Restrictions on Inhibitory Circuits Contribute to Limited Recruitment of Fast Inhibition in Rat Neocortical Pyramidal Cells

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To further define the operational boundaries on fast inhibition in neocortex, whole cell recordings were made from layer V pyramidal neurons in neocortical slices to evaluate evoked inhibitory postsynaptic currents (IPSCs) and spontaneous miniature IPSCs (mIPSCs). Stimulating electrodes were placed in layers VI and VII to determine whether simultaneous stimulation of deep and superficial laminae could extend the magnitude of maximal IPSCs evoked by deep-layer stimulation alone. The addition of superficial-layer stimulation did not increase maximal IPSC amplitude, confirming the strict limit on fast inhibition. Spontaneous miniature IPSCs were recorded in the presence of tetrodotoxin. The frequency of spontaneous mIPSCs ranged from 10.0 to 33.1 Hz. mIPSC amplitude varied quantally, with a range of 5.0–128.2 pA and a mean value of 20.7 ± 4.1 pA (n = 12 cells). The decay phase of miniature IPSCs was best fit by a single exponential, similar to evoked IPSCs. The mean time constant of decay was 6.4 ± 0.6 ms, with a range of 0.2–20.1 ms. The mean 10–90% rise time was 1.9 ± 0.2 ms, ranging from 0.2 to 6.3 ms. Evaluation of mIPSC kinetics revealed no evidence of dendritic filtering. Amplitude histograms of mIPSCs exhibited skewed distributions with several discernable peaks that, when fit with Gaussian curves, appeared to be spaced equidistantly, suggesting that mIPSC amplitudes varied quantally. The mean separation of Gaussian peaks ranged from 6.1 to 7.8 pA. The quantal distributions did not appear to be artifacts of noise. Exposure to saline containing low Ca2+ and high Mg2+ concentrations reduced the number of histogram peaks, but did not affect the quantal size. Mean mIPSC amplitude and quantal size varied with cell holding potential in a near-linear manner. Statistical evaluation of amplitude histograms verified the multimodality of mIPSC amplitude distributions and corroborated the equidistant spacing of peaks. Comparison of mIPSC values with published data from single GABA channel recordings suggests that the mean mIPSC conductance corresponds to the activation of 10–20 GABA_A receptor channels, and that the release of a single inhibitory quantum opens 3–6 channels. Further comparison of mIPSCs with evoked inhibitory events suggests that a single interneuron may form, on average, 4–12 functional synapses with a pyramidal cell, and that 10–12 individual interneurons are engaged during recruitment of maximal IPSC magnitude. It is possible that inhibitory circuits are much more restricted in both the size of the unit events and effective number of connections when compared with excitatory inputs.

INTRODUCTION

In CNS neurons, fast inhibitory postsynaptic currents (IPSCs) arise from the activation of the GABA_A receptor/channel complex (for review see Mody et al. 1994; Nicoll et al. 1990). In the neocortex this form of inhibition is mediated by inhibitory interneurons, which are ubiquitous (Huntley et al. 1994; Kawaguchi and Kubota 1993). These cells perform the important task of gating and shaping extrinsic and intrinsic excitatory inputs. Our prior work on inhibition focused on characterizing population inhibitory events evoked by extracellular stimulation. We found that inhibitory neurons were maximally recruited at relatively low stimulus levels, but that there was a fixed limitation on the magnitude of evoked fast IPSCs (Ling and Benardo 1995a). What underlies the limit on fast inhibition?

Although much recent work has been directed at investigating cortical fast inhibition (Benardo 1994; Chagnac-Amitaï and Connors 1989; Deuchars et al. 1995; Deuchars and Thomson 1995; Kawaguchi 1995; Kubota and Kawaguchi 1994; Salin and Prince 1996a), there is still insufficient information available about cortical local circuity to understand the precise functional processes mediating the limit on fast inhibition. Several very elegant studies in cortical slices combining paired recordings from synaptically coupled neurons with electron microscopic analysis have provided some physiological and anatomic data on the connectivity of discrete sets of interneuron-pyramidal cell pairs (Buhl et al. 1994; Tamás et al. 1997; Thomson et al. 1996). However, because obtaining paired recordings from the entire ensemble of interneurons connected to given pyramidal cells would be an impossible undertaking, the details regarding interneuron-to-pyramidal cell transmission and the distribution of inhibitory synapses on principal cells for entire inhibitory networks still remain to be quantified. Basic information such as this is needed to better define the finite nature of fast cortical inhibition. A fundamental approach to investigating this issue is to undertake an analysis of miniature fast inhibitory events as a means to obtain a first approximation of the number of inhibitory synapses involved in the mediation of maximal evoked IPSCs.

Miniature synaptic events are thought to arise from the spontaneous release of a single packet, or quantum, of transmitter at individual synaptic terminals. Previously, quantal analyses of miniature events have utilized complex mathematical methods, such as deconvolution, to extract peaks in amplitude distributions (Bekkers 1994; Korn and Faber 1991; Redman 1990). This was necessitated by the large variance in inhibitory postsynaptic potential (IPSP)/IPSC amplitudes imposed by the high noise of traditional intracellular recording methods. As such, previous estimates of IPSC quantal size and...
variability were premised, at least in part, on assumptions regarding release statistics, deconvolution analysis, and ana-
tomically based models of transmitter release (Korn and Faber
1991; Redman 1990).

However, Edwards et al. (1990) showed that whole cell
patch techniques can be used to obtain high resolution, low-
noise recordings of spontaneous synaptic currents in situ. Thus
direct measurement of miniature IPSC (mIPSC) amplitudes
amenable with sufficient precision for detailed analyses of
amplitude distributions. This has allowed for improved detec-
tion of peaks within amplitude histograms of miniature synaptic
currents, bypassing the need for more complex methods of
signal extraction. However, peaks in amplitude distributions
may nonetheless arise from other artificial sources, such as
statistical variations due to finite sampling, and this has
prompted the development of rigorous statistical tests to eval-
uate the reliability of histogram peaks (Stratford et al. 1997).

In this study, we utilized whole cell recording techniques in
slice preparations of rat somatosensory cortex to further exam-
ine limits on evoked IPSCs and to evaluate mIPSCs in layer V
pyramidal neurons. We have described the kinetics of mini-
ature inhibitory events in neocortical pyramidal cells and the
quantal nature of their amplitude distributions. Similar to other
investigations of inhibitory events (Edwards et al. 1990; Ropert
et al. 1990), we report skewed distributions of mIPSC amplitu-
des exhibiting multiple peaks that are unlike the unimodal,
skewed distributions often observed with miniature excitatory
events (Mody et al. 1994; Redman 1990). The reliability of the
peaks observed in these histograms was tested using both χ²
and autocorrelation scoring methods (Stratford et al. 1997).
Comparisons of these data to measurements made on evoked
IPSCs were used to derive practical estimates of the functional
properties and limits of cortical fast inhibitory circuits.

METHODS

Preparation and maintenance of slices

Cortical slices were prepared from Sprague-Dawley rats aged
postnatal day 18 to 30 (P18–P30), using methods previously described
(Benardo 1993; Edwards et al. 1989). Rats were deeply anesthetized
by either inhalation of halothane or by intramuscular injection of
ketamine (87 mg/kg) and xylazine (13 mg/kg) and killed by decapi-
tation. Their brains were quickly removed and placed in chilled
(0–5°C) physiological saline. Coronal slices (300–400 μm), which
included somatosensory cortex and dorsal hippocampus, were
prepared using one of two methods, depending on the recording tech-
nique used (i.e., “blind” or visualized whole cell patch). For blind
patch recordings, slices were cut using a McIlwain tissue chopper
and then placed in a warm (35.5 ± 1°C) recording chamber where they
rested on a nylon net at the interface between physiological saline and
a stream of humidified gas (95% O₂–5% CO₂).

For slices used in visualized patch recording, the somatosensory
cortical region was blocked off, dissected, and attached to a dissection
block with cyanacrylate glue. The tissue block was then transferred
to the ice-chilled bath of a Vibratome tissue slicer (Oxford) that
contained oxygenated saline. This saline contained low calcium (0.5
mM) and elevated magnesium (8 mM) to reversibly block synaptic
activity. Coronal slices were cut and then transferred to an antecham-
ber where they rested submerged in warm (35.5 ± 1°C), oxygenated
(95% O₂–5% CO₂) physiological saline. Slices were allowed to incu-
bate for at least 1 h before recording. Following incubation, a single
slice was transferred to a recording chamber placed on the stage of an
upright microscope (Zeiss Axioskop, Carl Zeiss, Thornton, NY), and
perfused with warm (34–35°C) oxygenated saline at ~5 ml/min.
Cells were visualized and accessed using infrared differential interfer-
ence contrast (IR-DIC) optics with a ×40 water immersion objec-

tive.

Normal, external physiological saline contained (in mM) 124 NaCl,
5 KCl, 26 NaHCO₃, 1.6 MgCl₂, 2 CaCl₂, and 10 glucose and was
continuously bubbled with a mixture of 95% O₂–5% CO₂ (pH be-
tween 7.35 and 7.4). All drugs were delivered through the perfusate.

Whole cell recordings and analysis

Whole cell recordings were obtained from layer V pyramidal cells in
slices using both the blind (Blanton et al. 1989; Ling and Benardo
1995a) and visualized (Edwards et al. 1989) patch techniques. Patch
electrodes were pulled to tip resistances of 2–5 MΩ and filled with a
solution composed of (in mM) 130 Cs-glucronate, 2 MgCl₂, 2 CaCl₂,
10 EGTA, 10 HEPES, 2 Na-ATP, and 10 QX-314, pH 7.25, adjusted
with CsOH. QX-314 was included to block voltage-dependent sodium
currents and thus reduce cell spiking. Currents were recorded under
voltage clamp with a Warner PC-501A patch-clamp amplifier
(Warner Instrument, Hamden, CT). Criteria for cell acceptability
were the same as previously detailed (Ling and Benardo 1995a), and all
cells meeting these criteria were included in the results presented,
without further bias. Briefly, cells accepted for study had resting input
resistances of ≥100 MΩ (typically ≥150 MΩ) and access resistances
≤20 MΩ (typically <15 MΩ). Cells were discarded if access resist-
ance increased significantly (>20%) during the experiment. Cell
membrane characteristics (input resistance, resting membrane poten-
tial) were the same with both whole cell recording methods. Signals
were digitized at 47 kHz via a 14-bit PCM interface (VR-10B
Digital Data Recorder, Instrutech, Elmont, NY) and stored on VHS videotape
for post hoc analysis. Recorded data were filtered off-line at 1–5 kHz
(–3 dB, 4-pole Bessel) and digitally sampled at 10–20 kHz with
pCLAMP 6.0 software (Axon Instruments, Foster City, CA) running
on a PC/AT-compatible Pentium-166 MHz microcomputer.

Synaptic events were evoked by extracellular stimulation with
coated, monopolar, tungsten electrodes placed in layer V lateral to
the recording electrode (proximal location). A second electrode
was placed in layer I/II (distal location) for simultaneous stimulation of
superficial laminae within the same vertical column as the first elec-
trode. Cathodal shocks (2–10 V; 200 μs duration) were delivered
through a digitally controlled stimulus isolation unit (World Precision
Instruments) at a low frequency (0.1 Hz) that does not induce depres-
sion of fast IPSC amplitude (Deisz and Prince 1989).

Spontaneous mIPSCs were detected and measured using Mini
Analysis 4.0 software (Synaptosoft, Leonia, NJ), which identifies
spontaneous currents on the basis of several criteria, including thresh-
old amplitude and the area under each event. As a routine check, we
visually inspected all mIPSCs detected by the software and rejected
any events that did not exhibit the general shape expected for synaptic
events. Background noise was measured from quiescent sections of
records (i.e., devoid of spontaneous events) or, in cases in which
picrotoxin was applied, from records acquired during picrotoxin
blockade of mIPSCs. The peak-to-peak noise level in our recordings
ranged from 2 to 5 pA.

Kinetic analysis of synaptic currents was performed according to
methods previously described (Ling and Benardo 1994). Exponential
curves were fit to the decaying phase of current traces by an iterative
least-squares method of regression based on the Simplex algorithm
(Nelder and Mead 1965), yielding values for time constants.

Histograms of the distribution of peak IPSC amplitudes were con-
structed from mIPSC measurements (amplitude vs. number of obser-
vations). Amplitude distributions were first assessed by eye to deter-
mine whether histogram peaks were readily apparent. This was
possible due to the high resolution (high signal-to-noise ratio) of
current recordings made with whole cell patch techniques. In all cases,
peaks in amplitude histograms were detectable by eye. Peak values
thus determined were then used as initial estimates for fitting of distributions with Gaussian curves. IPSC histograms were analyzed using the PSTAT analysis program (pCLAMP, Axon Instruments), which determines the best fit for a sum of Gaussian distributions using a least-squares method based on the Simplex algorithm (Nelder and Mead 1965).

All data throughout this report are expressed as means ± SE, unless indicated otherwise.

**Statistical evaluation of mIPSC amplitude distributions**

The reliability of the peaks observed in the amplitude histograms was evaluated using both the standard $\chi^2$ test and the recently developed autocorrelation (AC) scoring method of Stratford et al. (1997). Specifically, these tests were used to check whether multimodal distributions could have arisen from parent unimodal distributions. Multimodal and unimodal distributions were fit to amplitude histograms by an iterative least-squares method of regression based on the Simplex algorithm (Nelder and Mead 1965; Press et al. 1992). Histogram fits using sums of multiple Gaussians (i.e., multimodal) were compared with fits obtained with Weibull functions (i.e., unimodal).

Skewed unimodal fits were obtained with the Weibull function (Stratford et al. 1997; Zanakis 1979)

$$g(x) = (c/b) [(x/a)/b]^{-c} \exp \left\{ -[(x/a)/b] \right\}$$

where $a$ is the location parameter, $b$ the scale parameter, and $c$ the shape parameter, subject to the following constraints: $x \geq a$, $b > 0$, $c > 0$. In no case did single Gaussians provide adequate fits to amplitude distributions of mIPSCs recorded in normal saline.

$\chi^2$ TEST. Fits of amplitude distributions were compared using $\chi^2$ goodness-of-fit tests (Guttman et al. 1971; Press et al. 1992). The best multimodal fits of the amplitude data using sums of Gaussians were compared with the best unimodal fits obtained with the Weibull function. Bins with values <5 were pooled to avoid errors with small expected values (Guttman et al. 1971).

**AUTOCORRELATION SCORING.** Histogram fits were also evaluated using the AC scoring method as described by Stratford et al. (1997). This test involves a multistep procedure whereby the raw amplitude histogram is first smoothed using filtering algorithms derived from Fourier techniques traditionally used to filter time-varying signals. The filter strength was set to eliminate all peaks and valleys in the amplitude histogram. The smoothed plot was then subtracted from the raw histogram to obtain a plot of the difference (or residuals) between the smoothed and original data plots. The difference plot was then lightly smoothed and its autocorrelation calculated (Press et al. 1992) and plotted. The AC score of the amplitude distribution was taken from the autocorrelation plot, which has a characteristic damped-sinusoidal profile, as the difference in magnitude between the first peak (i.e., zero-lag) and the preceding trough.

Once the AC score was calculated for a given mIPSC amplitude data set, Monte Carlo trials were performed to generate simulated amplitude histogram data based on the best-fit unimodal (i.e., Weibull) distribution. To this end, a random number generator (RAN2) (Press et al. 1992) was used in conjunction with the fitted Weibull function to generate mIPSC amplitude values. Uniform random numbers (deviates, U) generated with the RAN2 algorithm were transformed to the Weibull distribution using the inverse of the Weibull function’s continuous cumulative distribution (Box et al. 1978; Stratford et al. 1997), which is given by

$$G^{-1}(U) = a + b \left\{ -\ln \left( 1 - U \right) \right\}^{\frac{1}{c}}$$

where $a$, $b$, and $c$ are the values of the Weibull function parameters determined from Simplex fits to the amplitude histogram above. The same number of samples were generated as there were in the real mIPSC data set. The AC scores of the resulting simulated amplitude histograms were then calculated using the same strength filters and bin sizes that were used for the real histogram. For each set of real data, a minimum of 100 Monte Carlo trials were performed and compared against the real amplitude histogram. The fraction of simulated histograms that yielded higher AC scores than the real mIPSC amplitude histogram provided an effective measure of the probability that histogram peaks arose from a parent unimodal distribution. For example, if 4 in 100 simulated trials yielded higher AC scores than the real mIPSC data set, this would correspond to a probability of 0.04 (and thus $P < 0.05$) and would suggest that the real data were significantly different from the unimodal model. In this way, estimates of $P$-values for the reliability of real mIPSC amplitude distributions were obtained.

Evoked IPSCs

One possible explanation for the previously reported limit on the recruitment of fast inhibition (Ling and Benardo, 1995a) is a restricted capacity of deep-layer stimulation to engage the entire inhibitory network. Simultaneous stimulation of regionally separate sites might activate additional inhibitory cells and, in turn, evoke maximal IPSCs of greater amplitude than those recruited with deep-layer stimulation alone. To test this possibility directly, electrical stimulation was applied in both superficial (layer I/II) and deep (layer VI) cortical laminae (Fig. 1A, left) to evoke fast IPSCs in layer V pyramidal cells. IPSCs were selectively recorded by holding target cells at the empirically determined reversal potential ($<0$ mV) for excitatory postsynaptic currents (EPSCs; Fig. 1A, right). The results showed that the addition of superficial-layer stimulation did not alter the peak amplitude of maximal IPSCs evoked with deep-layer stimuli. The reason for this can be deduced from a comparison of the respective input-output relationships for layer VI and layer I/II stimulation (Fig. 1B). When stimuli were applied in layer VI, the threshold intensity values were ±3.0 V, and maximal IPSCs were evoked at intensities 2.0–2.5 V above threshold, similar to our previously reported findings (Ling and Benardo, 1995a). When stimuli were delivered solely in superficial cortical layers, the threshold was significantly higher (~2-fold), and the slope of the input-output curve was less steep. The plot of the input-output functions revealed that layer VI stimulation evoked maximal IPSCs at intensities that were approximately equal to the threshold values for layer I/II stimulation. Thus, electrical stimulation delivered in deep cortical layers proximal to the recording site is apparently sufficient to recruit all available inhibitory neurons in the inhibitory network. The results also show that superficial stimuli likewise engage this inhibitory system, albeit at higher intensities.
Spontaneous mIPSCs

We examined spontaneous synaptic activity from whole cell recordings obtained from 12 cells (10 slices) that satisfied our criteria for inclusion in our analyses. Tetrodotoxin (TTX, 1–10 μM) was used to block spontaneous IPSCs arising from action potential–dependent release of GABA from presynaptic inhibitory cells, leaving mIPSCs resulting from the spontaneous release of GABA (Fig. 2A). mIPSCs were selectively recorded at the EPSC reversal potential (\( \eta = 0 \) mV). Application of 50 μM picrotoxin (PTX) completely blocked spontaneous mIPSCs, confirming that they were mediated by GABAA receptors (Fig. 2A).

The frequency of mIPSCs within single neurons ranged from 10.0 to 33.1 Hz, with a mean value of 19.5 ± 0.6 Hz (mean ± SE, \( n = 12 \)). The amplitude of mIPSCs varied considerably, with a range of 5.0–128.2 pA and a mean value of 20.7 ± 4.1 pA (determined by pooling 18,456 events from 12 cells). Given an IPSC reversal potential of \(-74.1 \) mV in these cells (Ling and Benardo 1995a), the mean mIPSC conductance, as given by Ohm’s Law, is \( \sim 0.3 \) nS (range \( \sim 0.1–1.8 \) nS).

The average mIPSC amplitude overlaps with the lower range of values we found for fast IPSCs evoked by minimal strength extracellular stimuli, which varied from 28 to 300 pA (mean, 168 ± 84 pA) (Ling and Benardo 1995b). The source of this wide range of magnitudes for minimally evoked IPSCs is unclear. Interestingly, when we restricted our examination to minimally evoked events with peak amplitudes <60 pA, the mean threshold value for evoked IPSCs was 28.7 ± 1.6 pA (\( n = 25 \) cells), close to the amplitude of mIPSCs.

Kinetic properties of mIPSCs

The decay phase of miniature IPSCs was best fit by a single exponential (Fig. 2B), similar to evoked IPSCs (Fig. 2C, same cell) as previously reported (Ling and Benardo 1995b). As shown in Fig. 2B, the decay kinetics did not appear to vary with mIPSC amplitude. The mean time constant of decay (\( \tau_d \)) was 6.4 ± 0.6 ms (\( n = 12 \) cells), with a range of 0.2–20.1 ms. The 10–90% rise time of mIPSCs ranged from 0.2 to 6.3 ms, with a mean value of 1.9 ± 0.2 ms (\( n = 12 \)).

Amplitude distributions of spontaneous miniature IPSCs

The distribution of mIPSC amplitudes displayed an overall skewing toward the lowest values (Fig. 3). However, multiple peaks could be visually detected in mIPSC histograms for all cells, and multipeak Gaussian curves could be fit to the amplitude distribution histograms (Fig. 3, A and B). Table 1 summarizes the results of fitting Gaussian distributions to the amplitude histograms generated for each cell. The mean separation of Gaussian peaks ranged from 6.2 to 7.8 pA. The standard deviations of the fitted Gaussians remained fairly constant within individual data sets, ranging from 1.0 to 3.4 pA and were consistently larger than the standard deviations of the
and frequency (41.5 ± 6.7%). The reduction in mIPSC magnitude was reflected in the amplitude histograms, which exhibited a decrease in the proportion of larger amplitude events and a reduction in the number of histogram peaks. In three cells, only one clear histogram peak remained after Ca\(^{2+}\) and Mg\(^{2+}\) concentrations were altered, whereas in the fourth cell two peaks remained. Figure 4A shows the amplitude distribution measured in one cell during bath perfusion of TTX-containing saline with the normal concentration of 2 mM Ca\(^{2+}\) and 1.6 mM Mg\(^{2+}\). The histogram was best fit by the sum of three Gaussians with a mean peak separation of 7.2 pA. After exposure to low Ca\(^{2+}\)/high Mg\(^{2+}\) saline, only one peak remained, which was best fit by a single Gaussian with a mean of 7.4 pA. In the second example (Fig. 4B), the amplitude histogram of mIPSCs recorded in TTX exhibited three peaks with a mean peak separation of 6.9. Perfusion with low Ca\(^{2+}\)/high Mg\(^{2+}\) saline eliminated the third peak, leaving two peaks with a mean separation of 7.1. Overall, these results are consistent with previous reports that alterations in calcium-magnesium concentration ratios affect the probability of divalent cation-dependent transmitter release, but not quantal size (Bekkers and Stevens 1994; Del Castillo and Katz 1954).

**Effect of altering cell holding potential on mIPSC amplitude**

In two cells, cell holding potential was increased from 0 to +40 mV, which translates to an ~50% increase in the driving force of the chloride-mediated fast IPSC \((E_{Cl} = -74.1\) mV\). The change in holding potential led to a 51.2% increase in mean mIPSC amplitude, from 16.6 ± 0.4 pA to 25.1 ± 0.6 pA (Fig. 5A). The amplitude distributions measured at both holding potentials were similarly best fit by the sums of several Gaussians (Fig. 5, B and C), but the peak separation was greater at the higher potential. At 0 mV, the mean separation between peaks was 6.3 pA (cell 9, Table 1), whereas at +40 mV, the peak separation was 10.5 pA, representing a 66.7% increase in quantal size. Thus mIPSC amplitudes increased in a near-linear fashion with respect to membrane potential. The observed increase in mIPSC amplitude and quantal size could not be attributed to increased background noise, which was -0.1 ± 1.4 pA at 0 mV and -0.1 ± 1.7 pA at +40 mV.

**Contribution of histogram bin size**

Because peaks in amplitude histograms can erroneously arise from sources such as data binning, variations in data subsets, and finite sampling from large populations, it was essential to subject the amplitude data to various tests to evaluate the reliability of the multipeak fits. One potential source of error with the least-squares method of fitting Gaussian distributions is the dependency of this algorithm on bin size (Edwards et al. 1990). Figure 6 shows the effect of varying bin size on data distributions. Bin width size had no significant effect on the fit of Gaussian curves to amplitude histograms, causing very little change in the overall amplitude distributions and positions of peaks.

**Consistency of distributions within a data set**

Another possible source of error might lie in variations in the sample populations taken. One check against this is to compare subsets of data taken within the same data set (Edwards et al.
If the quantal nature of the distributions is an artifact of either small sample size or the sampling procedure, then different subsets of the data would be expected to exhibit different peaks. Figure 7 shows the effect of displaying sequential subsets of data taken from the same data set. The peaks fall in the same locations, thereby ruling out the possibility of a sampling artifact or systematic change in mIPSC amplitude with time.

Statistical analysis of histogram distributions

The results of these initial tests supported the reliability of the multipeak fits. The data were then subjected to more rigorous, statistical tests to check whether histogram peaks could have arisen from errors in sampling from unimodal distributions. Both the $\chi^2$ test and autocorrelation scoring method were employed to assess this possibility.

We first evaluated amplitude distributions using the $\chi^2$ method. Here, the best fits of mIPSC amplitude histograms obtained with the unimodal (Weibull) function were compared against the fits obtained with a sum of Gaussians. The $\chi^2$ test was used to assess whether the amplitude distributions were significantly different from either multimodal or unimodal fits, i.e., if multimodal fits yielded significantly better fits than unimodal fits (Fig. 8). In all cases, multipeak fits obtained with the sum of Gaussians produced better fits than unimodal Weibull functions ($P < 0.05$), with Weibull fits failing in most cases to meet $\chi^2$ goodness-of-fit criteria. In no case did single Gaussians provide adequate fits to amplitude distributions.

However, there is evidence that suggests that the $\chi^2$ test (in
The results of fitting Gaussian distributions to the miniature inhibitory postsynaptic current (IPSC) amplitudes are listed. *n* is the number of mIPSC amplitudes used for the fit. Peak Number refers to consecutive peaks in the IPSC amplitude histogram. Peak Amplitude is the mean ± SD (in pA) value of Gaussians fitted to peaks of amplitude histograms. Peak separation *q* is the mean amplitude separation (interval) between peaks [*/q* calculated as mean of the interval between successive peaks; † *q* calculated according to the method of Busch (Edwards et al. 1990) from *q* = (1/k) * Σ[mlp], where *k* is the total number of peaks, *n* is the peak amplitude, and *p* is the peak number]. Noise is the mean ± SD value of the baseline noise. For consistency, a bin size of 1 pA was used to obtain values for all the cells listed in this table.

The next step was to determine whether unimodal distributions fit to the amplitude histograms could generate data sets that appear to be multimodal, i.e., yield AC scores equal to or higher than the original data. If so, this would suggest that the peaks in the amplitude histogram may have erroneously arisen from sampling artifacts. For each real data set, a unimodal curve was fit to the amplitude histogram, which in all cases was best-fit by a Weibull function. The Weibull distribution was then used in Monte Carlo simulations to generate at least 100 sets of amplitude data, each consisting of the same number of data depicted in Fig. 6 was 2,314. Notably, the first peak in the AC plot occurred at 7 pA, with subsequent peaks occurring approximately every 6–7 pA. This is in close agreement with the separation values between peaks of the multiple Gaussians fitted previously.

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samples as the original data set. Each simulated data set was
smoothed with the same strength filter employed for the real
amplitude distribution, and its AC score determined. The AC
scores were then compared against the real data, and the
number of scores exceeding the AC value of the real amplitude
histogram noted. If <5 in 100 simulated data sets exhibited AC
scores higher than the original data, this would suggest that the
probability that histogram peaks resulted from sampling errors
is <5% (i.e., \( P < 0.05 \)). In no case did more than 2% of the
simulation trials exceed the AC score for the real amplitude
data, suggesting that the amplitude histogram peaks were not
the result of sampling artifact.

Dendritic filtering of mIPSCs

Synaptic signals generated at distant dendritic locations, but
recorded somatically, would be expected to be distorted (Bur-
gard and Hablitz 1993; Rall 1969). However, past experiments

![Figure 4](http://jn.physiology.org/)

**FIG. 4.** Effect of altering extracellular calcium and magnesium concentrations on mIPSC amplitude. *A, top:* amplitude distribution of mIPSCs in TTX-containing (1 \( \mu M \)) physiological saline containing 2 mM Ca\(^{2+}\) and 1.6
mM Mg\(^{2+}\) \((n = 850)\). The mean peak separation is 7.3 pA. Dashed lines
represent mean \( \pm \) SD of the individual Gaussians (given in Table 1: cell 7).
*Bottom:* amplitude distribution measured in the same cell during perfusion with
saline containing 0.5 mM Ca\(^{2+}\) and 8.0 mM Mg\(^{2+}\) \((n = 475)\). Only one clear
peak remained, which was fit by a single Gaussian with a mean \( \pm \) SD of 7.4 \( \pm 
2.9 \) pA. *B, top:* amplitude distribution measured in another cell in normal
TTX-containing saline (Gaussian values given in Table 1: cell 6). The mean
peak separation is 7.3 pA. *Bottom:* amplitude distribution of mIPSCs recorded
in the same cell during perfusion with low Ca\(^{2+}\)/high Mg\(^{2+}\) saline \((n = 848)\).
The histogram was best fit by the sum of 2 Gaussians having means \( \pm \) SD of
7.9 \( \pm \) 2.8 and 14.8 \( \pm \) 2.7. The mean peak separation is 7.6 pA.

![Figure 5](http://jn.physiology.org/)

**FIG. 5.** Voltage dependence of mIPSC amplitude and quantal size. The cell
membrane holding potential was initially set at 0 mV and then changed to +40
mV. *A:* mean mIPSC amplitude was 16.5 \( \pm \) 0.4 pA at 0 mV \((n = 1,145)\) and
25.1 \( \pm \) 0.6 pA at +40 mV \((n = 1,203)\). *B:* amplitude distribution measured at
0 mV (same data set as shown in Fig. 3B; Table 1: cell 9). The peak separation
is 6.3 pA. The baseline noise was \(-0.1 \pm 1.4 \) pA. *C: amplitude distribution
measured in same cell at +40 mV \((n = 1,203)\). The mean peak separation is
10.5 pA. The baseline noise was \(-0.1 \pm 1.7 \) pA.
have indicated that neocortical pyramidal cells are electrotically compact (Larkman et al. 1992; Stafstrom et al. 1984), and previous studies have found little or no correlation between decay time constants and rise times, suggesting that miniature synaptic events are generated within a restricted region of the dendritic tree (Burgard and Hablitz 1993). Electrotonic filtering is usually signaled by a negative correlation between rise time and amplitude. Events that are generated more distally from the recording site would be both attenuated and delayed due to the cable properties of dendrites (Rall 1969).

We examined mIPSCs for evidence of dendritic filtering and found no negative linear correlation between mIPSC rise time and amplitude (Fig. 10A), suggesting that most mIPSCs were generated within a confined location of the dendritic tree. However, other investigators have raised valid concerns regarding the limitations of this type of analysis (Soltesz et al. 1995), arguing that the lack of a negative correlation may not definitively exclude electrotonic filtering. Therefore to further assess cable filtering effects, the population of mIPSCs was arbitrarily separated into two nonoverlapping subclasses of events with fast (≤1.0 ms) and slow (≥1.5 ms) rise times to

FIG. 6. Effect of different bin sizes on mIPSC amplitude distributions. Each successive panel shows the effects of increasing the binwidth (0.5, 1.0, 2.0 pA) on the mIPSC amplitude histogram (same data set as shown in Fig. 3A; Table 1: cell 1). There was no significant change in the separation between peaks of the Gaussian fits. Peak separations are as follows: 1.0 pA bin width, 7.2 pA; 0.5 pA bin width, 7.3 pA; 2.0 pA bin width, 7.5 pA.

FIG. 7. Effect of sampling subsets of data on the mIPSC amplitude distribution profile. A: histogram of amplitude distributions of mIPSCs (same data set as shown in Fig. 3A; Table 1: cell 1). Mean separation between peaks is 7.2 pA. B: subset of first 506 samples of data set. Peak separation is 7.3 pA. C: subset comprised of last 695 samples of data set. Peak separation is 7.1 pA.
Amplitude distributions of mIPSCs

It appears from our analyses that mIPSCs exhibit multimodal amplitude distributions. Similar work in other central neurons showed that mIPSCs display a skewed, unimodal amplitude distribution, with the peak corresponding to the first peak and decaying phases of mIPSCs. The extent to which the anatomy of the neuronal circuit or space-clamp limitations contribute to this finding is presently not known. Prior examinations of miniature excitatory events in hippocampal CA3 pyramidal cells showed that mEPSC rise time was correlated to \( \tau_R \) but not to amplitude, suggesting that, although mEPSC shape was partly determined by electrotonic filtering, variations in mEPSC amplitude were not (McBain and Dingledine 1993). Similarly, the locus of inhibitory synaptic terminals on layer V pyramidal cells may be too confined for passive cable properties to affect mIPSC amplitude, but may likewise contribute to shaping mIPSCs.

As a final check for evidence of electrotonic filtering, amplitude histograms were constructed using mIPSCs with only fast rise times (\( \leq 1.5 \text{ ms} \)) and then compared with the amplitude distribution of the parent data set (Fig. 11). If dendritic filtering influences mIPSC amplitude in a systematic way, then it could lead to a quantal-like distribution. Analyzing only those events with fast rise times, and hence minimal filtering, would obviate this potential problem. We found that histogram peaks were consistent across both data sets, further suggesting that dendritic filtering does not play a prominent role in determining the amplitude of mIPSCs recorded in these cells.

**Discussion**

IPSCs evoked with simultaneous stimulation in deep and superficial layers

Previous studies in cortex (Nicoll et al. 1996; Salin and Prince 1996b) have shown that stimulation of superficial layers can evoke inhibitory responses in layer V pyramidal neurons. This raised the question whether synchronous stimulation of both proximal and distal inhibitory neurons could extend the magnitude of maximal IPSCs evoked by deep-layer stimulation alone. Our findings confirmed that superficial-layer stimulation can evoke inhibitory responses in layer V target cells, albeit at higher threshold intensities. A comparison of input-output plots revealed that deep-layer stimulation evoked maximal amplitude IPSCs at intensities where superficially evoked responses were still relatively small. Stimuli delivered simultaneously to both layers I/II and VI did not evoke maximal IPSCs of greater amplitude than those obtained with layer VI stimulation alone, confirming the strict limit on recruitment of fast inhibition. Thus deep-layer stimulation ultimately engages all inhibitory elements presynaptic to layer V pyramidal cells. This interpretation is supported by previous work examining the relationship between stimulus placement and peak IPSC amplitude (Salin and Prince 1996b) that showed that stimuli applied <350 \( \mu \text{M} \) from the pyramidal cell soma produced the strongest inhibitory responses and by studies in our laboratory (Yang and Benardo 1997) that demonstrated that maximal inhibitory postsynaptic potentials recruited with deep-layer stimuli are reduced in magnitude (by \( \sim 25\% \)) when superficial circuit activity is pharmacologically eliminated.

Amplitude distributions of mIPSCs

Determine whether either class was more restricted in amplitude. The expectation is that if filtering is present, slower events should be generally smaller in amplitude than fast events (Soltesz et al. 1995). To the contrary, our findings showed that fast and slow mIPSCs encompass broad, overlapping ranges of amplitudes (Fig. 10B). Although mIPSCs may be generated within a confined region on an individual cell, we have no information on the relative distribution of individual synapses within this restricted locus. In fact, small positive correlations were observed between rise time and \( \tau_R \) (Fig. 10C, \( r = 0.39 \)) and between rise time and half-width duration (Fig. 7D, \( r = 0.52 \)), suggesting that mIPSCs may be generated from diffusely distributed sites within a confined region on individual cells (Fig. 10C). Some small degree of local dendritic filtering could account for the correlated changes in the rising
of amplitude distributions of evoked, unitary IPSCs (Bekkers 1994; Korn and Faber 1991). The mIPSCs recorded in our study also displayed gross skewing of the overall distribution toward the lowest amplitude values. However, closer inspection revealed that mIPSC amplitude histograms were multimodal and well fit by sums of Gaussian curves. mIPSC amplitudes appeared to be quantally distributed, similar to the findings of previous studies of mIPSCs (Edwards et al. 1990; Korn et al. 1993; Ropert et al. 1990). The amplitude distributions did not appear to an artifact of instrument (e.g., noise) or statistical variation. In low Ca\(^{2+}\)/high Mg\(^{2+}\), the majority of amplitude histograms fell to a single peak corresponding to the first Gaussian, which is consistent with decreased probability of quantal GABA release. Increasing the cell holding potential revealed a near-linear dependence of mIPSC mean amplitude and histogram peak separation (quantal size) on chloride-current driving force, suggesting that the amplitude distributions were not the result of instrument artifact. It also confirmed that the mIPSCs recorded were GABA\(_A\)-mediated events and that the majority were apparently under adequate voltage-clamp control. Statistical evaluation of mIPSC amplitude histograms supported the reliability of the multipeak Gaussian fits, suggesting that the observed peaks in the amplitude distributions are not artifacts of sampling or statistical errors. Moreover, the estimates of the spacing between peaks given by the autocorrelation method were in close agreement to the peak separation values between fitted Gaussians.

Between cells, the peak separations of mIPSCs were remarkably similar, suggesting a consistency within this inhibitory network for the determinants of quantal size such as GABA\(_A\)-receptor channel conductance, synaptic connectivity, number of release sites, postsynaptic receptor distributions, and the number and size of quanta (vesicle content) released (Mody et al. 1994). Whether this is related to homogeneity of the population of cells examined is unknown, but sample variation would be expected to decline even further with increased sample size.

The variance of the fitted Gaussians was larger than the variance of the background noise recorded, which is consistent with quantal variability. The variance in fitted Gaussian curves did not consistently increase over successive peaks as would be expected for superposition of independent quantal events, although in one neuron, the variance was generally larger for higher order peaks. Several mechanisms are believed to underlie variations in quantal size (for review see Frerking and Wilson 1996), which can be generally categorized between presynaptic and postsynaptic origins.

In the context of the “all-or-nothing” scheme (Redman 1990), variations in quantal size have been attributed to variations in the transmitter content in vesicles (Bekkers and Stevens 1995; Frerking et al. 1995), suggesting that the amplitude of synaptic events is determined by the amount of transmitter released. Alternatively, it has been proposed that quantal variation is determined by the number of postsynaptic receptors at each synapse, which assumes saturation of all postsynaptic receptors by the contents of a single vesicle. There is also increasing evidence that the variations in the amplitude of miniature synaptic events may derive from differences intrinsic to each synaptic contact (Bekkers et al. 1990; Liu and Tsien 1995).

Assuming that a single synaptic contact comprises several independent release sites, equidistant peaks in amplitude distributions could result from asynchronous vesicle release at multiple independent sites or activation of receptor aggregates by individual quanta (Behrends and Ten Bruggencate 1998; Edwards et al. 1990). Our observation that low Ca\(^{2+}\)/high Mg\(^{2+}\) saline eliminated multiquantal events is consistent with coincidental summation of events arising from synchronized multivesicular release of GABA, either within a single release site or between neighboring sites (Lewis and Faber 1996; Poisbeau et al. 1996). The former assumes that not all receptors at a given site are saturated by a single quantum of transmitter, which is in line with previous studies showing that the likelihood of receptor saturation may vary between synapses for a single cell (Frerking et al. 1995; Frerking and Wilson 1996; Nusser et al. 1997). Findings in cerebellar granule cells (Nusser

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**FIG. 9.** Evaluation of multimodal and unimodal fits to amplitude histograms using autocorrelation scoring method. A: mIPSC amplitude histogram (n = 2,198) was Fourier filtered to obtain smooth unimodal distribution (continuous line). B: difference function obtained by subtracting the smoothed unimodal distribution from the real amplitude histogram. Bars show the raw subtraction, and the continuous line shows the effect of lightly smoothing the difference data. C: the autocorrelation (AC) function of the smoothed difference function depicted in B. The first peak (i.e., nonzero lag) is at 7 pA. The AC score of 2,314 was given by the peak-to-peak difference in magnitude between the 1st peak and preceding trough.
et al. 1997) suggest that receptor occupancy at these synapses is heterogeneous and depends on the number of postsynaptic receptors present at each synapse.

However, in the present study, the variance of fitted Gaussian curves did not consistently increase over successive peaks, as would be expected for superposition of independent events that should lead to summation of the individual variances. This, however, may not necessarily preclude multivesicular release. A constancy of variance has been reported for evoked IPSCs in dentate granule cells (Edwards et al. 1990) and in goldfish Mauthner cells (Korn et al. 1987), and several explanations for this have been proposed. Edwards et al. (1990) showed that relatively small sample sizes could give rise to apparent invariant standard deviations. However, in this study, the lack of increase in variance was also observed with data sets comprised of considerably larger samples ($n > 1,000$). The constancy of variance may also indicate that larger amplitude, multiquantal mIPSCs result from the synchronous activation of multiple GABA$_A$-receptor aggregates at individual synaptic terminals by a single packet of transmitter (Edwards et al.1804 D.S.F. LING AND L. S. BENARDO

FIG. 10. Evaluation of influence of dendritic filtering on mIPSC amplitude. A: plots of mIPSC rise time vs. amplitude revealed no negative linear correlation between these 2 parameters, suggesting that most mIPSCs were generated within a confined dendritic locus. B: cable filtering was further assessed by sorting mIPSCs into 2 subclasses according to rise times: fast ($<1.0$ ms) and slow ($>1.5$ ms). Fast and slow mIPSCs encompass broad, overlapping ranges of amplitudes, as further illustrated by the cumulative amplitude distributions for both subpopulations (inset). C and D: plots of mIPSC rise time vs. decay time constant and rise time vs. IPSC duration (half-width) exhibited small positive correlations ($r = 0.39$ and $r = 0.52$, respectively), suggesting some degree of local dendritic filtering.
This might explain the amplitude distribution data in which two histogram peaks remained in low Ca\(^2+\)/high Mg\(^2+\) saline (Fig. 4B). In either case, the consistency of the quantal size suggests that the response of individual GABA\(_A\) receptors in these circuits are very similar.

**Characteristics of mIPSCs**

The general characteristics of mIPSCs presented in this study are in agreement with those reported for miniature inhibitory events recorded with similar techniques in both neocortex and hippocampus (De Koninck and Mody 1994; Edwards et al. 1990; Otis and Mody 1992; Salin and Prince 1996a). The mean mIPSC amplitude and conductance were in accord with values found in other cortical regions, and the average frequency of mIPSCs overlapped with that reported by Salin and Prince (1996a) for neocortex. As in those previous studies, mIPSCs were found to decay exponentially with a single time constant, and the values for rise times and decay time constants were similar to those reported for pyramidal cells in neocortex and dentate gyrus. The kinetics were faster than those found by Ropert et al. (1990) in hippocampal CA1 region, but in that study cooler temperatures were used, which could account for the slower kinetics reported.

**Comparison of mIPSCs with GABA-mediated currents**

A primary objective of this study was to increase our understanding of the properties of local cortical inhibitory circuits that underlie limited fast inhibition. The aim was to obtain estimates of the number of interneurons involved in generating maximal, ensemble inhibitory events. Because of the prohibitive difficulty in obtaining paired recordings from all interneurons connected to a single pyramidal cell, we elected to examine spontaneous miniature events as a means to estimate the number of interneurons in the circuit through comparisons with published data on evoked IPSCs and GABA-mediated currents.

We found that the mean mIPSC conductance was \(~0.3\) nS, which is close to values previously reported for cortex (0.42–0.48 nS) (Salin and Prince 1996a) and hippocampus (0.2–0.4 nS) (Otis and Mody 1992). Studies examining GABA\(_A\) receptor–activated conductances in excised membrane patches (De Koninck and Mody 1994; Edwards et al. 1990) and cultured neurons (Segal and Barker 1984) have reported single-channel conductance values in the range of 18–30 pS. Thus the mean mIPSC conductance would correspond to the activation of 10–20 GABA\(_A\) receptor channels. The quantal inhibitory current of \(~7\) pA (at 0 mV holding potential) is equivalent to a conductance of \(~0.1\) nS. Given this, the number of channels underlying a quantal event would range from three to six. These low numbers are in accord with previous estimates suggesting that mIPSCs and quantal GABA events in mammalian cortex involve the activation of only a small number of channels (De Koninck and Mody 1994; Edwards et al. 1990; Ropert et al. 1990; Salin and Prince 1996a).

**Comparison of mIPSCs with evoked IPSCs**

Previously, we found that the conductance of IPSCs evoked with minimal stimulation ranged widely, from 0.4 to 9.3 nS, with an average value of 2.4 \(\pm\) 1.2 nS (Ling and Benardo 1995b). This would suggest that for a given target cell, 4–90 quanta of transmitter are released in response to the minimal extracellular stimulus needed to evoke an IPSC. These minimally evoked IPSCs likely represent unitary IPSCs resulting from the activation of a single interneuron (Edwards et al. 1990; Jonas et al. 1993; Redman 1990). Assessing the lowest range of values, these events appear to be the result of release of a few quanta of GABA. Assuming no transmission failures or silent release sites, this suggests that a single interneuron forms at least a few (i.e., probably \(>4\)) synapses (or release sites) onto the postsynaptic target cell (cf. Buhl et al. 1994; Tamás et al. 1997; Thomson et al. 1996). Extending this analysis further, we can develop estimates, or a range of estimates, for the number of quanta released, synapses acti-
vated, and interneurons engaged for a maximally evoked population IPSC recorded in a single pyramidal neuron.

Although the values for miniature, unitary, and maximal IPSCs vary considerably, comparisons of mIPSC values with those of evoked IPSCs should yield preliminary, quantitative estimates to describe the functional properties of these local inhibitory circuits. Previously, we found that the mean peak conductance of the maximal IPSC evoked with extracellular stimulation was 25.9 ± 4.2 nS (range 10.5–41.2 nS) (Ling and Benardo 1995a,b). If the average minimal evoked IPSC conductance (2.4 ± 1.2 nS) is taken as a measure of the unitary inhibitory conductance (i.e., event associated with the activation of a single, presynaptic, inhibitory interneuron), then on average 10–12 individual interneurons are engaged during generation of maximal population IPSCs, very close to estimates in neocortex (Benardo 1994) and hippocampus (Miles and Wong 1984) made using current-clamp recordings. If we assume that all neurons connected to an individual pyramidal cell can be activated during the maximal evoked population IPSC (i.e., no “silent cells”), then this number would also correspond to the total number of interneurons that synapse onto a given principal cell in the slice preparation. Of course, this likely underestimates the neuronal connectivity in neocortex in vivo. Nonetheless, to extrapolate further, if the mIPSC is representative of transmitter release from a single synapse, we could estimate, given a mean mIPSC conductance of ~0.3 nS, that a single interneuron may form, on average, ~4–12 synapses with an individual pyramidal cell. This estimate is in line with findings from combined electrophysiological and anatomical studies of mammalian cortex that show that individual cells belonging to certain classes of inhibitory interneurons may form up to a dozen or more synapses with a single principal cell (Buhl et al. 1994, 1995; Tamás et al. 1997; Thomson et al. 1996). Thus the functional connectivity scheme for inhibition is low as compared with excitatory circuits (Thomson and Deuchars 1994), consistent with a high-fidelity throughput system in which the activation of only a small number of inhibitory cells is sufficient to exert a substantial regulatory influence on principal cell excitation (Miles 1990; Miles and Wong 1984).

Dendritic filtering of mIPSCs

There were no indications that dendritic filtering contributed to variations in mIPSC amplitude in our experiments. As stated above, investigations examining synaptic events in neocortex using standard intracellular techniques indicated that neocortical pyramidal cells are electrotonically compact (Larkman et al. 1992; Stafstrom et al. 1984). Furthermore, studies examining laminar differences in neocortical circuitry (Salin and Prince 1996b) suggest that the majority of inhibitory input to layer V pyramidal neurons may originate from presynaptic elements proximal to the principal cell soma. Thus the majority of fast IPSCs may be generated within an electrotonically restricted locus relative to the soma, which would explain the apparent lack of dendritic filtering in our recordings. However, this does not preclude the existence of distal inhibitory GABAergic synapses, because previous studies have provided evidence for distal sites of IPSC generation in cortical pyramidal cells (Benardo 1997; Salin and Prince 1996b). In the present study, access to these distal sites may be impeded on technical grounds owing to possible space-clamp limitations (Salin and Prince 1996a,b). However, the variations observed in mIPSCs rise times and decay time constants suggest that some cable filtering was present, which might contribute to determining the shape of mIPSCs. The importance of distally generated IPSCs in gating excitatory drive in layer V cells provides a further impetus to our attempts to better define fast inhibition in this cortical region.

In conclusion, our analysis provides electrophysiologically based estimates on the connectivity scheme of inhibitory cortical circuits. Although this purely electrophysiological analysis cannot provide complete, definitive information on the cell-to-cell connection patterns, it does reveal some important empiric parameters describing the functional properties of inhibitory connections and, in turn, the physiological limits of fast inhibition, and thus provides a functional correlate to anatomic data. As such, our findings help to further define the operational boundaries of cortical inhibition.

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