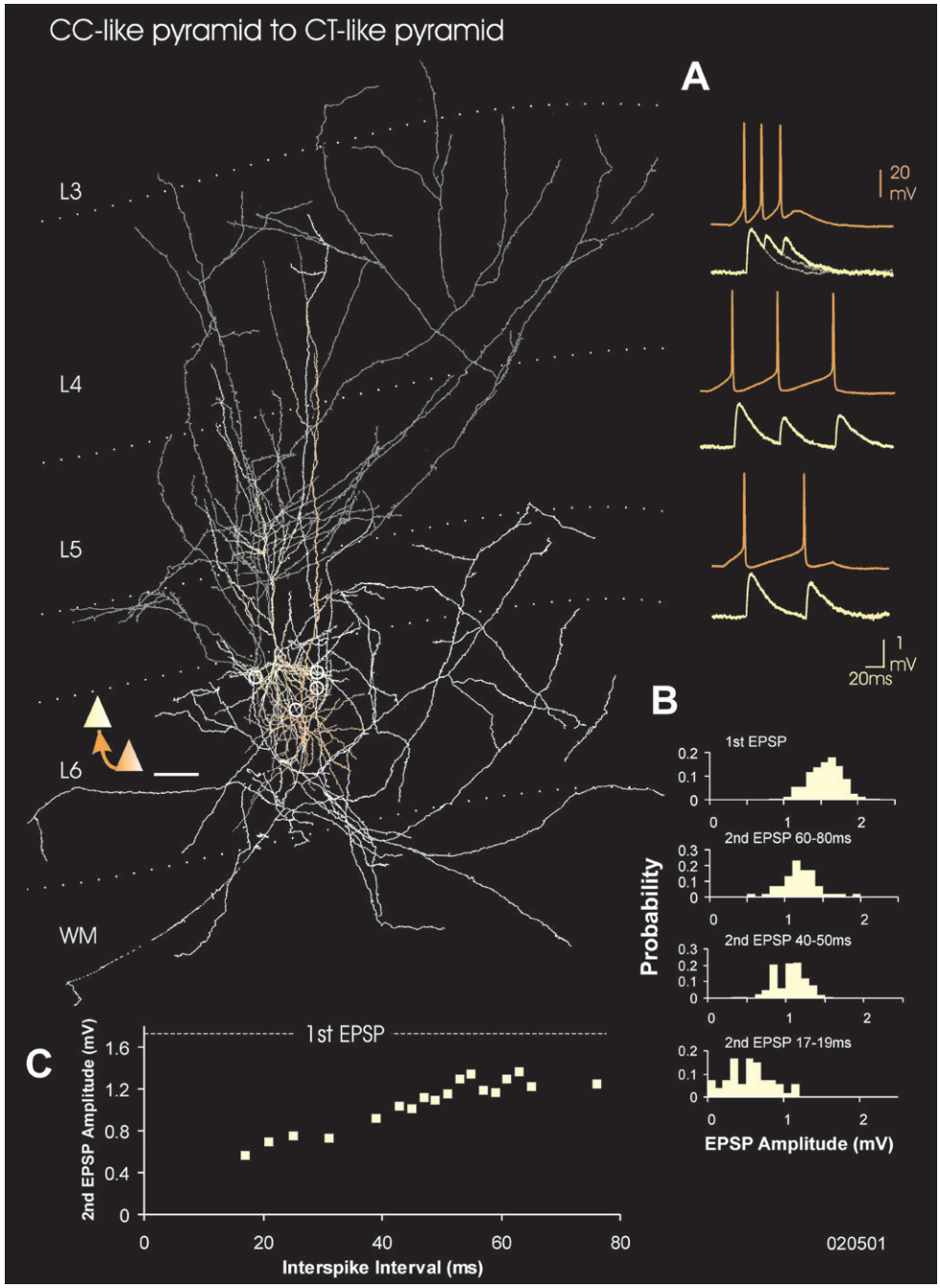

Figure 6. Two synaptically connected cat layer 6 pyramidal cells are reconstructed. The presynaptic pyramid (orange soma/dendrites, white axon) resembles a claustrally projecting cell with a slender apical dendrite reaching layer 3 and an axonal arbour confined to the deep layers. The postsynaptic pyramid is a CT-like pyramidal cell (soma/dendrites yellow, axon grey) with an apical dendritic tuft and extensive axonal arborization in layer 4. (*A*) The EPSP resulting from this connection exhibited paired pulse and brief train depression that were more pronounced and involved a larger proportion of apparent failures of transmission (*B*) at shorter interspike intervals. (*C*) Recovery from paired pulse depression was slow and was incomplete at 80 ms.

four pairs that did not, were compared. A higher paired pulse ratio indicates a lower release probability. At an interspike interval of 10 ms the paired pulse ratios were 0.5 ± 0.1 and 0.36 ± 0.08 respectively, for those that did and those that did not display PTP. At an interspike interval of 20 ms the paired pulse ratios were 0.7 ± 0.1 and 0.4 ± 0.1 respectively. Although these differences did not reach significance with this small sample ($P > 0.05$), connections displaying PTP had larger paired pulse ratios, suggesting a lower probability of release at low frequencies.

Frequency-dependent Depression

Previous studies have indicated that the number of vesicles constituting the readily releasable pool of transmitter at pyramidal axon terminals is small, ~30–40 vesicles, and that the immediately releasable pool is even smaller (4–5 vesicles). The fastest retrieval/recycle time following release for those vesicles that have been docked for some time is 1–2 s and for the majority in the readily releasable pool is 30–60 s (Murthy and Stevens, 1999; for review, see Harata *et al.*, 2001). This limits the rate at which each of these terminals can release transmitter

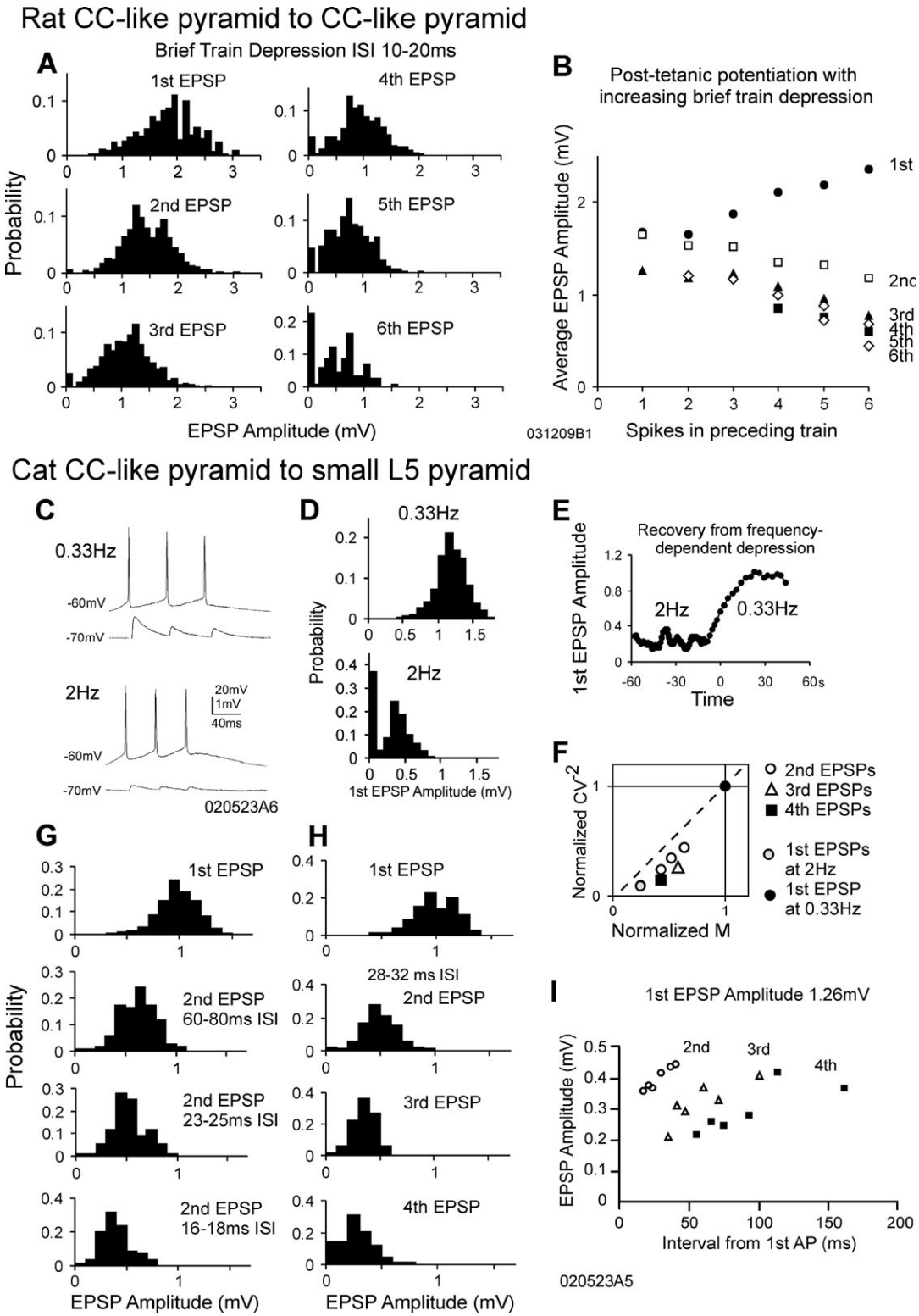

Figure 7. (*A*, *B*) EPSPs recorded from a rat CC-like pyramid to CC-like pyramidal pair. (*A*) EPSP amplitude distributions illustrate the decrease in mean EPSP amplitude and increase in the proportion of apparent failures of transmission from the first to the sixth EPSP in brief trains. (*B*) Average EPSP amplitude plotted against the number of spikes in the preceding train (inter-train interval 3 s). Although the amplitude of the first EPSP in the train is increasingly enhanced by preceding trains containing more spikes, paired pulse and brief train depression are increased, resulting in a more phasic response. (*C*–*I*) EPSPs elicited in a small layer 5 pyramidal cell by a presynaptic CC-like pyramidal cell in cat visual cortex (see Fig. 4 for reconstruction). (*C*, *D*) Frequency-dependent depression is apparent when trains of three spikes are repeated at inter-train intervals of 0.5 s versus intervals of 3 s and this depression is accompanied by an increase in the proportion of apparent failures of transmission. (*E*) Recovery from frequency-dependent depression is relatively rapid. (*F*) Both brief train and frequency-dependent depression appear to be mediated presynaptically since in the plot of normalised CV^{-2} against normalized *M* all points fell on a slope >1. (*G*) EPSP amplitude distributions for second EPSPs at different interspike intervals compared with first EPSPs in trains. (*H*) First EPSP amplitude distributions compared with those for second, third and fourth EPSPs elicited at interspike intervals between 28 and 32 ms. (*I*) Early phase of recovery of the second, third and fourth EPSPs from paired pulse/brief train depression.

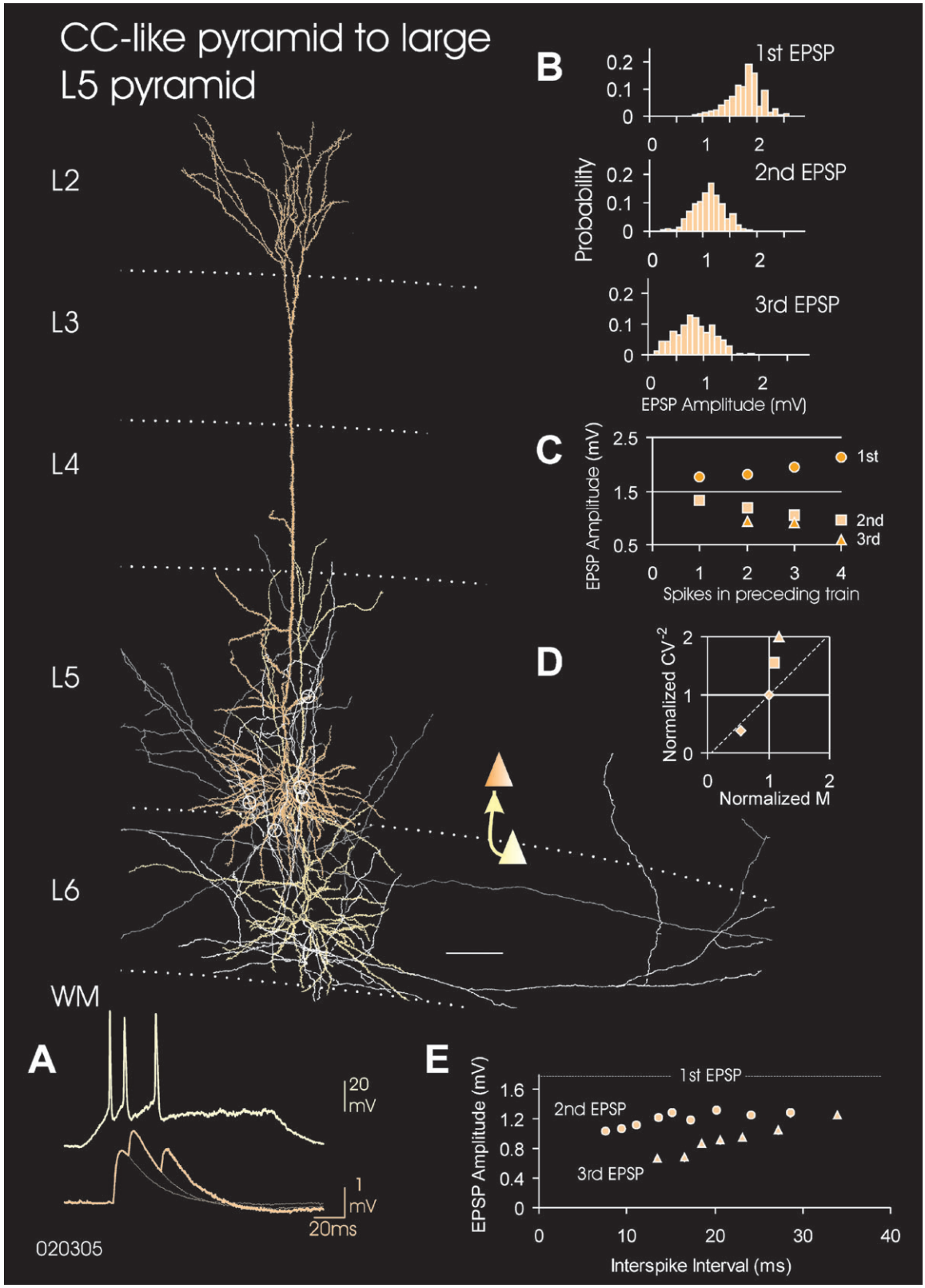

Figure 8. Reconstruction of a short, upright CC-like pyramidal cell in rat neocortex (soma/dendrites yellow, axon white) that was presynaptic to a large layer 5 pyramidal cell with an apical dendritic tuft reaching layer 1 (soma/dendrites orange, axon grey). (*A*) Averaged EPSPs elicited by brief trains of three spikes. (*B*) EPSP amplitude histograms for first, second and third EPSPs in brief trains. (*C*) Average EPSP amplitude plotted against the number of spikes in the preceding train (inter-train interval 3 s). Although the amplitude of the first EPSP in the train is increasingly enhanced by preceding trains containing more spikes, paired pulse and brief train depression are increased, resulting in a more phasic response. (*D*) Plots of normalized CV^{-2} against normalized *M* indicate that both the post-tetanic potentiation (PTP) of first EPSPs (square following three spikes, triangle following four spikes) and the paired pulse depression of second EPSPs (diamonds) are mediated presynaptically. (*E*) Average EPSP amplitude plotted against interspike interval illustrates recovery of second and third EPSPs from depression.

during sustained activity to ~1 release per 1–2 s and results in frequency-dependent depression even at frequencies <1 Hz (Thomson *et al.*, 1993).

To test for this phenomenon, short spike trains (1–3 APs) were elicited at repetition rates of 0.33 and 2 Hz. At 2 Hz, EPSP amplitudes declined gradually, reaching a plateau after ~60 s. In one CC to CT pair, the average first EPSP amplitude decreased from 1.7 to 1.0 mV. In a CC cell to layer 5 connection, the average first EPSP decreased from 1.1 mV to 0.3 mV (Fig. 8*D*,*E*). The relatively greater depression in the CC to layer 5 pyramid connection paralleled the stronger paired pulse depression that it displayed (paired pulse ratio 0.3 versus 0.7 at an interspike interval of 18 ms) indicating that release probability (at low frequencies) was higher. That frequency-dependent depression was also mediated presynaptically is indicated by the increase in the proportion of apparent failures of transmission at higher frequencies (Fig. 7*D*) and by plots of CV^{-2} against M in which points fell on lines with slopes >1 (Fig. 7*F*). On slowing the repetition rate again, recovery was relatively rapid, within 15–20 s (Fig. 7*E*).

Connections Between Cortico-cortical Layer 6 Pyramids and Interneurons

Fifty-nine pairs (21 in rat, 38 in cat) in which one of the cells was a layer 6 pyramid and the other a layer 6 interneuron were recorded. In 12 pairs (five in rat, seven in cat) a pyramid to interneuron EPSP was recorded, giving average probabilities of 1:4 in rat and 1:5 in cat. Most of the pyramidal cells involved in these tests were recorded only briefly and discarded when they were found not to be connected to the recorded interneuron and were not therefore recovered morphologically. It is not possible, therefore, to determine accurately the relative probabilities of CC-like and CT-like cells innervating layer 6 interneurons. It is, however, interesting to note that in only two of the 10 pairs in which the presynaptic cell was identified morphologically was it a CC-like cell. Eight of the pyramidal cells that were presynaptic to interneurons were CT-like cells (paper in preparation). This suggests that CC-like cells are up to four times less likely to innervate interneurons in layer 6 than CT-like cells. As reported in previous studies (Thomson *et al.*, 2002; Thomson and West, 2003), the EPSPs associated with CC pyramid–interneuron connections were considerably briefer than those involving postsynaptic pyramidal cells with rise times of 0.5 ms and widths at half amplitude of <5 ms. In one connection the average first EPSP amplitude was 2.5 mV and the connection displayed pronounced paired pulse depression, the second EPSP amplitude being on average 27% of the first at interspike intervals between 10 and 20 ms.

Discussion

The properties of EPSPs elicited in both CC-like and CT-like layer 6 pyramidal cells and in layer 5 pyramidal cells by action potentials in CC-like pyramids in layer 6 of adult rat and cat cortex are described. These EPSPs display many of the characteristics typically displayed by pyramid–pyramid EPSPs in other layers of the neocortex. They are partially NMDA receptor mediated, increase in amplitude with depolarization, and at depolarized membrane potentials are followed by an after-hyperpolarization (Thomson and West, 1993; Thomson *et al.*, 1985, 1989). Like many other cortical pyramid–pyramid EPSPs, they display paired pulse and brief train depression (Markram and Tsodyks, 1996; Markram *et al.*, 1997), frequency-dependent depression and post-tetanic potentiation that are all mediated, at least in part, presynaptically (Thomson *et al.*, 1993; Thomson and West, 2003; for review, see Thomson, 2003).

Connectivity in Layer 6

The present finding that CC-like pyramidal cells are 2–4 times more likely to be presynaptic to another closely neighbouring pyramidal cell than CT cells provides an explanation for the findings of Beierlein and Connors (2002). They observed paired pulse facilitation when EPSPs were elicited by presumed antidromic activation of CT cells from the thalamus, but paired pulse depression when pairs of synaptically connected layer 6 cells were recorded. The present study demonstrates that the inputs to other pyramidal cells in layers 5 and 6 from CC-like pyramids demonstrate paired pulse, brief train and frequency-dependent depression (for discussion of the multiple mechanisms contributing to the dynamic control of transmitter release, see Thomson, 2003). They also displayed post-tetanic potentiation, becoming more strongly phasic when the probability of release for the first EPSP of a train was increased. These connections did not, however, appear to display one characteristic commonly expressed by pyramid–pyramid connections in other layers, the 'notch filter' (Thomson and West, 2003). This indicates that despite their pronounced dynamic characteristics, they do not selectively filter gamma frequencies.

Taken together, these two studies also predict that the less common pyramid–pyramid connections in layer 6 involving presynaptic CT cells may display facilitation. To date, only one paired recording of a layer 6 pyramid–pyramid connection that exhibited facilitation (at some frequencies) has been published (Thomson and West, 2003). In that example the presynaptic cell could not be unambiguously identified morphologically, but its tonic firing characteristics suggested that it may have involved a presynaptic CT cell. In addition, in the present study, a CT-like cell that received a strongly depressing EPSP from a phasic (presumed CC) cell, also received what appeared to be frequent spontaneous EPSP doublets that exhibited facilitation (not illustrated). This observation suggests that this cell received depressing inputs from one class of pyramidal cells and facilitating inputs from another (facilitation in EPSPs involving presynaptic CT-like cells is described in a paper in preparation). In many previous studies, emphasis has been placed on the apparent ability of the postsynaptic target cell to signal its identity to the presynaptic terminal and prescribe the release characteristics of the inputs it receives. While the present study does not dispute this, with layer 6 CC-like and CT-like cells we may have an example of the presynaptic neuron ordaining some of the release characteristics of each connection it provides.

Phasic Characteristics of Layer 6 Cortico-cortical Cells and Their Outputs

The pronounced phasic firing characteristics of CC-like cells and the strong, slowly recovering and cumulative depression apparent in their outputs suggests that these cells primarily signal novelty. Their typical firing pattern in response to a sustained depolarization is a brief, high-frequency doublet or very brief burst of APs followed by no further firing (Fig. 1). Only when the injected depolarizing current was increased during the pulse and/or when hyperpolarizing pulses were superimposed on prolonged depolarizations could these cells be

driven to fire more tonically, albeit at a lower frequency. Thus, unless a prolonged excitatory input increases significantly in strength over time and/or involves alternating excitatory and inhibitory inputs, the firing of CC-like cells will decline very rapidly. Moreover, even when the CC-like cell is driven to fire repeatedly, its outputs become steadily less and less effective. In striking contrast, CT-like cells typically fired tonically. The suggestion (above) that their outputs may not exhibit depression, and may even exhibit facilitation at some frequencies, predicts very different information transfer functions for the two cell classes. It also supports a hypothesis put forward previously, that if it is important to give preference to the transmission of certain frequencies or patterns at a given stage of information transfer, the circuitry employs more than one mechanism to ensure this preference. It should, however, be noted that firing patterns elicited *in vitro* by square wave current pulses, even when these are combined with ramps and brief hyperpolarizing pulses as here, do not reproduce all the patterns that may occur *in vivo*. Until, therefore, we have detailed information about how cells belonging to each of these classes of pyramidal cells fire during a range of behaviourally relevant responses, we can only speculate as to the functional relevance of the transfer functions predicted by *in vitro* data.

Correlations between conduction delays and separation of connected cell pairs indicated that conduction velocities in the largely unmyelinated axons of CC-like cells were slow ($\leq$0.1m/s). Since, therefore, CC cell horizontal axon collaterals can be long (up to 4 mm even in the slice), the conduction delays to their more distant connections would be tens of milliseconds in duration. CC pyramidal cells are therefore suited to the provision of relatively sparse, delayed, long-range and highly phasic excitatory input to other infragranular layer pyramidal cells, do not apparently discriminate between the different subclasses of pyramids innervated in these layers, but demonstrate a strong preference for pyramidal cells over inhibitory interneurons.

Notes

Our grateful thanks to Dr Andrew Ramage, Department of Pharmacology, Royal Free and UCL Medical School, London for providing the cat tissue.This work was supported by the Medical Research Council (UK) and Novartis Pharma (Basel).

Address correspondence to A.M. Thomson, Department of Pharmacology, The School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX, UK. Email: alex.thomson@ulsop.ac.uk.

References

Arimatsu Y, Ishida M (2002) Distinct neuronal populations specified to form corticocortical and corticothalamic projections from layer VI of developing cerebral cortex. Neuroscience 114: 1033–1045.

Beierlein M, Connors BW (2002) Short-term dynamics of thalamocortical and intracortical synapses onto layer 6 neurons in neocortex. J Neurophysiol 88:1924–1932.

Brumberg JC, Hamzei-Sichani F, Yuste R (2003) Morphological and physiological characterization of layer VI corticofugal neurons of mouse primary visual cortex. J Neurophysiol 89:2854–2867.

Harata N, Pyle JL, Aravanis AM, Mozhayeva M, Kavalali ET, Tsien RW (2001) Limited numbers of recycling vesicles in small CNS nerve terminals: implications for neural signaling and vesicular cycling. Trends Neurosci 24:637–643.

Hirsch JA, Gallagher CA, Alonso J-M, Martinez LM (1998a) Ascending projections of simple and complex cells in layer 6 of the cat striate cortex. J Neurosci 18:8086–8094.

Hirsch JA, Alonso JM, Reid RC, Martinez LM (1998b) Synaptic integration in striate cortical simple cells. J Neurosci 18:9517–9528.

Hughes DI, Bannister AP, Pawelzik H, Thomson AM (2000) Double immuno-fluorescence, peroxidase labelling and ultrastructural analysis of interneurones following prolonged electrophysiological recordings *in vitro*. J Neurosci Methods 101:107–116.

Gil Z, Connors BW, Amitai Y (1999) Efficacy of thalamocortical and intracortical synaptic connections: quanta, innervation, and reliability. Neuron 23:385–397.

Katz LC (1987) Local circuitry of identified projection neurons in cat visual cortex brain slices. J Neurosci 7:1223–1249.

Lund JS (1987) Local circuit neurons of macaque monkey striate cortex: I. Neurons of laminae 4C and 5A. J Comp Neurol 257:60–92.

Markram H, Tsodyks M (1996) Redistribution of synaptic efficacy between neocortical pyramidal neurons. Nature 382:807–810.

Markram H, Lubke J, Frotscher M, Sakmann B (1997) Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. Science 275:213–215.

McGuire BA, Hornung JP, Gilbert CD, Wiesel TN (1984) Patterns of synaptic input to layer 4 of cat striate cortex. J Neurosci 4:3021–3033.

Murthy VN, Stevens CF (1999) Reversal of synaptic vesicle docking at central synapses. Nat Neurosci 2:503–507.

Thomson AM (2003) Presynaptic frequency- and pattern-dependent filtering. J Comput Neurosci 15:159–202.

Thomson AM, West DC (1993) Fluctuations in pyramid–pyramid EPSPs modified by presynaptic firing pattern and postsynaptic membrane potential using paired intracellular recordings in rat neocortex. Neuroscience 54:329–346.

Thomson AM, Bannister AP (1998) Postsynaptic pyramidal target selection by descending layer III pyramidal axons: dual intracellular recordings and biocytin filling in slices of rat neocortex. Neuroscience 84:669–683.

Thomson AM, Morris OT (2002) Selectivity in the inter-laminar connections made by neocortical neurones. J Neurocytol 31:239–246.

Thomson AM, West DC (2003) Presynaptic frequency filtering in the gamma frequency band; dual intracellular recordings in slices of adult rat and cat neocortex. Cereb Cortex 13:136–143.

Thomson AM, West DC, Lodge D (1985) An N-methylaspartate receptor mediated synapse in rat cerebral cortex: a site of action for ketamine? Nature 313:479–481.

Thomson AM, Girdlestone D, West DC (1989) A local circuit neocortical synapse that operates via both NMDA and non-NMDA receptors. Br J Pharmacol 96:406–408.

Thomson AM, Deuchars J, West DC (1993) Large, deep layer pyramid–pyramid, single axon EPSPs in slices of rat motor cortex display paired pulse and frequency dependent depression mediated presynaptically and self facilitation mediated postsynaptically. J Neurophysiol 70:2354–2369.

Thomson AM, West DC, Wang Y, Bannister AP (2002) Synaptic connections and small circuits involving excitatory and inhibitory neurones in layers 2 to 5 of adult rat and cat neocortex: triple intracellular recordings and biocytin-labelling *in vitro*. Cereb Cortex 12:936–953.

Wang Y, Ramage AG (2001) The role of central 5-HT1A receptors in the control of B-fibre cardiac and bronchoconstrictor vagal preganglionic neurones in anaesthetized cats. J Physiol 536:753–767.

Wiser AK, Callaway EM (1996) Contributions of individual layer 6 pyramidal neurons to local circuitry in the macaque primary visual cortex. J Neurosci 16:2724–2739.

Zhang ZW, Deschenes M. (1998) Projections to layer VI of the posteromedial barrel field in the rat: a reappraisal of the role of corticothalamic pathways. Cereb Cortex 8:428–436.