High-Probability Uniquantal Transmission at Excitatory Synapses in Barrel Cortex

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The number of vesicles released at excitatory synapses and the number of release sites per synaptic connection are key determinants of information processing in the cortex, yet they remain uncertain. Here we show that the number of functional release sites and the number of anatomically identified synaptic contacts are equal at connections between spiny stellate and pyramidal cells in rat barrel cortex. Moreover, our results indicate that the amount of transmitter released per synaptic contact is independent of release probability and the intrinsic release probability is high. These properties suggest that connections between layer 4 and layer 2/3 are tuned for reliable transmission of spatially distributed, timing-based signals.

Synaptic weight is thought to be a primary determinant of neural computation. According to the quantal hypothesis (1, 2) the efficacy of a connection is determined by the product of the probability of release, the number of release sites, and the size of the postsynaptic response to a quantum of transmitter. Quantal parameters determine how reliably information is transmitted (3, 4), with higher rates of information transfer possible at connections with larger numbers of release sites and higher (sustained) release probabilities (3). However, basic synaptic properties, such as number of functional and anatomical release sites per connection and the release probability per site, remain uncertain at most cortical synapses. Moreover, the number of vesicles released at individual synaptic contacts is controversial. Correlation of functional release sites and anatomically

Fig. 1. EPSP recordings from an L4 to L2/3 cell pair in rat barrel cortex under different release probability conditions at 36°C. (A) Current protocols for AP stimulation in the presynaptic spiny stellate cell (top trace) and for testing input resistance stability in postsynaptic pyramidal cell (second trace). Third trace shows the AP in the spiny stellate cell. The bottom trace shows voltage responses to current injection followed by individual EPSPs (gray lines) and the mean EPSP (black solid line) in the postsynaptic pyramidal cell. (B) Mean EPSPs recorded in different extracellular Ca2+/Mg2+ concentrations at a potential of –73 mV. (C) Input resistance during different extracellular Ca2+ and Mg2+ concentrations.

Fig. 2. Quantal parameters estimated from evoked EPSPs under low probability conditions. (A) The top trace shows the mean action potential evoked by a brief depolarizing current pulse at –72 mV; the bottom trace shows 14 individual EPSPs (gray lines), the mean of the 44 EPSP successes (black solid line), and the mean of the 279 failures (dashed line) recorded at –72 mV in 1 mM [Ca2+]/1001 and 5 mM [Mg2+]. (B) The amplitude histogram of EPSPs (bins) and scaled baseline noise (dashed line). The coefficient of variation of the quantal EPSP amplitude (CVQ) was calculated from the background-subtracted variance. (C) Relation between the variance of the EPSP peak amplitude (corrected for background variance) and mean peak EPSP amplitude for an individual L4 to L2/3 cell pair. Each data point shows a different probability condition. Error bars indicate the theoretical standard error in the estimate of the variance. Solid line shows the fit to a multinomial model giving Q = 0.09 mV, Nf = 5.25 and α = 19800.
identified synaptic contacts in inhibitory neurons of goldfish lead to the hypothesis that a maximum of one vesicle is released per active zone ("the single vesicle hypothesis") (5). This hypothesis was subsequently supported at connections between hippocampal pyramidal cells and interneurons (6). However, recent studies indicate that the concentration of glutamate in the synaptic cleft changes with release probability, which implies that multiple vesicles are released at each synaptic contact per action potential (AP) (7, 8).

We investigated the number of functional and anatomical release sites, the release probability, and the mode of release at synaptic connections between pairs of layer 4 (L4) and layer 2/3 (L2/3) excitatory neurons in rat barrel cortex. Paired whole-cell recordings were made from acute slices from 17- to 23-day-old rats at near-physiological temperature (9, 10). In the presence of 2 mM [Ca$^{2+}$] and N-methyl-D-aspartate (NMDA) receptor antagonists, single APs in L4 spiny neurons evoked unitary excitatory postsynaptic potentials (EPSPs) in L2/3 pyramidal cells with a mean peak amplitude of 0.54 ± 0.06 mV at −70 mV (n = 32 cells; Fig. 1A). When stimulated at low frequency (0.1 to 0.033 Hz), EPSP amplitudes remained stable for long periods (~2 to 3 hours). The amplitude of unitary EPSPs could be increased (in eight of nine cells) or reduced by altering the [Ca$^{2+}$] and [Mg$^{2+}$], which suggests that transmitter release probability was not maximal under our control conditions (Fig. 1B). Such alterations in the [Ca$^{2+}$]/[Mg$^{2+}$] ratio had little effect on the input resistance, and thus on the voltage response to a given synaptic conductance (Fig. 1C).

We first determined the mean postsynaptic response to a single quantum of transmitter (Q) by evoking release under conditions of low release probability. In the presence of 1 mM extracellular Ca$^{2+}$, the proportion of presynaptic APs that failed to release transmitter was high (0.82 ± 0.02; 0.8 to 1.0 mM Ca$^{2+}$; n = 5), and thus, the vast majority of the EPSPs were single quantum events (Fig. 2A) (10). Quantal EPSPs were identified on the basis of their characteristic shape, after each trace was digitally filtered. We checked that small-amplitude EPSPs were not missed by averaging the failures (Fig. 2A). On average, the mean amplitude of quantal EPSPs was 0.15 ± 0.02 mV at −70 mV (n = 5 cells). The mean number of quanta contributing to the evoked EPSP (quantal content) under control conditions (2 mM Ca$^{2+}$) was thus ~3.6.

The number of quanta events generated per synaptic contact can be determined by comparing the number of sites that can generate a quantal event (functional release sites, $N_f$) and the number of anatomically identified synaptic contacts ($N_A$). Because the quantal content does not provide information about $N_f$ or about the average release probability across these sites ($P_r$), we estimated these quantal parameters by applying multiple-probability fluctuation analysis (MPFA) (11, 12), also known as variance-mean analysis (13). We first estimated the total nonuniformity in Q from amplitudes of quantal EPSPs under low $P_r$ conditions, using the same peak and baseline measurement windows used for MPFA (Fig. 2B) (10). This gave a coefficient of variation (standard deviation/mean; $CV_Q = 0.43 ± 0.06$; $n = 5$ cells) similar to that reported for other excitatory synapses (11, 14). The variability in quantal size at an individual release site (intrasite variability) has been measured directly at single site cerebellar synapses at physiological temperature ($CV_Q = 0.21$) (15). If we assume the same value for intrasite quantal variability at L4 to L2/3 synapses (see verification below), the variation arising from differences in the mean quantal amplitude across sites (intersite variability) can be calculated ($CV_{Q}^{10} = 0.37$) from the total quantal variability (10). These estimates of quantal

**Fig. 3.** Relation between the number of anatomically identified synaptic contacts and functional release sites. (A) Camera lucida reconstruction of a cell pair, L4 stellate cell with L2/3 pyramidal cell. The soma and dendrites of the presynaptic L4 cell are given in red, the axonal arbor in blue. The somatodendritic configuration of the postsynaptic L2/3 pyramidal cell is given in black, the axonal arbor in green. Lower inset shows the distribution of putative, light microscopically identified synaptic contacts. (B) Variance-mean plot and multinomial fit for the cell pair shown in (A), where Q and $N_f$ are the estimated quantal size and number of functional release sites, respectively. (C) Electron micrographs (EMs) of the synaptic contacts identified by light microscopy in (A), where the letters next to the blue dots in (A) indicate the position of each EM image shown in (C). Labeling in (C): b, presynaptic bouton with clearly visible synaptic vesicles; d, postsynaptic dendrites.
variability were used to constrain a nonuniform quantal model, which allowed more accurate estimates of $Q$, $N_e$, and $P_R$. Time-stable epochs of EPSP amplitudes were identified for each release probability condition with a Spearman rank-order analysis (15). The mean and variance of the peak amplitude were calculated and corrected for deviations in driving force from $-70$ mV, with an assumed reversal potential for AMPA receptors of $0$ mV (9). EPSP variance-mean plots were constructed after subtracting background variance and were fit with a multinomial model with fixed values of quantal variance (10); goodness of fit was assessed with the chi-square test. A variance-mean plot and multinomial fit are shown in Fig. 2C that gave a $Q$ of $0.09$ mV and an estimate of $5.3$ functional release sites for this cell pair. The mean $Q$ varied little across cell pairs ($0.14 \pm 0.02$, $n = 9$) and was not significantly different from that measured directly from quantal EPSPs under low $P_R$ conditions ($P = 0.55$, unpaired $t$ test, unequal variance). The $N_e$ across cells was $5.1 \pm 0.9$ ($n = 9$) and varied between $2.3$ and $6.5$ except for one outlying cell in which $N_e = 11.1$ (the mean value excluding this cell was $4.4 \pm 0.5$, $n = 8$).

Simulations suggest that part of the variance in $N_e$ for the eight clustered cells arose from uncertainty in estimating the variance from a limited number of EPSPs (10). Under control conditions, the $P_R$ at each site was high ($0.79 \pm 0.04$ in $2$ mM $\text{Ca}^{2+}$, $n = 9$), consistent with a previous study (16). An upper limit for the quantal variability at an individual site was calculated by dividing the variance remaining when $P_R$ was maximal ($1.02 \pm 0.05$, $n = 9$) by $N_e$. This gave a value of $CV_q$ close to that assumed from measurements at single-site synapses ($0.26 \pm 0.02$ versus $0.21$) (15). Estimation of the coefficient of variation of release probability across sites gave a value ($0.28 \pm 0.07$ in $2$ mM $\text{Ca}^{2+}$, $n = 9$), similar to that obtained in hippocampal synapses in culture (17).

Anatomical identification of axondendrite intersections with high-resolution light microscopy of biocytin-filled cell pairs (10) indicated between four and six putative synaptic contacts per connection located on the proximal dendritic segments of the postsynaptic neuron (blue dots, Fig. 3A, inset). Across cells, an average of $4.6 \pm 0.2$ ($n = 14$) putative synaptic connections were found, a value not significantly different from the $N_e$ ($P = 0.58$ unpaired $t$ test, unequal variance). In four cell pairs, we were able to recover the anatomy after performing quantal analysis. A camera lucida reconstruction of a cell pair is shown in Fig. 3, for which we estimated an $N_e$ of $5.5$ and an $N_e$ of $5$ (Fig. 3, A and B). For cell pairs in which both quantal analysis and anatomical reconstruction were performed, $N_e$ and $N_e$ were not significantly different ($P = 0.21$, paired $t$ test, $n = 4$; table S1). We further examined whether these close appositions were indeed synaptic contacts by making serial ultrathin sections through the entire dendritic domain of the postsynaptic neuron and examining them at the electron microscopic (EM) level. Synaptic contacts were identified on the basis of a clear presynaptic bouton-like structure containing closely packed synaptic vesicles together with closely apposing (\approx 20 nm) pre- and postsynaptic membranes (Fig. 3C). The presence of electron-dense biocytin reaction product both pre- and postsynaptically and the long duration of the recordings precluded the use of other anatomical identifiers such as the pre- and postsynaptic densities. Full EM analysis of two quantal analysis pairs of neurons and one additional cell confirmed identification of $13$ out of $14$ putative synaptic contacts, which gave a detection rate close to that observed at other cortical synapses (18, 19). Synaptic contacts were located directly on dendritic shafts (Fig. 3C) and occasionally on spines. Applying the observed optical detection rate for synaptic contacts ($93\%$) across the four pairs of neurons, we estimated $N_e N_e$ to be $0.90 \pm 0.16$, which suggests a one-to-one relation between these synaptic properties (table S1). The all-or-none behavior of excitatory synapses could arise either presynaptically, from the release of a single quantum per AP, or postsynaptically, from saturation of postsynaptic receptors (20, 21) following multivesicular release (22, 23). We therefore examined whether the clef glutamate concentration changes with $P_R$ by assaying the level of block of the EPSPs by low-affinity glutamate receptor antagonists (7, 12, 24). The effect of $1$ mM $\gamma$-d-glutamylglycine ($\gamma$-DGG) on the mean EPSPs recorded from a cell pair in normal $\text{Ca}^{2+}$ ($2$ mM) and in the presence of a low $\text{Ca}^{2+}$ ($1.25$ mM) concentration is shown in Fig. 4, A and B. Normalization to the first EPSP amplitude illustrates that the fractional block of the EPSP by $\gamma$-DGG was similar under these two conditions (Fig. 4C). On average, the fractional block of the EPSP by $\gamma$-DGG was not significantly different in $2$ mM $\text{Ca}^{2+}$ and $1.25$ mM $\text{Ca}^{2+}$ ($0.6 \pm 5\%$, respectively; $P = 0.43$; $n = 5$). Moreover, the paired-pulse ratio in $2$ mM $\text{Ca}^{2+}$ was unchanged in the presence of $\gamma$-DGG ($0.59\pm 0.05$, $P = 0.36$; $n = 10$), which indicates a similar fractional block on each pulse. We tested these results further by carrying out a series of experiments.

Fig. 4. Fractional block of the EPSP by low-affinity glutamate antagonists is independent of release probability. (A) The upper trace shows the presynaptic APs in a L4 spiny neuron; middle traces show mean EPSPs recorded in $2$ mM $\text{Ca}^{2+}$, $1$ mM $\text{Mg}^{2+}$ under control conditions and in $1$ mM $\gamma$-DGG (red trace). The bottom trace shows mean EPSPs recorded in $1.25$ mM $\text{Ca}^{2+}$ under control conditions (blue) and in $1$ mM $\gamma$-DGG (green). (B) Plot of EPSP amplitudes as a function of time through the experiment. (C) EPSPs in (A) normalized to the peak of the first control EPSPs. (D) Relation between percentage block of the EPSP and extracellular $[\text{Ca}^{2+}]$ for $0.5$ mM kynurenic acid and $1$ mM $\gamma$-DGG.
Anterior-Posterior Guidance of Commisural Axons by Wnt-Frizzled Signaling

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Commissural neurons in the mammalian dorsal spinal cord send axons ventrally toward the floor plate, where they cross the midline and turn anteriorly toward the brain; a gradient of chemotactant(s) inside the spinal cord controls this turning. In rodents, several Wnt proteins stimulate the extension of commissural axons after midline crossing (postcrossing). We found that Wnt4 messenger RNA is expressed in a decreasing anterior-to-posterior gradient in the floor plate, and that a directed source of Wnt4 protein attracted postcrossing commissural axons. Commissural axons in mice lacking the Wnt receptor Frizzled3 displayed anterior-posterior guidance defects after midline crossing. Thus, Wnt-Frizzled signaling guides commissural axons along the anterior-posterior axis of the spinal cord.

Axonal connections are patterned along the anterior-posterior (A-P) and dorsal-ventral (D-V) neuraxes. Guidance molecules that play essential roles in the D-V guidance of axons have been identified, whereas the nature of the A-P guidance cues has remained an enigma (1, 2). The dorsal spinal cord commissural neurons form several ascending somatosensory pathways. During embryonic development, they project axons to the ventral midline (Fig. 1A). At the floor plate, commissural axons cross the midline, enter the contralateral side of the spinal cord, and make a sharp anterior turn toward the brain (Fig. 1B) (3). The initial ventral growth of the commissural axons is directed by a collaboration of two chemotactants, netrin-1 (4–6) and Sonic hedgehog (Shh) (7), and chemorepellents of the bone morphogenetic protein (BMP) family (8). As the axons cross the midline, they lose responsiveness to these chemotactants (9) but gain responsiveness to several chemorepellents, including Slit and semaphorin proteins (10), which guide axons from the D-V axis into the A-P axis (10).

To determine why axons turn in an anterior direction, we studied the turning of commissural axons after midline crossing in “open-book” spi-