Dynamic Properties of Excitatory Synaptic Connections Involving Layer 4 Pyramidal Cells in Adult Rat and Cat Neocortex

To investigate the properties of excitatory connections between layer 4 pyramidal cells and whether these differed between rat and cat, paired intracellular recordings were made with bicucullin filling in slices of adult neocortex. These connections were also compared with those from layer 4 spiny cells to layer 3 pyramids and connections between layer 3 pyramids. Connectivity ratios for layer 4 pyramid–pyramid pairs (1:14 cat, 1:18 rat) appeared lower than for the other types of connections studied in parallel, but excitatory postsynaptic potential (EPSP) amplitudes and time course were not significantly different either between species or across types of connection. Layer 4 pyramids targeted postsynaptic basal dendrites in both species, whether the pyramidal target was in layer 4 or layer 3. Within layer 4, relationships between mean EPSP amplitude, numbers of putative contacts, and distance between connected pairs indicated a rapid decline in connectivity strength with distance, equivalent to 3.4 mV and 10 synapses per 100 μm separation, from a maximum of 4 mV and 10 synapses at 0 μm. However, a subset, of burst-firing layer 4 pyramids, appeared to make no connections with other layer 4 spiny cells. Second EPSPs were depressed by 36% in rat and 28% in cat relative to first EPSPs at interspike intervals <15 ms. Subsequent EPSPs in brief trains were further depressed. Depression was predominantly presynaptic in origin. Recovery from depression could not be described adequately by a simple exponential for individual connections; it included peaks and troughs with periodicities of 10–15 ms. Complex relationships between the first 2 interspike intervals and third EPSP amplitude were also apparent in all connections so studied. Large third EPSPs followed specific combinations of first and second interspike intervals so that increasing, or decreasing, one without changing the other resulted in a smaller third EPSP. Finally, the outputs of layer 4 spiny cells to layer 3 exhibited partial recovery from depression during longer high-frequency trains, a property not apparent in the other connections studied.

Keywords: EPSP (excitatory postsynaptic potential), layer 4, neocortex, oscillations, pyramidal cell, synapse, synaptic dynamics

Introduction

Despite a large volume of excellent anatomical data and an increasing number of elegant dual and multiple recording studies with parallel anatomy, there are still many types of connections in neocortex for which we have few examples or where we have data only from one species or one developmental stage. Attempts to build biologically realistic models of cortical circuitry are highlighting how many pieces of the jigsaw are still missing. This is particularly the case in the light of increasing evidence for a high degree of selectivity; a selectivity that is expressed both in the properties of each class of connection (e.g., Markram et al. 1998) and in the targets innervated by each class of neurone in each of the layers in which its axon arborizes (e.g., Thomson and Bannister 2003; Mercer et al. 2005; West et al. 2006).

Spiny cells in layer 4 have extensive axonal arbors within layer 4 as well as focused axonal projections to layer 3 and less dense projections to the deeper layers 5 and 6 (Lund et al. 1979; Burkharter 1989; Anderson et al. 1994; Lübke et al. 2003). In addition to the major excitatory input they provide to layer 3 and their less powerful inputs to the deep layers, layer 4 spiny cells can be predicted to provide a significant excitatory input to other layer 4 cells. In juvenile rat barrel cortex, boutons supplied by layer 4 spiny stellate axons contact the small caliber dendrites and dendritic spines of other spiny neurones, the preferred synaptic targets being tertiary dendrites, close to the postsynaptic cell body (Lübke et al. 2000). In layer 2/3, the preferred targets of layer 4 spiny cell axons are pyramidal basal dendrites (Thomson et al. 2002; Lübke et al. 2003). Whether their preferred targets in layer 4 pyramids are also basal dendrites remains to be determined.

With the exception of 2 dual recording studies that reported, respectively, 3 layer 4 pyramid-to-pyramid connections in adult cat visual cortex (Tarczy-Hornoch et al. 1999) and one in adult rat (Thomson et al. 2002), previous studies of layer 4 spiny cell connections have been performed in young rats, between 12 and 23 days old (Feldmeyer et al. 1999, Lübke et al. 2000; Petersen and Sakmann 2000; Petersen 2002). In these studies, the majority of recorded connections between spiny layer 4 cells involved pairs of spiny stellate cells. This suggests 2 possibilities, first that pyramidal cells constitute only a minor proportion of spiny cells in layer 4. Alternatively, their connectivity rates within layer 4 could be low compared with spiny stellate cells, as suggested by previous anatomical studies in which spiny stellates were found to have richer projections within layer 4 than star pyramids (Martin and Whitteridge 1984) and were proposed therefore to be the major local source of excitation within that layer (Anderson et al. 1994). No comparative connectivity ratios for spiny stellates versus pyramids have been reported. Morphometric analysis of immature layer 4 cells suggested that the probable connectivity rates amongst spiny cells of all types was 1:5 (Feldmeyer et al. 1999), similar to the connectivity ratios reported for layer 4 connections in adult cat (Stratford et al. 1996; Thomson et al. 2002). The “hit rates” found for randomly selected spiny cell pairs at postnatal days 13–15 in rat were, however, lower (approximately 1:14, Petersen 2002).

Excitatory postsynaptic potentials (EPSPs) in immature and mature cortex have been reported to differ in the slower time course of the recovery from paired-pulse depression in young...
brain. For a range of types of connections in adult rat and cat cortex, the time constants for decay of depression were typically <30 ms and fell within a relatively narrow range (mean 12.4 ± 8.1 ms; Thomson and West 2003). For layer 4 EPSPs at postnatal days 13–15, time constants were more variable (20–1000 ms), but typically longer than in adults, with an average of nearly 500 ms (Petersen 2002). The wide range may indicate that synapses are maturing toward their adult characteristics over a period of days to weeks.

A recent study of the other major group of thalamoreceptive pyramidal cells demonstrated that, unlike most types of pyramidal cells, layer 6 corticothalamic pyramids elicited facilitating EPSPs both in other pyramids and in all classes of interneurones studied. However, the development of facilitation and the recovery from short-term facilitation and from depression of later EPSPs in train did not follow a smooth exponential but exhibited distinct periodic peaks and troughs (West et al. 2006). Whether such complex dynamics occur in other types of pyramid–pyramid connections and particularly in those that exhibit synaptic depression remains to be determined.

The present study was therefore undertaken to investigate the local connections between mature layer 4 pyramidal cells and to compare these in the 2 most commonly studied species, rat and cat, and with projections from layer 4 to layer 3 and with connections within layer 3, allowing data to be correlated with previous studies. Of particular interest were circuitry details such as connectivity rate and target preference, as well as whether the synaptic dynamics match those previously reported for mature connections between other excitatory cell classes or immature connections in the same layer.

**Methods**

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

Male Sprague–Dawley rats 116–230 g in body weight (n = 37) were anesthetized with inhaled Fluothane and intraperitoneal pentobarbitone sodium (Sagatal or Euthetal; Rhone Merieux, Harlow, Essex, UK, > 60 mg kg\(^{-1}\)). Male cats (n = 8, 2.5–3.4 kg) were anesthetized intravenously with a mixture of x-chloralose (70 mg kg\(^{-1}\)) and pentobarbitone sodium (6 mg kg\(^{-1}\)) 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\(^{-1}\)) 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 to 500-μm thick, were cut (Vibroslice, Camden Instruments, London, 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\(_2\)PO\(_4\), 1.0 MgSO\(_4\), 2.5 CaCl\(_2\), 15 n-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 microelectrodes, containing 2 M KMeSO\(_4\) and 2% (v/v) biocytin, tip resistance 90–150 MΩ. Presynaptic neurones 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 (Spide-2, Cambridge Electronic Designs, Cambridge, UK). Previous studies have indicated that the time course of recovery from synaptic depression may not be adequately described by a simple exponential. Nor can it be fully explored using a finite number of well-spaced interspike intervals that are identical, in any one trial, for all spikes in the train (Thomson and West 2003; West et al. 2006). In these experiments, therefore, the size and shape of the current pulse was continuously changed to elicit as wide a range of interspike intervals and firing patterns as possible.

Cells were filled with biocytin and slices fixed, resectioned at 50 μm, and processed histologically (Avidin-horseradish peroxidase/diamino-benzidine) for identification of recorded neurones, as described previously (Hughes et al. 2000). Connected cell pairs were drawn in their entirety at 1000× magnification using a drawing tube.

As a first step in 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 fast component of rising phase of each AP. Sweeps including artifacts 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 analyzed similarly. In figures, all illustrated EPSPs are averages and composite averages of between 20 and 200 sweeps. Where postsynaptic responses exhibited an adequate signal to noise ratio, single-event sweeps were also measured (by hand with cursors). To measure short-interval second and subsequent EPSP amplitudes, an average of all single-spike EPSPs was scaled to match the amplitude of the first EPSP in each sweep and second EPSP amplitude measured from its peak to the appropriate point on the decay of the scaled first EPSP average.

Single-spike EPSP amplitudes were plotted against interspike interval and smoothed (running average of 10–30 points) to reveal trends (Figs 4–8). To compare the contributions made by the first EPSP amplitude and interspike interval, respectively, toward determining second EPSP amplitude, second EPSP amplitude was plotted against these 2 parameters. Where enough single-sweep measurements with a suitable range of interspike intervals for both second and third EPSPs had been collected, the combined effects of the interval between the first and second APs (first interspike interval) and the interval between the second and third APs (second interspike interval) on the amplitude of the third EPSP were tested by plotting third EPSP amplitude against these 2 interspike intervals. The resulting 3-dimensional (3D) surface plots were then rendered as contour maps with second or third EPSP amplitudes indicated by color (PSI-Plot, Poly Software International Inc., Pearl River, NY, Figs 4–8).

In Figures 2 and 4–8, averaged EPSPs elicited by second and subsequent spikes in trains are color coded according to their amplitude (blue indicates a low and red a large amplitude). In composite averaged traces comprising averaged responses to each of several spikes in trains, fine lines represent the extrapolated decay of each EPSP derived from the decay of a similar amplitude EPSP. Capacitance-coupling artifacts associated with the presynaptic spike were removed graphically.

**Statistical Analysis**

In comparisons of the morphological parameters of the layer 4 pyramidal cells across species and of EPSP properties across species and across types of connections, data were pooled and an unpaired Student's t-test was used to determine whether any differences in the means reached significance.

Statistical analysis was performed to determine whether the "peaks and troughs" apparent in plots of EPSP amplitude against interspike interval (see Figs 2 and 4–8) resulted from populations of EPSPs with significantly different mean amplitudes. Subsets of single-sweep EPSP amplitudes were selected as narrow preceding interspike interval ranges (2–5 ms wide) were selected. This resulted in between 5 and 10 subsets of second or third EPSP amplitudes for each connection corresponding to peaks and troughs in the plots. To test the significance of the peaks and troughs apparent in the 3D contour plots of third EPSP amplitude, subsets of single-sweep third EPSP amplitude measurements were selected, each associated with narrow ranges of both of the 2 preceding interspike intervals. This resulted in between 5 and 11
subset of third EPSP amplitudes for each connection. The means of these data subsets were compared using analysis of variance (1-way ANOVA) after testing for homogeneity of variance and multiple pairwise unpaired t-tests subjected to the Bonferroni correction.

Results

This study describes the excitatory connections made and received by layer 4 pyramidal cells and compares their properties with those of connections between layer 3 pyramidal cells and connections from layer 4 spiny cells to layer 3 pyramidal cells. Some data from the layer 3 pairs used in the population comparisons in Figure 3B,C have been published previously, although the analysis presented here is new. However, new recordings are also included, and importantly, it is these that are illustrated as a direct comparison with the new layer 4 data collected in the same experimental series.

Electrophysiological Properties of Layer 4 Pyramidal Cells

The firing patterns of layer 4 pyramidal cells in both species fell into 2 broad categories. 63% exhibited adapting firing patterns and 37% were burst firing (BF). Sixty-two percent of the adapting cells showed rapid spike frequency adaptation to zero within the first 50 ms of a 500 ms pulse and are more appropriately termed phasically firing cells. The BF cells typically produced short bursts of 3-5 APs at the start of a long depolarizing pulse, followed by single spikes with progressively increasing interspike intervals. Intrinsically bursting cells similar to those described in layer 5 (Connors et al. 1982; McCormick et al. 1985) were rarely seen in layer 4. All the layer 4 pyramidal cells that were synaptically connected to another layer 4 pyramidal were of the adapting type. BF cells were found to innervate layer 3 pyramids, but in the present study, none was found to be synaptically connected to another layer 4 pyramid in 120 pairs tested in which one or both of the layer 4 pyramids was a BF cell.

Morphology of Layer 4 Pyramidal Cells

Fourteen layer 4 pyramidal cells were reconstructed from cat and 13 from rat neocortical slices. Their somata and the most proximal portions of their 4-7 primary dendrites were smooth and aspiny, becoming spiny 5-20 μm from the cell body to their extremities. Basal dendrites bifurcated up to 7 times, generating between 11 and 32 last-order branches. Apical dendrites extended perpendicular to the pial surface generating between 2 and 10 oblique branches with between 3 and 24 terminal or last-order branches. Apical dendrites terminated in layer 1 or 2. The distal apical dendrites of all but one rat pyramid bifurcated at least once to form an apical tuft with between 3 and 14 terminal or last-order branches in the most superficial layer it reached. The numbers of dendritic branches of each type for rat and cat layer 4 pyramidal cells are summarized in Figure 1G and

![Diagram](image-url)

**Figure 1.** Two pairs of synaptically connected layer 4 pyramidal cells in rat somatosensory cortex (A–C, D–F). Single-sweep presynaptic firing and composite averaged postsynaptic responses for these 2 pairs of layer 4 pyramidal cells are shown in (A) and (F), with trace colors matching the soma/dendrite reconstructions in (B) and (D). The pair illustrated in (D–F) was reciprocally connected. Reconstructions of the 2 pyramidal cell pairs are shown in (G) and (D) and enlarged portions in (C) and (E). The circles, colored to match the presynaptic axon (black soma–green axon, red soma–blue axon), indicate locations of putative synaptic contacts. In (E), the blue squares indicate the positions of putative autapses made by the axon of the red pyramid on its own parent dendrites. In (G), the mean numbers of dendritic branches for 14 cat (filled bars) and 13 rat (open bars) layer 4 pyramidal cells are shown: the number of primary dendrites (primary); the number of second-order basal, apical oblique, and apical tuft branch points (first branch); and the number of terminal or last-order branches for basal, apical oblique, and apical tuft dendrites. Error bars indicate the SDs for the populations.
demonstrate a striking similarity in gross structure across these species. Neurone dimensions were also similar. This is perhaps surprising given the much larger size of cat cortex. The average soma diameter of cat layer 4 pyramidal cells was 15.7 ± 5.1 μm (mean ± standard deviation [SD]), with an average horizontal dendritic span of 305 ± 70 μm and vertical span perpendicular to the pial surface of 616 ± 174 μm. The average soma diameter of rat layer 4 pyramids was 14.9 ± 5.1 μm, with a horizontal dendritic span of 307 ± 49 μm and vertical span of 658 ± 80 μm. There was no significant difference in any of these morphological parameters between the 2 species (P > 0.05, unpaired t-test).

A partially myelinated primary axon projected toward the white matter. From nodes between myelinated portions, axon collaterals that were typically unmyelinated projected either horizontally or obliquely toward the superficial layers. Recovered axon collaterals were not observed to project more superficially than the cells’ own apical dendrite. Horizontally, the majority of collaterals in the superficial layers terminated within a cylinder whose horizontal span equaled that of the cell’s basal dendrites. In a minority, the axon’s horizontal span extended to twice that of the basal dendrites, but very long horizontal axonal processes extending laterally up to 750 μm were observed in only a few cells from both species. Descending axons typically generated short (200–300 μm) horizontal collaterals in layer 5 and to a lesser degree in layer 6. It should, however, be noted that many of the longer collaterals, particularly those running fronto-caudally for more than 500 μm, will have been pruned in the slicing procedure.

Multiple putative autapses, that is, close membrane appositions between the axon and its parent dendrites, were observed in adult layer 4 pyramidal cells in both species (Figs 1, 2, 3A, and 4A). In all cases, they occurred either more than once (on average 2.8 autapses per cell, range 2–4) or not at all.

**Probability, Distribution, and Strength of Connections between Layer 4 Pyramidal Cells**

In 37 experiments in rat slices and 8 in cat, 528 pairs of rat and 139 pairs of cat layer 4 excitatory cells were tested. Twenty-two tested pairs in rat and 10 in cat yielded excitatory connections, giving average connectivity ratios (connected pairs: pairs tested) of 1:24 in rat and 1:14 in cat. One pair in rat (Fig. 1D) and 2 in cat (Fig. 4) were reciprocally connected. Of the 528 pairs tested in rat, 120 involved BF pyramids that were never found in this study to be connected to other layer 4 pyramids. The connectivity ratio for the type of layer 4 pyramids that more commonly connected to other layer 4 pyramids (i.e., those with an adapting firing pattern) was therefore 1:18.5. Since no layer 4 pyramid–pyramid pairs involving BF cells were tested in cat, these ratios are relatively similar.

In cat, the distance between synaptically connected pairs of layer 4 pyramidal somata (calculated in 3 dimensions) ranged from 50 to 171 μm (mean 107 ± 6 μm). Lateral separation was between 0 and 123 μm (50 ± 43 μm). In rat, the distance between connected layer 4 pyramidal cells was smaller, but not, with this sample size, significantly so (P > 0.05, unpaired t-test), between 5 and 158 μm (87 ± 43 μm). Lateral separation was between 0 and 86 μm (35 ± 33 μm). Between 1 and 7, close membrane appositions between the presynaptic axon and postsynaptic dendrites were identified at the light level for 6 of these pairs (2 in cat, 4 in rat). All identified putative contacts were onto spiny portions of primary, secondary, and tertiary basal dendrites (Figs 1C, E, and 4E). A correlation (correlation coefficient 0.88) was found between the number of putative synapses and the amplitude of the resultant average EPSP with a slope of 0.35 mV per contact.

In Table 1, the properties of EPSPs elicited by layer 4 pyramidal cells in other layer 4 pyramidal cells in both rat and cat are compared with those of EPSPs elicited by layer 4 spiny cells in layer 3 pyramidal cells and by layer 3 pyramidal cells in other layer 3 pyramidal cells. There were, in these samples, no significant differences between the amplitudes, 10–90% rise times or the widths at half amplitude for these populations of EPSPs, either between species or across types of connection (P > 0.05, unpaired t-test).

**Presynaptically Mediated Paired-Pulse and Brief Train Depression**

In Figures 2 and 4–8, data from one connection (or reciprocal connection) are illustrated together on a cell pair by cell pair basis to allow the representations of data obtained from plots of single-sweep measurements and from averaging responses to be compared. Several figures are therefore cited in relation to each of the properties discussed below. Consistency in the style of presentation has been attempted to aid comparisons across pairs. To aid rapid assessment of EPSP amplitude by eye, second and subsequent EPSPs in composite averages of responses to trains of spikes are color coded, those with amplitudes that were large in comparison with the population in red and the smallest in dark blue.

In all layer 4 pyramid–pyramid pairs in which responses to pairs of presynaptic spikes with interspike intervals ≤15 ms could be analyzed (9 in rat, 5 in cat), the average amplitude of the second EPSP was smaller than that of the first. Between 15 and 40 ms, 3 of the cat pairs exhibited slight paired-pulse facilitation (1–12%), but all rat and the remaining 8 cat pairs exhibited depression for at least 50 ms. Third and subsequent EPSPs at similar intervals were further depressed (Figs 2C, 5D, 5F).

**Table 1**

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<th>Properties of EPSPs elicited by layer 4 pyramidal cells in other layer 4 pyramids in rat and cat are compared with those of EPSPs elicited by layer 4 spiny cells in layer 3 pyramids and by layer 3 pyramids in other layer 3 pyramids</th>
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Note: New layer 4 data are presented. Means and SDs for layer 4 to layer 3 connections include both new and previously published data (Thomson et al. 2002) and for layer 3 connections new data are compared with previously published data (italics in square brackets), L1–6, layer 1–6.
In all 10 pairs in which there were apparent failures of transmission, the proportion of failures increased from the first to the fourth EPSP (e.g., Fig. 2D). In addition to this evidence for a presynaptic site for paired-pulse and brief train depression, the majority of points in plots of the normalized inverse square of the coefficient of variation (CV$^2 = np^2 / n$) of second and subsequent EPSPs against their normalized mean amplitudes ($M = npq$) fell on slopes $>1$, indicating that $p$ had declined (where $n$ is the number of release sites, $q$ the quantal amplitude, and $p$ the probability of release, and assuming a simple or compound binomial model for release, values normalized against those for the first EPSP) (Fig. 3D) (for discussion of assumptions and applicability of this method, see Faber and Korn 1991). Also included in this plot are values obtained from layer 3 pyramidal pairs and for connections from layer 4 spiny cells to layer 3 pyramids.

Figure 2. Recordings and analysis from a layer 4 pyramid-to-pyramid pair in rat visual cortex. (A) Averaged responses to pairs of presynaptic APs at different interspike intervals are superimposed and their average amplitude is color coded so that the largest second EPSPs are shown as red and the smallest as dark blue. (B) Averaged responses to trains of 3 presynaptic spikes with the third EPSP amplitude similarly color coded. (C) Averaged responses to trains of 4 (left) and 5 (right) presynaptic spikes with average fourth and fifth EPSP amplitudes color coded. (D) Single-sweep amplitudes of second and third to sixth EPSPs in brief trains plotted against interspike interval and smoothed. Both this plot and the relative amplitudes of averaged EPSPs demonstrate that the recovery from depression does not follow a smooth curve, and peaks and troughs are apparent in the plots. Average first EPSP amplitude for this connection was 0.77 mV. (D) EPSP Amplitude distributions for first to sixth EPSPs in trains. The proportion of apparent failures of transmission increases from the first to the sixth as average EPSP amplitude declines indicating that depression is primarily presynaptically mediated.
Time Course of Recovery from Paired-Pulse Depression

When second EPSP amplitude measurements from single pairs were binned finely, that is, within narrow interspike interval ranges, averaged, and plotted against interspike interval, the recovery from depression did not follow a sufficiently smooth decay (see below) to allow any simple exponential curve to be fitted (correlation coefficients < 0.7). To gain an indication of the average time course of recovery, therefore, broad interspike interval bins were selected (5–10, 10–15, 15–20, 20–30, 30–40, and 40–60 ms). These encompassed components of both the peaks and troughs visible in the finely binned plots of EPSP amplitude against interspike interval and allowed any underlying more slow decaying component(s) to be identified. The means obtained for these broad bins were normalized against mean first EPSP amplitude, data pooled and means ± SDs for each population calculated. Normalized second EPSP amplitudes for 10 rat and for 11 cat layer 4 pyramid–pyramid pairs were then plotted against interspike interval and single exponential curves fitted (Fig. 3A). It was not possible to study all intervals in all pairs, and the number of pairs included in each of these means ranges from 4 to 9. Time constants obtained from these fits were 17 ms for rat and 8 ms for cat, and projected amplitudes at infinity were 85% and 94% of first EPSP amplitude, respectively.

Although depression was typically less pronounced in cat layer 4 pairs at all intervals, variation between pairs was large (see SDs in Fig. 3), the sample size small, and the difference did not reach significance ($P > 0.1$, unpaired $t$-test). For comparison, similar analysis is illustrated in Figure 3B,C for layer 3 pyramid–pyramid (8 rat and 10 cat pairs, time constant 12 ms in rat and 22 ms in cat) and for layer 4 spiny cell to layer 3 pyramid pairs (5 rat and 7 cat pairs, time constant 4.4 ms in rat and 21 ms in cat). No significant differences in the degree of depression between these populations were found, whether the same class of connection was compared between rat and cat or whether different classes of connections within a species were compared ($P > 0.1$, unpaired $t$-test).

Complex Relationships between EPSP Amplitude and Interspike Interval

The finer details of the time course of EPSP recovery from paired-pulse and brief train depression at connections between layer 4 pyramids are described here and compared with those of connections between layer 3 pyramids and those from layer 4 to layer 3 pyramids.

In 3 rat and 7 cat layer 4 pyramidal cell pairs, single-sweep data covering a sufficiently wide range of interspike intervals were collected to allow the time course of recovery from depression to be studied in more detail. It is clear from Figures 2 and 4–7 that these data are not adequately described by a single component that is maximum at short interspike intervals and recovers smoothly with increasing intervals. Peaks and troughs are superimposed on the recovery from paired-pulse and brief train depression. To investigate the relative contributions made by the amplitude of the preceding EPSP(s) and the interspike interval to determining the amplitudes of the second or third EPSP in brief trains, 3D surface plots were generated and are illustrated here as contours. The most striking feature of these plots is the dominance of interspike interval, with banding representing the peaks and troughs (Fig. 4B, see also Figs 6B and 7B). When third EPSP amplitude was plotted against first and second interspike interval, a complex relationship between these intervals and third EPSP amplitude became apparent (Fig. 4D, see also Figs 5C, 6D, and 7D) as described previously for the outputs of layer 6 corticothalamic neurones (West et al. 2006). The amplitude of the third EPSP could not be predicted from a simple relation with either the first or the second interval or with the sum of the two.

In Figures 5–7, three layer 3 to layer 3 pyramidal connections are illustrated. Again, the dominance of interspike intervals in determining the amplitude of EPSPs, the peaks and troughs apparent in the decay of depression, and the complex relationship between the first 2 intervals and the amplitude of the third EPSP can be seen. In Figure 7, a layer 4 pyramid to layer 3 pyramid connection is also illustrated and compared with a layer 3 pyramidal connection that involved one of the same layer 3 pyramidal cells. This layer 4 to layer 3 recording was relatively brief, but characteristics similar to those described for connections within layer 4 or within layer 3 are apparent from the composite averaged EPSPs.

To determine whether the previously undocumented characteristics exhibited by connections between layer 4 pyramidal...
Figure 5. A connection between 2 layer 3 pyramidal cells in cat visual cortex. (A) EPSPs activated by pairs of presynaptic APs at different interspike intervals, color coded according to average EPSP amplitude. (B) EPSPs activated by trains of 3 presynaptic APs at different interspike intervals. Composite averages in which the first interspike interval was the same, but the second interval varied, are superimposed and third EPSP amplitude color coded. (C) A 3D plot of third EPSP amplitude against first and second interspike interval rendered as a color contour. Points indicate single-sweep measurements. (D) Single-sweep amplitudes of second to seventh EPSPs in brief trains plotted against interspike interval and smoothed. (E) EPSPs activated by trains of 4 to 6 presynaptic APs at different interspike intervals. Average amplitudes of fourth, fifth, and sixth EPSPs are color coded.

Figure 4. A reciprocal connection between 2 layer 4 pyramidal cells in cat visual cortex. (A) EPSPs activated in the red pyramid (blue axon) by pairs of presynaptic APs in the black pyramid (green axon) at different interspike intervals. EPSP amplitude is color coded; the largest average second EPSPs being shown as red and the smallest as dark blue. (B) A 3D surface plot of second EPSP amplitude against interspike interval and first EPSP amplitude rendered as a color contour. The largest second EPSP amplitudes are coded red and the smallest dark blue. The points indicate individual single-sweep measurements. (C) Average third and fourth EPSPs at different intervals color coded according to average amplitude. (D) Third EPSP amplitude plotted against first and second interspike intervals as a 3D plot rendered as a color contour. Points indicate individual single-sweep measurements. (E) Reconstruction of the 2 pyramids. The inset illustrates putative points of contact, blue circles indicating inputs from blue axon to black dendrites, the green circle a putative input from green axon to red dendrites, and the blue squares putative autapses. Although one blue circle appears to mark a dendrite superficial to the basal dendritic tree, this branch originates from a large basal dendrite issuing from the soma toward the viewer and not from the main apical dendrite. (F) EPSPs activated in the black pyramid by pairs of presynaptic APs in the red pyramid at different interspike intervals recorded at \(-70\) mV. Superimposed on the first spike EPSP average is an average of single-spike sweeps in which the presynaptic neurone appeared also to activate a disynaptic inhibitory postsynaptic potential (IPSP) that curtails the EPSP. (G) Brief trains of APs elicit EPSPs that sum when no IPSP is activated (dark gray), but which do not sum when the IPSP is activated near the peak of the EPSP (light gray). IPSPs can therefore suppress summation even when the soma is close to \(Cl^-\) reversal potential. To generate these averages, those single-sweep traces in which EPSPs appeared to be curtailed by IPSPs and those traces in which the shape of the EPSP more closely resembled that of EPSPs from other similar connections were selected by eye and averaged separately.
cells are also expressed by the outputs of layer 4 spiny stellate cells, 4 spiny stellate to layer 3 pyramidal cell (Fig. 8) and one spiny stellate to spiny stellate connection were analyzed. The characteristic found in this study to typify connections made by layer 4 and layer 3 pyramids with other pyramidal cells, that is, peaks and troughs in the time course of recovery from depression, were also found in spiny stellate outputs.

Significance of Peaks and Troughs in Plots of EPSP Amplitude against Interspike Interval

Statistical analysis was performed to determine whether the peaks and troughs apparent in plots of EPSP amplitude against interspike interval resulted from subpopulations of EPSPs with significantly different mean amplitudes (see Methods). Significant differences between subsets were indicated by ANOVA (for second EPSPs \( P < 0.0001 \) for 4 of the pairs studied, \( P < 0.02 \) for one pair, and \( P < 0.05 \) for one pair, 1-way ANOVA). The higher levels of significance resulted from paired recordings for which the larger sets of data were available and in which all subsets included more than 50–100 sweeps. To determine which subsets differed significantly, pairwise \( t \)-tests were conducted. When subsets corresponding to successive peaks or subsets corresponding to successive troughs were compared, the means were not significantly different (\( P > 0.05 \)). However, when pairs of data subsets corresponding to a peak and the preceding or following trough were compared, the means differed significantly (\( P < 0.05 \) to \( P < 1 \times 10^{-9} \), 31 pairwise tests from 5 cell pairs). When these comparisons were subjected to more rigorous testing (Bonferroni correction), only those multiple comparisons (>1 pairs of subsets tested) resulting from the larger data sets (Figs 2, 6, and 7) consistently reached significance (\( P < 1 \times 10^{-8} \) to \( P < 0.05 \)).

In similar analysis of third EPSPs from the larger data sets, significant differences between the more prominent peaks and troughs were found (\( P < 0.05 \), 1-way ANOVA and 11 pairwise \( t \)-tests from 3 cell pairs subjected to the Bonferroni correction). The significance of the peaks and troughs indicated by color in the 3D contour plots of third EPSP amplitude against the first and second interspike interval (Figs 4–8) was also tested. For the 5 cell pairs tested, significant differences between subsets were indicated by ANOVA (\( P < 0.0002 \), 1-way ANOVA). Pairwise \( t \)-tests demonstrated that the levels of significance for data subsets that were widely different in color, for example, dark blue and yellow, were high (\( P < 0.00000002 \) to \( P < 0.005 \)). Where the color difference was intermediate, for example, dark blue to green or yellow to red, the level of significance was lower (\( P = 0.0002 \) to 0.03). For subsets with similar colors, for example, dark and light blue or yellow and light green, the differences in the means were not significant (\( P > 0.05 \)).

Figure 6. A connection between 2 layer 3 pyramidal cells in cat visual cortex. (A) EPSPs activated by pairs of presynaptic APs at different interspike intervals and the average second EPSP amplitude color coded. (B) 3D plot of second EPSP amplitude against interspike interval and first EPSP amplitude rendered as a color contour. (C) Single-sweep amplitudes of second, third, and fourth EPSPs in brief trains plotted against interspike interval and smoothed. (D) 3D plot of third EPSP amplitude against first and second interspike intervals rendered as a color contour. Points indicate single-sweep measurements. (E) EPSPs activated by trains of 3 presynaptic spikes at different interspike intervals. Composite averages in which the first interspike interval was the same, but the second interval varied, are superimposed and third EPSP average amplitude color coded.
Partial Recovery from Depression during Repetitive Firing at the Outputs of Layer 4 Cells

The connection illustrated in Figure 8.4 also demonstrates a novel phenomenon that is less commonly observed. Although the average EPSP amplitude declines from the first to the fourth, fifth, or sixth EPSP in a train, some recovery of later EPSPs is apparent over a range of relatively high frequencies. In pairs tested with trains of at least 10 presynaptic spikes, this recovery was apparent in all four layer 4 to layer 3 EPSPs studied (all in cat and including 2 presynaptic spiny stellate cells), but in no layer 4 pyramid-to-pyramid connections (4 cat, 3 rat) and in only one rat, but not in the 6 cat layer 3 pyramid-to-pyramid connections (4 cat, 3 rat).
connections tested. The more typical gradual decline in average amplitude throughout a long train of similar interspike intervals in a layer 3 pair is illustrated for comparison in Figure 8F.

Discussion
This study provides the first detailed analysis of synaptic connections between mature layer 4 pyramidal cells and compares these in 2 species, rat and cat. For the parameters studied, there was very little difference in the properties and gross morphology of the neurones (Fig. 1G) or in the connections between them in these 2 species. Layer 4 pyramid-pyramid connections were then also compared with connections made by layer 4 spiny cells with layer 3 pyramids and with connections between layer 3 pyramidal cells in both species.
Again, apart from connectivity ratios, the differences did not reach significance. Importantly, these findings suggest that some significant features of the circuitry are generic and independent of the species.

It should be noted that the extracellular [Ca\(^{2+}\)] used in these experiments is higher (2.5 mM) than is typically reported for in vivo concentrations. This probably results in a higher release probability at low firing frequencies and possibly in more profound synaptic depression than might apply in vivo (Markram and Tsodyks 1996). Higher than in vivo [Ca\(^{2+}\)] is commonly used in vitro because it helps to stabilize membranes and thereby long recordings and allowed the current data to be directly compared with those from this and other laboratories. Moreover, previous studies have demonstrated that whereas the release probability at pyramid–pyramid synapses is reduced by reducing extracellular [Ca\(^{2+}\)], the essential differences between low “p" facilitating and high “p" depressing connections are maintained over a range of [Ca\(^{2+}\)]s (Thomson et al. 1993, 1995; Thomson 1997).

**Connectivity Rates for Connections between Layer 4 Pyramidal Cells**

In the present study, the connectivity ratios for connections between layer 4 pyramidal cells were relatively low (1:18.5 in rat and 1:14 in cat) when compared with those previously reported for connections between mature cat layer 4 spiny cells (which included a majority of spiny stellate cells, Stratford et al. 1996), or between mature rat layer 3 pyramids (Thomson et al. 2002, 1:4), but similar to that found for immature layer 4 spiny cell connections (1:14, Petersen 2002) and for pyramid–pyramid connections in the deeper layers (Thomson et al. 2002; Mercer et al. 2005). Because a large proportion of the neurones filled and recovered in this study were pyramidal cells, these data support the suggestion that the richer projections of spiny stellate cells within layer 4 result in their higher connectivity rates when compared with layer 4 pyramids (Martin and Whitteridge 1984) and that it is for this reason that spiny stellates appear to be the major local source of excitation within that layer (Anderson et al. 1994). In addition, the subclass of layer 4 pyramidal cells that fired bursts of APs appeared to contribute little or not at all to connectivity between layer 4 spiny cells, although these pyramidal cells were found to be involved in connections with layer 3. This appears to parallel recent findings in layer 6 where cortico-cortical pyramidal neurones innervate other layer 6 pyramids very much more commonly than do corticothalamic pyramidal cells (Mercer et al. 2005). This again underlines the separation of information processing streams even amongst the pyramidal cells of a given layer, which first became apparent in studies of layer 5 pyramids (for review, see Thomson and Bannister 2003).

**Layer 4 Pyramidal Cells Target the Basal Dendrites of Other Layer 4 Pyramidal Cells**

Layer 4 spiny cell innervation of layer 3 pyramids preferentially targets their basal dendrites (Thomson et al. 2002; Lübke et al. 2003). That this is also the case for within layer 4 pyramid–pyramid connections was demonstrated in the present study because all putative synapses identified at the light microscope level were onto basal dendrites. Although these close appositions were not formally identified as synapses at the ultrastructural level, there was an absence of contacts involving apical dendrites. Thus, despite a spatial overlap between the apical dendrites and ascending axonal arbors of layer 4 pyramids in layer 3, synaptic connections between these cells appear to be restricted to their basal dendrites and largely to layer 4. Because layer 3 pyramidal cells rarely innervate layer 4 pyramids, the source of excitatory input to the well developed, spiny layer 4 pyramidal apical dendritic arbors remains to be identified.

**Properties of Connections between Layer 4 Pyramidal Cells**

Other properties of layer 4 pyramid–pyramid connections, such as EPSP amplitude, rise time and width at half amplitude were also similar when connections in the 2 species were compared and when other connections between layer 3 and 4 spiny cells were compared (Table 1.).

In layer 4 of rat barrel cortex, the strength of EPSPs activated by electrical stimulation at the center of a barrel was dependent on the distance of the recorded neurones from the stimulating electrode (Petersen and Sakmann 2000), dropping significantly at the edge of the column. This finding appears to be supported by paired intracellular recordings from spiny cells in layer 4 of cat striate cortex in which EPSP average amplitude decreased with increasing distance between the connected cells (Tarczy-Hornoch et al. 1999). These observations can be explained by a model of axonal bouton “clouds” that overlap with the dendritic arbors of postsynaptic cells, the implication being that if random connectivity can be assumed, the number of release sites involved in a connection decreases as a function of the overlap and therefore of the distance between the connected neurones (Douglas et al. 1995). However, in immature cortex, no significant correlation between the number of putative synaptic contacts and the amplitude of the resultant EPSP was found (Feldmeyer et al. 1999). This perhaps surprising finding may, however, reflect the immaturity of the circuitry studied; a significant proportion of “silent” or very low probability synapses might, for example, contribute to these connections at this stage of development.

In the present study, a correlation between the number of putative synapses connecting layer 4 pyramidal cells and the amplitude of the resultant average EPSP was indicated. The slope of this line (0.35 mV per contact) would suggest that with an average release probability of 0.8 (similar to that proposed by Tarczy-Hornoch et al. 1999, for layer 4 spiny cell connections: 0.69–0.98), each contact would contribute 0.44 mV. Weak correlations were found between EPSP amplitude and the number of putative contacts and the distance between the 2 connected layer 4 pyramidal somata (10 connections, 7 pairs). However, when the weaker of each pair of connections involved in reciprocal connections was omitted, these correlations were increased (correlation coefficients 0.92 and 0.86). The slopes of these lines indicated a rapid decline in mean EPSP amplitude of 3.4 mV and 10 synapses per 100 μm, from a maximum of approximately 4 mV and 10 synapses at 0 μm separation. The numbers included were small, and the contacts were not confirmed at the ultrastructural level but are consistent with previous studies in adult cortex. That reciprocal connections may be weaker, both in EPSP amplitude and number of contacts, than would be predicted from separation might indicate that these connections survive developmental pruning because of a relatively powerful 1-way
connection that promotes near synchronous firing even when other wiring “rules” are not satisfied.

**Paired-Pulse and Brief Train Depression of Layer 4 Pyramid-to-Pyramid Connections**

All pyramid–pyramid connections in this study exhibited paired-pulse depression at short interspike intervals. Overall, depression was typical of all connections included in this study at interspike intervals less than 50–60 ms and typically became increasingly more pronounced for third, fourth, and subsequent EPSPs in trains. Both an increase in the proportion of apparent failures of transmission (Fig. 2D) and the steeper than unity slopes of plots of normalized CV against normalized mean EPSP amplitude (Fig. 3D) indicated that this depression was at least predominantly presynaptic in origin. When data were binned broadly and pooled across similar connections, the time course of decay of depression following the first EPSP for the population of mature layer 4 connections was similar to those reported previously for adult neocortex in both rat and cat (Thomson and West 2003) and more rapid than those reported previously for immature layer 4 connections (Petersen 2002). Once again, there was no significant difference in the time course between adult rat and cat layer 4 connections nor did the differences between the different types of connections studied here reach significance. Extrapolation of the curves fit to these pooled data identifies a much more slowly decaying component of depression because at infinity, these curves extrapolate only to 85–94% of mean first EPSP amplitude. As discussed previously (Thomson 2003), this slowly decaying form of depression, which appears to accumulate with successive EPSPs in trains, probably results from partial depletion of the very small immediately releasable pool of vesicles. This pool is estimated to be no larger than 5–9 vesicles at each terminal and to replenish at a maximum rate of 1/s.

**Complex Relationships between Interspike Interval and EPSP Amplitude**

The recovery from depression could not be adequately described by a simple exponential when single-sweep data were binned finely for individual connections. Peaks and troughs with periodicities of 10–15 ms and similar to those recently described for layer 6 connections (West et al. 2006) were superimposed on the decay of second to sixth EPSPs. The mechanism underlying these peaks and troughs remains to be determined. However, their appearance in plots of EPSP amplitude against interspike interval for all EPSPs in trains, even when the preceding interval varies, suggests that it involves a mechanism that is reset by each successive AP in a train. The complex relations between third EPSP amplitude and the first 2 interspike intervals in the train suggest that the probability of release is not simply determined by the immediately preceding interval nor by the timing of the third spike relative to the first spike of the train. Certain combinations of preceding intervals, that is, certain firing patterns, result either in larger or in smaller EPSPs in spike trains.

**Conclusions**

Comparisons of the basic characteristics of layer 4 pyramidal cells and the connections between them indicated no significant differences between mature rat and cat neocortex. Nor were these connections significantly different in their amplitudes, time course, or in the degree of synaptic depression from those of ascending connections from layer 4 spiny cells to layer 3 pyramidal cells or the intralaminar connections between layer 3 pyramidal cells, except in the connectivity ratios that appear to be relatively low for layer 4 pyramid–pyramid pairs. Connections between pyramidal cells in layer 4, therefore, although they constitute a significant component of the intralaminar excitation to spiny cells, probably constitute less than that supplied by spiny stellate cells, but appear to follow the same basic principle: that the number of synapses involved and the strength of a connection decline steeply with increasing distance. This will restrict the region activated by recurrent collaterals and the spread of activity to cells with different input characteristics. A complex relationship between interspike interval and mean EPSP amplitude that appears to involve an oscillating mechanism with a 10- to 15-ms periodicity that is reset by each successive spike was apparent in all connections studied. The underlying mechanism and its outcome in circuit behavior remain to be determined but could contribute to tuning the firing of pyramidal cells to certain local frequencies or patterns.

**Notes**

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