Distinct AMPA-type glutamatergic synapses in developing rat CA1 hippocampus

Elizabeth A. Stubblefield and Tim A. Benke
Departments of Pharmacology, Pediatrics, Neurology and Neuroscience Program
University of Colorado, Denver, School of Medicine
Aurora, CO 80045 USA

* Corresponding author:
Tim Benke, MD, PhD
University of Colorado Denver, Anschutz Medical Campus
RC1 North, Room 6104
12800 E. 19th Ave., MS 8102
PO Box 6511
Aurora, CO 80045
tim.benke@ucdenver.edu

Running title: Development of distinct hippocampal AMPAR synapses
Abstract: (246/250 words)

We assessed synaptic $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPAR) properties during synaptogenesis in order to describe the development of individual glutamatergic synapses on rat hippocampal CA1 principal neurons. Pharmacologically isolated AMPAR-mediated glutamatergic synaptic currents (evoked by stimulation of the Schaffer Collateral pathway, EPSCs), had significantly greater inward-rectification at ages P5-7 compared to P8-18. These inward rectifying EPSCs demonstrated paired-pulse dependent unblocking at positive holding potentials, consistent with voltage-dependent internal polyamine block. Measurements of paired-pulse facilitation did not support altered presynaptic properties associated with inward rectification. Using asynchronous EPSCs (aEPSCs) in order to analyze populations of individual synapses, we found that quantal amplitudes (Q) increased across early postnatal development (P5-P18) and were directly modulated by increases in the number of activated receptors. Quantal AMPAR decay kinetics (aEPSC $\tau_{\text{decay}}$s) exhibited the highest coefficient of variation (CV) from P5-P7 and became markedly less variable at P8-P18. At P5-7, faster quantal kinetics coexisted with much slower kinetics; only slower quantal kinetics were found at P8-18. This supports diverse quantal synaptic properties limited to P5-7. Multivariate cluster analysis of Q, CV$_{\text{decay}}$, and median $\tau_{\text{decay}}$ supported a segregation of neurons into 2 distinct age groups of P5-7 and P8-18, similar to the age-related segregation suggested by inward rectification. Taken together, these findings support synaptic, calcium permeable AMPARs at a subset of synapses onto CA1 pyramidal neurons exclusively at P5-7. These distinct synapses coexist with those
sharing the properties of more mature synapses. These synapses disappear after P7 as activated receptor numbers increase with age.

Keywords: Development, synapse, glutamate, hippocampus, noise-analysis
Introduction

Changes in synaptic $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR) activation in the CA1 region of the hippocampus underlie important processes such as learning and memory formation. Thus, a better understanding of how the biophysical properties of AMPARs change during synaptogenesis would give insight into the mechanisms for developmental regulation of synaptic maturation. The AMPAR forms as a tetramer composed of homomorphic and heteromeric combinations of 4 subunits (GluA1, 2, 3, and 4) (18) and functions as a synaptically activated channel after postnatal day (P) 0 in rat (30; 81). It is thought that heteromeric assemblies of these subunits form preferentially in adults rats (P30-40) (83) and in heterologous expression systems (44), but preferred GluA assemblies in postnatal development have not yet been fully established. Electrophysiological evidence suggests that heterogeneity in AMPAR subunit populations dominate at most developing rat synapses, in that AMPAR GluA1 homomers co-exist at the same synapses as GluA1/2 and/or GluA2/3 heteromeric combinations, as well as different splice-variants of GluA subunits (73).

Biochemical evidence suggests that GluA subunit transcript levels (66) and protein levels (56) change drastically during synaptogenesis in rodents. For example, GluA4 levels increase from birth to P7, and then decrease thereafter to lower-than-birth levels in rat CA1. GluA1 levels appear to be higher than GluA2 during the first week of life (29; 84), but then GluA2 and GluA3 levels dramatically increase around P7-10 (22; 56; 57; 66). The total number of AMPARs at each synapse (N) is thought to increase during this period (3; 57; 81), and supporting this, spontaneous EPSC amplitude has been
shown to increase from P1 up to P8 (28). At rat thalamocortical synapses, it has been
demonstrated that the number of activated AMPARs modulating quantal amplitude (Q)
adjusts with the AMPAR single-channel conductance (γ) to result in a uniform Q during
early development (2).

A wide range of AMPAR activation and deactivation kinetics (EPSC \( \tau_{\text{rise}} \) and
\( \tau_{\text{decay}} \)) and single-channel conductances (γ) have been previously reported (17; 24; 49),
and it is likely that these differences arise due to developmental differences in AMPAR
expression (9; 73). Evidence in the rat cerebellum suggests a speeding of AMPAR-
mediated EPSC kinetics as age increases. For example, AMPAR kinetics are slower at
ages ≤ P10 versus ages ≥ P40 (9; 82). Previous studies in CA1 reveal faster \( \tau_{\text{decay}} \) at P45
compared to P15, supporting the idea that EPSC \( \tau_{\text{decay}} \) are faster in the adult (73).
Recombinant studies (51) and evidence in rat hippocampal culture (78) demonstrate
much faster channel kinetics for GluA2-lacking receptors compared to channels that
contain the GluA2 subunit, suggesting these changes are influenced by subunit
composition. Hippocampal AMPAR-mediated γ values range from 2 pS to 20 pS (25).
These values could vary depending upon differing subunit populations of AMPAR
channels, since (recombinant) GluA2-lacking receptors have a higher γ compared to
GluA2-containing AMPARs (51). GluA2-lacking AMPARs (or unedited GluA2) are
calcium permeable (7; 26; 38) and highly developmentally regulated. Calcium permeable
AMPARs have been found in the rat CA3 region mainly during the first 3 weeks of life
(34), in neocortical pyramidal neurons until P16, coincident when GluA2s become more
prevalent (40), and transiently in primary auditory neurons before the onset of
hearing(20). In CA1, the developmental expression of calcium permeable synaptic
AMPARs has been explored, revealing the presence of inward rectifying channels mainly in interneurons (36). Detailed analysis of inward rectifying AMPAR on CA1 principal neurons has not been studied during early developmental ages, except to note that they are typically not active around P14 (58).

It has been shown that AMPAR subunits can be targeted to specific glutamatergic synapses (15; 31; 71) and, in the cerebellum this can be due to the activity of calcium permeable AMPARs that direct GluA2-containing receptors into synapses (43). In CA1 hippocampus it has been demonstrated that specific targeting of calcium permeable, GluA2-lacking AMPARs aids in the induction and expression of LTP (58), (but see (1)). Similar targeting also occurs in response to pharmacological blockade of AMPARs (78) or in response to hypoxia-induced seizures (64). This suggests that selective AMPAR properties including calcium permeability are intrinsically necessary in processes that involve precise strengthening or weakening of synaptic neurotransmission. However, it remains unknown if biophysically different AMPARs are segregated to different synapses during basal levels of neurotransmission. We therefore studied both multiquantal and quantal AMPAR-mediated synaptic responses during the formation of synapses in CA1 hippocampus when AMPAR mediated calcium fluxes might affect synaptogenesis. We used whole-cell electrophysiological recordings to investigate synaptic AMPAR-mediated multiquantal EPSCs to study inward rectification and quantal aEPSCs (in the presence of extracellular strontium) to study Q, quantal $\tau_{\text{decay}}$, $\gamma$, and number of activated receptors ($N^*P_o$). Our data suggest that neurons at P5-7 expressed two distinct types of synapses: one type expressed inward rectifying AMPARs associated with faster quantal kinetics, while the other type expressed non-rectifying...
(linear) AMPARs associated with slower quantal kinetics, similar to synapses at P8-18. The older neurons (P8-18) had more uniform biophysical properties, yet the number of activated AMPARs significantly increased at the synapse. We therefore speculate that there are AMPAR-mediated calcium fluxes at distinct synapses at P5-7 during early synaptogenesis; as these synapses mature, they then become more biophysically uniform.

**Methods**

**Ethical approval**

All experiments were approved by the University of Colorado at Denver Health Science Center Institutional Animal Care and Use Committee. Data from 122 rat pups were used in this study.

**Slice preparation**

Sprague-Dawley rats, male and female, P5-18 days old, were decapitated and the whole brain was rapidly removed and placed in ice-cold cutting solution comprising (in mM): 124 NaCl, 26 NaHCO₃, 3 KCl, 1.2 NaH₂PO₄, 5 MgSO₄, 10 D-glucose, 2 Na ascorbate and bubbled with 95% O₂, 5% CO₂. Sagittal slices were made (300-400 μm thick) using a Leica VT-1200 microtome (Nussloch, Germany) and additional cuts were made with a scalpel to remove the CA3 area of the hippocampus. Hippocampal slices were transferred to a holding chamber and stored for at least 1 hour prior to recording in recovery solution (as above, except including 2 mM CaCl₂ and substituting 1 mM MgSO₄) at room temperature (18-22°C).
Electrophysiological recording

CA1 pyramidal neurons were visually identified by characteristic position and shape in submerged slices using infrared differential interference contrast (DIC) optics under magnification (40x) (BX51-WI, Olympus) and patch-clamped in the whole-cell configuration. Borosilicate glass (Harvard Instruments, Holliston, MA) recording electrodes were pulled with a Sutter P-97 electrode puller (Sutter Instruments, Novato, CA, USA) with resistance 4-6 MΩ and filled with internal patch solution comprising (in mM) 135 CsMeSO₄, 10 HEPES, 0.5 EGTA, 0.3 Na-GTP, 5 QX314, 4 ATP-Na₂, 4 MgCl₂, 0.1 spermine, pH 7.25 with 1 M CsOH. For recording, picrotoxin (100 μM), and D-APV (50 μM) were added to the recording solution to block γ-amino butyric acid (GABA) receptor and N-methyl-d-aspartate (NMDA) receptors, respectively (recording solution).

For rectification experiments, a platinum/iridium bipolar stimulating electrode (FHC, Bowdoin, ME) was placed in the stratum radiatum in order to evoke a near maximal, synchronous, multiquantal excitatory post-synaptic current (EPSC) by using a constant voltage source (Digitimer, DS2A, England) (7-9 V, 100 μs) every 5 s. In the remainder of experiments, near minimal stimulation of axons in the stratum radiatum to evoke aEPSCs was performed with a glass stimulating electrode (resistance of 6-7 MΩ when filled with recording solution) and placed in a consistent location (30-50 μM distally from the soma and 15-30 μM laterally from the primary apical dendrite) to the recording electrode. A constant voltage (Digitimer, DS2A, England) or constant current source (WPI, Sarasota, Fl) source (1-3.0 V or 8.5-9.9 mA, 100 μs duration) was applied
Calcium was replaced with 8 mM Sr²⁺ in the recording solution to cause asynchronous release of glutamate (4; 27) and was introduced following membrane rupture. Resulting asynchronous excitatory post-synaptic currents (aEPSC) were collected at a holding potential of -70 mV in a 2.25 second time-frame after each single stimulus. EPSCs and aEPSCs were confirmed to be mediated by AMPA receptors by addition of GYKI 52466 (100 μM) across all ages resulting in complete block of current (n=8). Spontaneous events prior to stimulation were only seen in two of 122 recordings; these recordings were not analyzed further; this suggests that aEPSCs in the 2.25 s window after stimulation were not spontaneous events. Series resistance associated with the recording electrode was monitored in every sweep by a -2.0 mV voltage-step; recordings were terminated if changes in series resistance of greater than 10% of baseline occurred (See Supplemental Fig. 1). Cell capacitance was also determined from this voltage-step (2) (See Supplemental Fig. 2). Responses were amplified and filtered at 5 kHz (Axopatch 200B, MDS, Sunnyvale, CA) and digitized at 20 kHz (Digidata 1322A and Clampex 9.2, MDS, Sunnyvale, CA). No series resistance cancellation was performed. All drugs were purchased from Sigma Aldrich (St Louis, MO) or Tocris Bioscience (Ellisville, MO).

Data Analysis

For calculation of rectification index (RI), neurons were voltage clamped at -70 mV for a set of 10-12 EPSCs and then voltage-clamped at +40 mV for a set of 10-12 EPSCs. RI was calculated as the average of absolute peak current at -70 mV divided by absolute peak current at +40 mV for each neuron. In a subset of recordings, voltage was
varied from -70mV to -40mV, 0mV, +20mV, and +40mV for at least 10-12 EPSCs each, in order to obtain peak current-voltage (I-V) relationship, verify minimal voltage offsets (no corrections were performed) and show separation of smaller EPSCs from stimulus artifacts. The voltage-dependent unblocking index (UBI) was calculated by obtaining the paired-pulse ratio (PPR) of averaged amplitudes (at least 25 currents) EPSC2/EPSC1 (obtained within a 70ms inter-stimulus interval) at +40mV and divided by the PPR of averaged amplitudes at -70mV for each neuron (69). The averaged current for EPSC1 at -70 mV was normalized by the averaged amplitude of EPSC1 at +40 mV and compared (Fig. 2C1,2) to demonstrate degree of unblock for EPSC2 at +40mV for inward rectifying AMPARs. Since EPSCs are potentially a mixture of inward rectifying and linear AMPARs, then RI is a reflection of that percentage (Suppl. Text). Since presynaptic-mediated paired pulse ratios will be the same at different post-synaptic holding potentials, UBI can be expressed in terms of the fractional percentage of inward rectifying AMPARs ($PRI$) as

$$UBI = \frac{PRI(F2 - F3)}{PRI(F1 - F3) + F3}$$

where, for a pure population of only inward rectifiers, $F1$ is the maximal block at +40 (EPSC1 at +40 mV/EPSC1 at -70 mV) and $F2$ is the unblock at +40 (EPSC2 at +40 mV/EPSC1 at -70 mV); unblock at -70 ($F4 = EPSC2$ at -70 mV/EPSC1 at -70 mV) was assumed to be near unity (see Results, Fig. 2B). $F1$ was extrapolated to be approximately 0.05 (based on (70), their Fig. 4). For a linear AMPAR conductance, $F3$ is 0.57 (40/70). $F1$, $F2$ and $F4$ apply only to pure inward rectifiers; $F3$ applies only to linear AMPARs. The unknown $F2$ would be expected to be less than or equal to $F3$ (70), based on the
degree of unblock (70). The unblock ratio, the amount of unblock of inward-rectifying AMPARs after single paired pulses, \(F2/F3'\), where \(F3' = 40/70\), would be expected to be less than unity for single paired pulses compared to a train of pulses when it would more closely approach unity (70). Furthermore, using

\[
PRI = (1 - RI \times F3)/(RI \times (F1 - F3)),
\]

allows an estimate of PRI from RI. This analysis assumes that presynaptic function, as it affects the activation of either pool of AMPARs, is equal.

AMPAR-mediated aEPSCs were visually detected as peaks arising at least 2.5 times the noise (\(\sigma_n^2\), average variance 2.04 pA\(^2\), range 0.81-7.84 pA\(^2\), \(n = 38\)). aEPSCs were analyzed for amplitude (Q) and quantal kinetics (\(\tau_{\text{rise}}\) and \(\tau_{\text{decay}}\)) using purpose-written software in which responses were identified based on the point of onset (5; 6). Responses were selected as long as they were not overlapping with another aEPSC in a 10-25 ms window, depending on the rate of aEPSC decay. Each aEPSC peak amplitude was measured as the difference between the average peak value from the middle of a 5-sample window (0.25 ms) and a similar window prior to the onset of the aEPSC used to measure the noise. \(\tau_{\text{rise}}\) and \(\tau_{\text{decay}}\) for each aEPSC were obtained by fits to the rising phase and decaying phase of individual aEPSCs according to \(I(t) = a \times \exp(-t/\tau_{\text{rise}}) - a \times \exp(-t/\tau_{\text{decay}})\) using a non-linear least-squares iterative method (see Fig. 3). Median values were calculated from binned cumulative histograms for amplitude, \(\tau_{\text{rise}},\) and \(\tau_{\text{decay}}\) and used for subsequent comparisons across development. Coefficient of variation for amplitude and kinetics for the events from each neuron was estimated as \(CV = \sigma/\mu\), where \(\sigma\) is the standard deviation and \(\mu\) is the arithmetic mean.
Single-channel conductance was determined from an ensemble of aEPSCs from a neuron by using peak-scaled non-stationary fluctuation analysis (NSFA) (79). This analysis was based on a subset of hand-selected responses (minimum of 14) based on precise alignment around the onset and peak, non-overlapping aEPSCs (50-70 ms window, depending on the rate of aEPSC decay) and return to baseline in this window (2; 5; 6). These limitations prevented analysis on some neurons due to an inadequate number of responses (6). Selected individual aEPSCs were averaged to obtain a mean response waveform. This mean aEPSC response waveform was then scaled to the peak of each individual aEPSC peak and subtracted and squared. The variance of fluctuation around the mean was calculated for 10-100 bins of equal current decline from the peak of the mean current until within a time frame 5-6 times $\tau_{\text{decay}}$. The binned variance was plotted against the mean current amplitude and the single-channel current was estimated by fitting the data using a least-squares algorithm according to:

$$\sigma^2 = i*I - I^2/(N*P_o) + b_1$$  

(2)

where $\sigma^2$ is the variance, $I$ is the mean current, $N*P_o$ is the number of AMPAR activated at the peak of the mean current, $i$ is the single-channel current, and $b_1$ is the background variance (75; 79). The single-channel conductance, $\gamma$, is then $\gamma = i/V$ where $V$ is the driving force (holding potential of -70 mV, assumed AMPAR reversal potential of 0 mV). To obtain the most accurate estimate for $\gamma$, the data were fitted from 0 to 50% of the maximum current (5; 6) with goodness of fit assessed with the Spearman Rank-Order correlation coefficient ($R^2_{sp}$) (2; 60). Mean current ($I$) peak amplitude and single-channel conductance infer total AMPAR numbers and peak opening probability according to:
\[ I = N \cdot P_o \cdot \gamma \cdot V = Q_{\text{calc}} \quad (3) \]

where \( I \) is the mean current amplitude, \( N \) is the average total number of AMPARs across the population of AMPAR clusters activated, \( P_o \) is the peak open probability of AMPAR opening, \( \gamma \) is the single-channel conductance, and \( V \) was the holding potential.

NSFA permits calculation of \( N \cdot P_o \) for individual neurons; peak-scaled NSFA does not allow direct calculation of \( P_o \). In the case of quantal aEPSCs, peak quantal current (\( Q_{\text{calc}} \)) and peak mean current (\( I \)) are theoretically equivalent.

**Statistics**

For grouped comparisons, data are expressed as mean ± standard error of the mean (sem) and \( n \) = number of recordings from individual neurons; Mann-Whitney Rank Sum tests were used for comparisons of two groups, with significance set at \( p \leq 0.05 \) (Sigmaplot, Systat, Point Richmond, CA). Kruskal-Wallis one-way ANOVA on ranks was used to compare 3 groups with Dunn’s Method for pair-wise testing of multiple comparisons. Significance was set at \( p \leq 0.05 \). Linear interpolation was used to determine trends with \( r^2 \) used to assess trend (1 = perfect) and \( p \) to assess significance.

Median values were interpolated from binned cumulative data distributions as the point at which half of the distribution was above and half below that point. Grouped cumulative distributions were binned along the y-axis for averaging. For comparisons of averaged cumulative distributions, the Kolmogorov-Smirnov (K-S) test was used with significance set at \( p \leq 0.05 \) (60). Hartigan’s Dip test was used to test for multimodal distributions (significance set at \( p \leq 0.1 \) (33; 61; 63)). Cluster analysis (JMP, SAS, Cary, NC) was
used to suggest segregational break points for group comparisons. Levene’s test of equal variance (JMP, SAS, Cary, NC) was used for some group comparisons.

Results

Inward rectifying AMPARs exist very early in development at CA1 synapses

In order to establish the existence of inward rectifying AMPAR during early development, we conducted whole-cell recordings and obtained current-voltage relationships from electrically evoked EPSCs in individual pyramidal neurons. It is known that inward rectifying AMPARs exhibit a voltage-dependent decrease in open probability at positive holding potentials compared to negative holding potentials when in the presence of intracellular polyamines such as spermine (7; 38; 70). This means inward rectifying channels mediate less current at positive holding potentials compared to negative holding potentials. Fig. 1 demonstrates the voltage-dependent properties of EPSCs in the presence of intracellular spermine. An example neuron at P6 (Fig. 1A1) demonstrated minimal outward EPSCs at +20mV and +40mV compared to the robust inward EPSCs at -70mV to indicate the presence of inward rectifying, synaptic AMPARs. This was not always the case, however, since synaptic AMPARs also demonstrated robust current at both positive and negative holding potentials (Fig. 1A2, example neuron) at this age. I-V curves for neurons between ages P8-18 consistently revealed linear synaptic AMPAR conductances (Fig. 1A3, example neuron). Since the largest RI was found at P7 (RI = 21.0), all values of RI after P7 were less than 5.0, and cluster analysis of RI across all age groups segregated all RI greater than 6.0, we grouped all neurons at P5-7 and P8-18 (Fig. 1B). Mean RI at P8-18 (2.67 ± 0.15, n = 38) was
significantly smaller than mean RI at P5-7 (5.39 ± 0.77, n = 38; p=0.005, Mann-Whitney Rank Sum) suggesting the presence of inward rectifying, synaptic AMPARs restricted to P5-7.

Since this method of calculating RI can be biased towards higher RI values due to the presence of relatively small EPSCs in developing neurons, especially in the presence of small voltage offsets, full I-V relationships were obtained in a subset of neurons (Fig. 1A). At P5-7, we segregated RI greater than 6 from the remainder (Fig. 1C) based on cluster analysis and comparison of group means at P5-7 and P8-18 (cf. Fig. 1B); all data sets for which full I-V relationships were available were included. Inward rectifying EPSCs at P5-7 (subset mean RI=13.13 ± 3.61, range 6.57 to 21.57, n = 5) were compared to non-rectifying EPSCs at P5-7 (subset mean RI=2.51 ± 0.38, range 1.50 to 3.76, n = 7) and all (non-rectifying) EPSCs at P8-18 (subset mean RI=2.20 ± 0.18, range 1.57 to 3.23, n = 9). Inward rectifying EPSCs at P5-7 had significantly less total outward current recorded at more positive holding potentials (+40mV, **p=0.004; +20mV, *p=0.032 Kruskal-Wallis one-way ANOVA on ranks, Dunn’s post-hoc). Inward rectifying EPSCs at P5-7 were also significantly different from non-rectifying EPSCs (+40mV, **p=0.006; +20mV *p=0.032, Kruskal-Wallis one-way ANOVA on ranks, Dunn’s post-hoc). EPSCs at P5-7 that were non-rectifying were not statistically different at any holding potential, compared to EPSCs from P8-18 neurons (Fig. 1C), and they both demonstrated the same linear profile. Therefore, at younger ages (P5-7), neurons expressed synaptic, inward rectifying AMPARs or non-rectifying (linear) AMPARs, whereas the older age group (P8-18) expressed only linear, synaptic AMPARs. The segregation at P5-7, based on a cut-off of RI greater than 6, was not significant (Hartigan’s Dip test, D= 0.039, N=38, p ≈...
A parsimonious interpretation is that neurons express synaptic, inward rectifying AMPARs in varying percentage across a continuum, rather than in a truly segregated fraction.

In order to examine this further, we employed the known voltage- and use-dependent properties of intracellular polyamine-mediated inward rectification (69). Delivering a paired-pulse stimulation relieves the intracellular polyamine block of inward rectifying AMPAR channels and to an even greater degree at positive holding potentials (8; 70). Comparison of paired-pulse ratios (PPR) at positive and negative holding potentials thus reflects the relative contribution of inward rectifying AMPARs to total peak synaptic current. To do this, it was necessary to first calculate the PPR at a negative holding potential for comparison. Similar PPR at -70 mV was found at P5-7 (1.71 ± 0.17, n=14) compared to P8-18 (1.85 ± 0.17, n = 20, p = 0.43, Mann-Whitney Rank-Sum)(Fig. 2A). At P5-7, there was no correlation of PPR at -70 mV with rectification index ($r^2 = 0.009$)(Fig. 2B). Since PPR is largely mediated by presynaptic release probability, this suggests that presynaptic function was similar at synapses containing inward rectifying and non-rectifying AMPARs. While there was similar PPR at -70 mV for both inward and non-rectifying EPSCs, inward rectifying EPSCs at P5-7 had more unblock (facilitation) at +40mV (Fig. 2C1) compared to non-rectifying neurons (Fig. 2C2) (e.g. P8-18) with the second pulse. The bottom panels of Fig. 2C1,2 show overlapped, normalized currents to demonstrate more unblock at +40mV (vs. -70mV) for inward rectifying EPSCs with the second pulse. We then compared the unblocking index (PPR at +40 mV/ PPR at -70 mV) to rectification index (Fig. 2D) for neurons at P5-7 and P8-18. As expected for the mechanism of polyamines on inward rectifying AMPARs, at
P5-7 the unblocking index correlated with the rectification index of each individual neuron (Fig. 2D, n = 18 neurons, Methods equation 1, $F_2 = 0.34$, $r^2 = 0.7$, $p < 0.0001$).

This correlation predicted an unblocking ratio ($F_2/F_3 = 0.34/0.57$) of less than unity (0.6), as expected for only two pulses (70). At P8-18, only lower unblocking index and non-rectification were found (Fig. 2D). Minimal unblock was seen at P8-18 (n = 14, $F_2 = 0.09$, $r^2 = 0.03$, $p = 0.54$) suggesting a much smaller unblocking ratio ($F_2/F_3 = 0.09/0.57 = 0.16$). The relationship of rectification index to unblocking index suggested that neurons possibly expressed inward rectifying AMPARs across a continuum. The largest rectification index utilized here (18.8) would be consistent with that for a nearly pure population of synaptically activated inward rectifying AMPARs (70). We used the relationship of UBI to RI (Methods equation 1a) to support our estimate of the fraction of inward rectifying AMPARs (Methods equation 1b) contributing to each EPSC (Fig. 2E).

This relationship suggests that for an inward rectifying fraction of AMPARs less than 0.5, measurements of RI are relatively insensitive, compared to fractions greater than 0.5. We conservatively estimate from this relationship that an unblocking index greater than 2 and a rectification index greater than 6 is consistent with greater than 75% inward rectifying AMPARs (Fig. 2E). This suggests that 26% (10/38) of neurons at P5-7 express synaptic AMPARs containing greater than 75% inward rectifying AMPARs. However, it is not clear from this analysis whether or not linear and inward rectifying types of AMPARs are mixed together or segregated at individual synapses.

Isolation of quantal AMPAR-mediated EPSCs at immature CA1 hippocampal synapses
Since AMPAR rectification properties changed significantly at P8 in CA1 pyramidal neurons and in order to address whether AMPAR subtypes might be segregated or mixed at synapses, we further assessed synaptic AMPARs to elucidate other developmental differences to support our findings. We measured peak quantal current (Q), kinetics of deactivation ($\tau_{\text{decay}}$), and the underlying single-channel conductance ($\gamma$). Calcium was substituted with strontium in the extracellular recording medium to cause asynchronous release (i.e., one quantum at a time) of glutamatergic vesicles from presynaptic axonal terminals (4; 52; 85), (Fig 3A, B). Near-minimal stimulation allowed the individual quantal activation of a small population of synapses containing AMPARs. By stimulating at a uniform distance from the somatic recording electrode, we approximate that responses emanated from a similar position in the dendritic tree to minimize the contribution of different electrotonic filtering to measurements. It was previously demonstrated that aEPSCs, in contrast to mEPSCs, eliminated discrepancies associated with the activation of synapses throughout the dendritic tree which would likely be differentially filtered (4). aEPSCs were AMPAR-mediated responses since, in the presence of D-AP5, they were completely blocked by the AMPAR-selective antagonist GYKI 52466 (100 μM) across all ages. (See Methods). Response amplitudes of aEPSCs remained stable for up to 35 minutes (Fig 3C) following membrane rupture. Response stability allowed us to apply both quantal analysis and peak-scaled non-stationary fluctuation analysis (NSFA) to aEPSCs in order to report a weighted mean estimate (16) of the underlying AMPAR $\gamma$. 


Quantal AMPA-mediated aEPSC amplitude during early development

We analyzed AMPAR-mediated aEPSC quantal amplitudes (Q) from each neuron across ages P5-P18. We used density and cumulative distribution histograms to assess median Q, mean Q, and relative variability (CV₀) for each neuron (Figs. 4A,B). Q did not appear to be normally distributed in individual neurons at the younger ages (Fig. 4A1, A2, P5 representative neuron), which necessitated analysis of cumulative distributions and median Q from each neuron. While suggestive, there was not a significant bimodal component for this distribution (Hartigan’s Dip test, D = 0.030, 117 events, p ≈ 0.7); pooled event data from all P5-7 neurons (not shown) also did not have a significant bimodal component (Hartigan’s Dip test, D = 0.008, 732 events, p ≈ 0.99). In comparison, distributions of Q from the older age group (P8-18) (P17 representative neuron, Figs. 4B1, 4B2) typically appeared more uniform (Hartigan’s Dip test, D = 0.014, 290 events, p ≈ 0.5); pooled event data from all P8-18 neurons (not shown) also did not have a significant bimodal component (Hartigan’s Dip test, D = 0.005, 1985 events, p ≈ 0.99).

Due to the significant differences in rectification properties in neurons at ages P5-7 compared to P8-18 (see Fig. 1), we then grouped median Qs at the respective age groups accordingly for direct comparison (Figs. 4C,D,E). Even though distributions of Q were (suggestively) more broadly distributed at P5-7, when segregated into groups at P5-7 and P8-18, we found that group-averaged Q distributions for P8-18 were significantly shifted towards larger amplitudes compared to group-averaged Q distributions at P5-7 (Fig. 4C, **p=0.0026, K-S test). Furthermore, median Qs from each individual neuron...
Quantal AMPAR-mediated aEPSC kinetics during early development

Inward rectifying AMPARs display faster kinetics ($\tau_{\text{rise}}$ and $\tau_{\text{decay}}$) than linear AMPARs (51). Collective activation time of an AMPAR synapse is represented in the $\tau_{\text{rise}}$ phase of an aEPSC, while the time it takes for AMPARs to deactivate and desensitize is evident in the $\tau_{\text{decay}}$ phase of an aEPSC. We measured quantal $\tau_{\text{rise}}$ and $\tau_{\text{decay}}$ for each aEPSC from each individual neuron across the developmental time points P5-18 and hypothesized the kinetics would be faster for P5-7 when grouped and compared to older animals. Density (Fig. 5A1,B1) and cumulative (Fig. 5A2,B2) distributions of quantal $\tau_{\text{decay}}$ for each aEPSC (Fig. 3AB) from each neuron were analyzed. Analysis of $\tau_{\text{rise}}$ suggested that these distributions were potentially influenced by filtering while $\tau_{\text{decay}}$ was not (Suppl. Figs. 1 & 2). Similar to our findings of Q per neuron, aEPSC quantal $\tau_{\text{decay}}$ in
each neuron demonstrated wider distributions at P5-7 (representative P7 neuron shown in Fig. 5A1,2) compared to individual neurons at P8-18 (representative P11 neuron shown in Fig. 5B1,2). While suggestive, there was not a significant bimodal component for this distribution at P7 (Hartigan’s Dip test, $D = 0.022$, 83 events, $p \approx 0.9$); pooled event data from all neurons at P5-7 (see Suppl. Fig. 4F) also did not have a significant bimodal component (Hartigan’s Dip test, $D = 0.011$, 732 events, $p \approx 0.9$). Given the variability of Q and aEPSC $\tau_{\text{decay}}$ at this age, we compared Q and aEPSC $\tau_{\text{decay}}$ for each aEPSC at both ages; no correlations were found (Suppl. Fig. 3). In comparison, distributions of $\tau_{\text{decay}}$ from the older age group (P8-18) (P11 representative neuron, Fig. 5B1, 5B2) typically appeared unimodal (Hartigan’s Dip test, $D = 0.023$, 107 events , $p \approx 0.99$); pooled event data (not shown) did not have a significant bimodal component (Hartigan’s Dip test, $D = 0.007$, 1985 events, $p \approx 0.99$).

We averaged cumulative probability distributions of quantal $\tau_{\text{decay}}$ for all P5-7 neurons and compared them to all P8-18 neurons. This revealed that distributions of quantal $\tau_{\text{decay}}$ for all P5-7 neurons was significantly different from all P8-18 neurons (Fig. 5C, P5-7 $n=15$, P8-18 $n=23$, **$p=0.0022$ K-S test), most likely due to significantly slower channels at P8-18. Median quantal $\tau_{\text{decay}}$s were much faster for all P5-7 (mean of median $\tau_{\text{decay}}$s=$3.48 \pm 0.467$ ms ($n = 15$) compared to all P8-18 (mean of median $\tau_{\text{decay}}$s=7.03 $\pm 0.22$ ms ($n = 23$), **$p<0.001$ Mann-Whitney Rank Sum test). Median quantal $\tau_{\text{decay}}$s were clearly segregated into two groups at P5-7 (Hartigan’s Dip test, $D = 0.097$, modal break point $= 2.6$ ms, $n = 15$, $p < 0.1$). To further determine the source of variability of quantal $\tau_{\text{decay}}$s, we compared the CV$_{\tau_{\text{decay}}}$ of each neuron from the two age groups. Consistent with a greater degree of variability in inter-synaptic parameters at P5-
7, neurons at P5-7 had a significantly higher CV\(_{\text{decay}}\) when grouped together and compared to all neurons at P8-18 (Fig. 5E, \(*p=0.023,\) Mann-Whitney Rank Sum).

Multivariate cluster analysis of Q, CV\(_Q\), CV\(_{\text{decay}}\), and median quantal \(\tau_{\text{decay}}\)s also segregated neurons into groups of P5-7 and P8-18, similar to the grouping suggested by inward rectification.

**Single-channel conductance (γ) of AMPARs in developing CA1 neurons**

It is known that inward rectifying AMPARs have a higher \(\gamma\) compared to linear AMPARs (51). We used peak-scaled non-stationary fluctuation analysis (NSFA) to estimate AMPAR \(\gamma\) across P5-18 to determine if \(\gamma\) might decrease during the developmental time points tested. To perform this analysis, a subset of the original aEPSCs (Fig. 6A1, A2) was chosen from each neuron meeting the necessary criteria (see Methods (Fig. 6B1, B2). The average \(\gamma\) at P5-7 compared to P8-18 was not statistically different. Similar to rectification and quantal \(\tau_{\text{decay}}\), the greatest differences in \(\gamma\) occurred at P5-7 (Fig. 6C, P5-7: \(\gamma=9.46 \pm 3.10\) pS (\(n=6\)); P8-18: \(\gamma=7.52 \pm 0.37\) pS (\(n=15\)), Levene’s \(F=10.59, p < 0.0042\)). Independent of age, \(\gamma\) was relatively uniform with the corresponding AMPAR median Q for each neuron tested (Fig. 6D). At P6, one neuron demonstrated a very high \(\gamma\) (24.3 pS) and correspondingly low Q (5.0 pA). It is possible that neurons may, indeed, have AMPAR channels that conduct more single-channel current than this plot indicates. However, since only a weighted mean conductance per neuron is possible using peak-scaled NSFA, it is likely that if there are only a few high-conducting channels, then they are most likely obscured by a comparatively greater number of lower-conducting channels. Therefore, AMPAR \(\gamma\) was not likely the dominant
mechanism regulating AMPAR Q; thus, the modest increase in Q during development (P5-18) cannot be explained by an increase in AMPAR single-channel conductance.

\[ N*P_o \text{ increases to directly mediate larger AMPAR quantal amplitudes} \]

At thalamocortical synapses, Q is tightly controlled across development by regulation of \( \gamma \) and the number of activated receptors (N*P_o) (2). Since AMPAR \( \gamma \) demonstrated no direct correlation with Q (Fig. 6D), it was possible that Q was directly associated with the number of activated receptors (N*P_o). This can also be determined from peak-scaled NSFA (Methods equation 3). When N*P_o was examined over the developmental ages P5-18, there was no clearly defined correlation (Fig. 7A). However, N*P_o was directly correlated with Q (Fig. 7B, linear regression, \( r^2 = 0.74, p<0.001 \)). Thus, regulation of AMPAR number and/or AMPAR activation most directly influenced Q at these synapses during early postnatal development. Increasing AMPAR number and/or its activation best supports increases in Q from P5-7 to P8-18.

**Discussion**

**Development of AMPAR synapses**

In the central nervous system, the hallmark of synaptogenesis is the formation of contacts between presynaptic axons and postsynaptic densities to form a synapse (11). During this formative phase which includes the ages studied here (P5-P18), anatomical data and microscopy studies have demonstrated significant neuronal growth, both in size
and shape (21), as well as an increase in dendritic spine density (14) and total number of AMPAR clusters per spine (81). We hypothesized that synaptic AMPAR properties also change significantly during this time period. Under our experimental conditions, Q increased across the developmental time points P5-18. This is an extension of the previous finding that spontaneous EPSCs recorded in this brain region also increase from birth up to P8 (28). We determined that these increases in amplitude are directly mediated by increases in the number of activated AMPARs (N*P_o) across development. Importantly, our findings suggest that the increases in AMPAR numbers occur across development in a heterogeneous fashion. Inward rectification and faster channel kinetics exclusively at P5-7 suggest that AMPARs mediating these properties are restricted to this developmental window. Furthermore, our data suggest that heterogeneous AMPARs are segregated to distinct synapses prior to P8. After P8, AMPAR synaptic properties are more uniform. These findings have important and novel implications for understanding how AMPAR synapses develop and raise important questions about the role of these distinct synapses in early synaptogenesis.

Neuronal versus synaptic variability in early development

We questioned first whether developing neurons express uniform synaptic AMPAR properties and second, if non-uniform, whether these were expressed distinctly or as mixtures across synapses. We suggest that during P5-7, developing hippocampal neurons segregate distinct AMPARs to different synapses leading to pronounced inter-synaptic variability. From P8, synaptic properties change with neuronal development but are more uniform across synapses. Synaptic variability prior to P8 was supported by
several findings. First, measurement of the apparent rectification index demonstrated inward rectification only before P8 (Fig. 1B). Comparison with inward rectification after P8 suggested a cut-off value for better visualization (Fig. 1C). Analysis of apparent inward rectification compared to the unblocking index revealed that inward rectifying AMPARs are likely segregated to synapses across a continuum, but with some synapses containing nearly all inward rectifying AMPARs. We approximated 26% of synapses contain more than 75% inward rectifying AMPARs at P5-7. We therefore investigated quantal aEPSCs to further quantify inter-synaptic variability. Second, the range of CVQ at P5-7 was greater than what would be expected for intra-synaptic variability (kinetic variability at an individual synapse) (42; 46). Therefore, there was additional inter-synaptic variability in the quantal amplitude, indicating a great degree of variability across synapses. These findings of bimodal Q are reminiscent of those found in the developing Xenopus visual system (59). Prior studies have found that CVQ is low near birth but increases to similar levels to what we have found around P5-7 (28; 30); we have extended this to show that CVQ then suggestively decreases and stabilizes after P7.

Third, median quantal $\tau_{\text{decay}}$ at P5-7 demonstrated two distinct populations. Furthermore, $CV\tau_{\text{decay}}$ was greater at P5-7 than other ages (similar to increased CVQ). This suggests that neurons at P5-7 can simultaneously contain two distinct populations of synapses. If synapses at P5-7 simply contained mixtures of functionally different AMPARs, it would be expected that the median quantal $\tau_{\text{decay}}$ would be a weighted mean of the relative proportion of each without necessarily increased $CV\tau_{\text{decay}}$ (Suppl. Fig. 4). While statistically we did not find evidence of multimodal distributions with the use of Hartigan’s Dip test, this test has low power for closely spaced modes or pronounced tails.
as seen with modeled data (Suppl. Figure 4.). We therefore suggest that analysis of
CV $\tau_{\text{decay}}$ is more sensitive for detecting inter-synaptic variability. The variability of
quantal $\tau_{\text{decay}}$ in individual neurons resulting in $\text{CV}\tau_{\text{decay}}$ greater than 1 at P5-7 can most
parsimoniously be explained by the co-existence of biophysically distinct AMPAR
synapses; indeed, some mixture of different AMPARs at a synapse was also likely
present to some degree. Nevertheless, the finding that there was markedly faster quantal
$\tau_{\text{decay}}$ at P5-7 suggested that younger synapses were biophysically different than older
synapses. Taken together, this supports our finding that different AMPAR synapses co-
exist at younger ages. Combining our similar estimates from inward rectification and
pooled distribution of quantal $\tau_{\text{decay}}$ (Suppl. Fig. 4F), we conservatively estimate that 30%
of synapses prior to P8 may contain greater than 75% inward rectifying AMPARs.

Functionally, inward rectification was not associated with differences in paired-
pulse facilitation (Fig. 2AB). This suggested that pre-synaptic function did not contribute
to inter-synaptic variability and that each population of synapses, though different in
numbers, was likely activated with equal probability. Our subsequent analysis to
estimate the fraction of inward rectifying AMPARs relied on this assumption. A prior
study found two populations of synapses at P6 with different release probabilities linked
to different mechanisms of synaptic plasticity (53). This study utilized a minimal
stimulation protocol compared to our maximal stimulation protocol used to detect inward
rectification. Maximal stimulation was necessary in order to minimize errors associated
with the classification of synaptic failures as inward rectification. Thus, the incidence of
synapses with low release probability was potentially obscured by the protocol we
employed. The prior study did not find that synapses with lower release probability
contained higher conductance AMPARs (53), which might have suggested that these synapses contained inward rectifiers, even rarely, as found here (Fig. 6). If taken on its own, however, our estimate from analysis of quantal $\tau_{\text{decay}}$ at P5-7 suggesting 30% of synapses with inward rectifying AMPARs (Suppl Fig. 4) could also be consistent with a lower probability of release at these synapses.

AMPARs can have a markedly faster $\tau_{\text{decay}}$ at P5-7. While we have attributed this to the presence of inward rectifying AMPARs, this is potentially influenced by multiple factors (12), such as subunit flip/flop variants (54; 62), glutamate release/uptake effects (10; 42) on agonist-receptor desensitization/dissociation (80) and subunit composition (51). Previous studies demonstrated a uniform effect of cyclothiazide across the developmental ages studied here (73), suggesting that flip/flop variability and desensitization effects are minimally contributing at these ages which has been supported by expression studies (48). AMPAR-mediated EPSC CV $\tau_{\text{decay}}$ has not been previously examined during early development. A previous study at cerebellar inhibitory GABAergic synapses (50), showed an increased CV $\tau_{\text{decay}}$ (0.1 – 0.3) which was associated, through modeling studies, with fluctuations in the time-course of GABA in the synaptic space of greater than 55%. CV $\tau_{\text{decay}}$ measured here was substantially greater than this, supporting our conclusion that the variability was due to the simultaneous presence of functionally different receptor clusters and thus inter-synaptic variability. It is unlikely that glutamate concentration cleft dynamics are fluctuating on a trial-to-trial basis to a degree to explain our findings. In support of this, strontium is thought to stabilize synaptic vesicle fusion to favor full, versus partial, release (19). Correlation with inward rectification thus favors that faster kinetics are most likely due to inward
rectifying AMPAR subunits at the synapse at P5-7(51). While inward rectifiers were present at P5-7, they were never detected after P8 (Fig. 1B and C).

However, not all P5-7 neurons exhibited inward rectification, as some behaved like P8-18 neurons with non-rectifying (linear) AMPARs (Fig. 1C) and slower channel kinetics (Fig. 5C and D). AMPAR single-channel conductance ($\gamma$) was not significantly higher at the younger P5-7 ages (Fig. 6C), though, to support inward rectification at the younger ages. The variance of $\gamma$ at P5-7 was significantly higher than at P8-18 (Fig. 6C), consistent with the presence of inward rectifying AMPAR channels in some but not all of the P5-7 neurons. As noted, $CV_{\tau_{\text{decay}}}$ was also comparatively much higher at P5-7 (Fig. 5E) to support the wide variability of rectification at these ages both across neurons as well as synapses within a neuron. Quantal amplitude significantly increased from P5-7 compared to P8-18 (Fig. 3C and D). This was most consistent, overall, with the developmental increase in AMPAR number ($N^*P_o$) (Fig. 6B). This increase likely overshadowed any differences in $CV_Q$ that might have been expected due to the presence of different AMPAR subtypes. Taken together, while two very different AMPAR synapses exist at younger ages (P5-7), the number of active AMPARs that increase at the synapse up to P18 are less variable in their biophysical properties ($CV_{\tau_{\text{decay}}}$ and $\gamma$), slower, ($\tau_{\text{decay}}$) and are never inward rectifying (lower RI). Thus, the biophysical properties of increasing numbers of AMPARs across neurons at P8-18 are much more uniform across synapses compared to the younger P5-7 age group.

Inward rectification, because of its association with calcium permeability of AMPARs (7; 26; 38), and faster kinetics, suggests that younger synapses have uniquely
different electrical and chemical signaling properties compared to older synapses. AMPARs lacking GluA2 uniquely have faster kinetics (51) and greater relative calcium permeability/inward rectification (26; 38; 51). These properties matched the features of synapses seen only at P5-7. Therefore, we speculate that prior to P8, many synapses completely lack GluA2, but other synapses prior to P8 seem to contain GluA2; after P8, synapses generally contain GluA2. Immature synapses are thought to initially develop as “silent” synapses, containing relatively calcium permeable NMDA receptors (41).

AMPAR are acquired through calcium-mediated, activity-dependent signaling (31; 37; 45; 55). Our findings temporally link calcium-permeable AMPARs with the synaptic development of principal neurons in hippocampal CA1. However, our findings could be explained by synapse specific clustering of transmembrane AMPAR regulatory proteins (TARPs) to achieve a similar clustering of AMPAR function (39; 77).

**Developmental increase in size of AMPAR synapses**

Although the total number of activated receptors at the peak ($N^*P_o$) of quantal amplitude ($Q$) was greater to modulate a larger $Q$, it was not possible to determine which factor of $N^*P_o$ contributed to the increases in median amplitude with age over the developmental range tested (Fig.6). Either receptor numbers ($N$) and/or peak-open probability ($P_o$), as influenced by such factors as cleft glutamate concentration (42), could be changing with development. This is due to the nature of peak-scaled NSFA, in that receptor number ($N$) and peak-open probability ($P_o$) cannot be directly calculated and thus, cannot be separated. However, studies suggest that greater numbers of AMPARs
are inserted postsynaptically with developmental age (14; 55; 57) favoring that increases in Q were primarily mediated by increases in N.

It has been shown that channel conductance can depend on glutamate concentration (25; 68; 76), though under conditions in which desensitization is minimized. Glutamate concentrations in the synaptic cleft may increase during synaptogenesis (65), which would also cause an increase in the quantal amplitude (23) via increased $P_o$ (47) if AMPARs were not already saturated. Under our conditions of presumed mono-vesicular, full release in strontium (19), this would have to occur by either greater vesicular filling with transmitter, or alterations in cleft volume and geometry. If this were the case, then increases in cleft glutamate levels could also then lead to an increase in the number of glutamate molecules bound to AMPAR and thus influence and favor higher AMPAR conductance levels (9). Therefore, if our measurements of relatively stable $\gamma$ across development were an indication of stable cleft glutamate concentrations, this would suggest that $P_o$ did not change significantly during the developmental range tested.

Significance of distinctly different AMPAR synapses at very early postnatal ages

Developmental changes in AMPAR subunit composition (48; 57) have been demonstrated in the neocortex (40; 74) and CA3 hippocampus (34), but developmental regulation in which these subunits are segregated to different synapses has not been reported. Transient AMPAR subunit composition changes have been described with LTP (58), following hypoxia induced seizures (64), and with synaptic inactivity (78). Developmental changes in AMPAR subunits have been associated with dendritic branch-
point maturation in the spinal cord (35). It is assumed that these composition changes result in mixtures of AMPARs at synapses. Therefore and in contrast, the transient expression of different AMPAR at distinct synapses suggested here from P5-7 likely underlies an important developmental process of network maturation in CA1 hippocampus. Indeed, the expression mechanisms of synaptic plasticity have been reported to change around P5-7 (53). Further studies will be necessary in order to determine the exact impact at P5-7 of the faster, inward-rectifying AMPAR synapses that exist with the slower, non-rectifying, mature AMPAR synapses. We speculate in the developing hippocampus that the faster synapses are both GluA2-lacking and calcium permeable, leading to greater calcium accumulations and possibly more finely-tuned dynamic signaling, compared to synapses with mixtures of AMPARs. This could have a substantial impact on not only our understanding of synaptic development, but also disorders primarily affecting immature synapses (13).

Acknowledgements
This was supported by NIH NINDS NS056090 (TAB), T32 GM007635 (EAS), institutional support from the Department of Pediatrics and The Childrens Hospital Research Institute. Thanks to Drs. John Isaac, Paul Bernard and Katie Rennie for critical reading of this manuscript.
References


8. **Brill J and Huguenard JR.** Sequential changes in AMPA receptor targeting in

9. **Cathala L, Holderith NB, Nusser Z, DiGregorio DA and Cull-Candy SG.**
Changes in synaptic structure underlie the developmental speeding of AMPA receptor-

10. **Clements JD, Lester RAJ, Tong G, Jahr C and Westbrook GL.** The time

11. **Cohen-Cory S.** The developing synapse: construction and modulation of

12. **Conti F and Weinberg RJ.** Shaping excitation at glutamatergic synapses. *Trends

13. **Cornejo BJ, Mesches MH, Coultrap S, Browning MD and Benke TA.** A
single episode of neonatal seizures permanently alters glutamatergic synapses. *Ann

14. **Cottrell JR, Dube GR, Egles C and Liu G.** Distribution, density, and clustering
of functional glutamate receptors before and after synaptogenesis in hippocampal

15. **Craig AM, Blackstone CD, Huganir RL and Banker G.** The distribution of
glutamate receptors in cultured rat hippocampal neurons: postsynaptic clustering of

16. **Cull-Candy SG, Howe JR and Ogden DC.** Noise and single channels activated
by excitatory amino acids in rat cerebellar granule neurones. *J Physiol* 400: 189-222,


32. **Harris KM and Sultan P.** Variation in the number, location, and size of synaptic vesicles provides an anatomical basis for the nonuniform probability of release at hippocampal CA1 synapses. *Neuropharmacol* 34: 1387-1395, 1995.


57. Pickard L, Noel J, Henley JM, Collingridge GL and Molnar E. Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA


Figure 1. Inward rectifying AMPARs were only present in some P5-7 pyramidal neurons. A1. Inward rectifying EPSCs at P6 showed less outward current at +40 mV holding potential (11 traces averaged) compared to inward currents at -70 mV (10 traces averaged) resulting in a high RI. A2. Non-rectifying EPSCs at P6, with greater outward current at +40mV (10 traces averaged) and at +20mV (10 traces) had a lower RI. A3. Non-rectifying EPSCs at P15 with robust outward current at +40mV (35 traces averaged) and at +20mV (34 traces averaged). B. Mean RI at P8-18 (2.67 ± 0.153, n = 38) was significantly smaller than mean RI at P5-7 (5.39 ± 0.77, n = 38; p=0.005, Mann-Whitney Rank Sum). C. I-V plots averaged from P5-7 inward rectifiers (light grey triangles, n = 5, RI > 6.5) were significantly different from older P8-18 (black circles, n=9) (+40mV, **p=0.004; +20mV, *p=0.032 Kruskal-Wallis one-way ANOVA on ranks, Dunn’s post-hoc). The P5-7 inward rectifiers were also significantly different from their non-rectifying littermates (dark grey circles, n=7, RI < 2.9) (+40mV, **p=0.006; +20mV *p=0.032, Kruskal-Wallis one-way ANOVA on ranks, Dunn’s post-hoc). Linear P5-7 neurons were not statistically different at any holding potential, compared to the older P8-18 neurons (p>0.05 Kruskal-Wallis one-way ANOVA on ranks, p>0.05 Dunn’s Method).

Figure 2. Polyamine-mediated voltage-dependence of paired-pulse stimulation was restricted to P5-7. A. PPR at -70 mV at P5-7 (1.71 ± 0.18, n=14) compared to P8-18 (1.85 ± 0.18, n = 18, p = 0.45, Mann-Whitney Rank-Sum) were similar. B. At P5-7, there was no correlation of PPR at -70 mV with rectification index (solid line, r² = 0.009, 95% confidence intervals dashed lines). C. EPSCs evoked by paired-pulse stimulation at +40mV and -70mV were compared (top panels), in which (PPR at +40mV)/(PPR at -70mV) = the unblocking index. C1. Inward rectifying AMPARs (P6, RI = 6.81 shown) demonstrated unblocked current in the second pulse at + 40 mV (peak current after first pulse = 10.54pA, after second pulse = 65.23pA, 70ms interstimulus interval, compared to the -70mV peak current after first pulse = -79.54pA, after second pulse = -123.59pA; unblocking index = 3.98). The bottom panels show the normalized currents from the upper panels overlapped to reveal more unblock at +40mV (vs. -70mV) for inward rectifying AMPARs. C2. Non-rectifying AMPARs (P15, RI = 1.12 shown) demonstrated little unblock on second pulse at +40 mV, compared to C1 example (peak current after first pulse = 45.66pA, after second pulse = 60.92pA, 70ms interstimulus interval, compared to the -70mV peak current after first pulse = -53.80pA, after second pulse = -92.86pA; unblocking index = 0.77). D. The degree of polyamine unblock correlated with the rectification index of neurons at P5-7 (Methods equation 1a, n=18, r²=0.7, p<0.0001). This correlation predicted an unblocking ratio (F2/F3 = 0.34/0.57) of less than unity (0.6). Minimal unblock was seen at P8-18 (n = 14, F2 = 0.09, r² = 0.03, p = 0.54, line not shown) suggesting a much smaller unblocking ratio (F2/F3 = 0.09/0.57 = 0.16). E. Measurements of RI from (D) are replotted (Methods equation 1b) to demonstrate the relationship of RI to the fraction of inward rectifying AMPARs activated; a fraction of 1 is equivalent to the synaptic activation of only inward rectifying AMPARs. Measurements of RI are relatively insensitive to changes in the fraction of inward rectifying AMPARs.
rectifying AMPARs less than 0.5 (dotted line). Fractions greater than 0.75 correlate with RI greater than 6 (solid intersecting lines).

Figure 3. AMPAR-mediated aEPSCs in the presence of extracellular Sr\textsuperscript{2+}. A1, A2. Successive traces of AMPAR-mediated aEPSC (a,b,c,d) evoked following an electrical stimulus (blanked at *) in the presence of 8mM Sr\textsuperscript{2+}/0 added Ca\textsuperscript{2+}/1Mg\textsuperscript{2+} in an example CA1 pyramidal neuron (P13). Series resistance and capacitance were monitored by the current response to the -2 mV voltage step. B, AMPAR-mediated aEPSCs (a,b,c,d from A) were distinguished from noise ($\sigma_n = 2.77$ pA, $\sigma_n^2 = 7.67$ pA\textsuperscript{2}) and fitted with 2 exponentials (red, superimposed, see Methods); Ba: $\tau_{\text{rise}} = 2.1$ ms $\tau_{\text{decay}} = 8.5$ ms, Bb: $\tau_{\text{rise}} = 2.1$ ms $\tau_{\text{decay}} = 9.2$ ms, Bc: $\tau_{\text{rise}} = 1.5$ ms $\tau_{\text{decay}} = 10.2$ ms, Bd: $\tau_{\text{rise}} = 3.1$ ms $\tau_{\text{decay}} = 5.8$ ms. C. aEPSC amplitudes remained stable for the duration of data acquisition (data from neuron in A, B shown).

Figure 4. AMPAR-mediated aEPSC quantal amplitudes (Q) across development.

A1. Probability density of aEPSC amplitudes (Q) from an individual neuron at P5 showed a wide CV\textsubscript{Q} (0.68) and non-normal, discontinuous distribution of amplitudes (bin width 1 pA). Darker vertical bars centered at zero represent the noise ($\sigma_n = 1.39$ pA). B1. Probability density of Q from an individual neuron, at P17 separated from noise ($\sigma_n = 1.59$ pA), exhibited a smaller CV\textsubscript{Q} (0.26) compared to A1. A2, B2. Median Qs (dotted line at 0.5) were obtained from cumulative distributions of Q for each neuron at P5 and P17, corresponding to probability densities shown at left for each neuron in A1 and B1, respectively. C. Cumulative probability distributions of Q at P5-7 (cf. A2) were group-averaged and compared to group-averaged distributions at P8-18 (cf. B2). The average distribution of Q was significantly different (**p=.0026, K-S test) for all P5-7 (grey circles, n = 15) neurons compared to all P8-18 (black circles, n=23). D. Median Qs obtained from each individual neuron (cf. A2 and B2) increased during development and were significantly higher for all P8-18 neurons (black circles) (mean of median Qs (dark line) = 11.44 ± 0.64 (n = 23)) compared to all neurons at ages P5-7 (grey circles) (mean of median Qs (dark line) = 8.22±0.56 (n = 15), **p<0.001 Mann-Whitney Rank Sum test). E. CV\textsubscript{Q} was not significantly different between the two age groups (P5-7, grey circles: mean of CV\textsubscript{Q} (dark line) = 0.46 ± 0.065 (n=15), P8-18, black circles: mean of CV\textsubscript{Q} (dark line) = 0.386 ± 0.031 (n=23), p=0.385, Mann-Whitney Rank Sum).

Figure 5. Quantal AMPAR-mediated aEPSC kinetics during early development.

A1. Probability density of quantal aEPSC decay kinetics ($\tau_{\text{decay}}$) from a representative neuron at P7 showed a wide CV\textsubscript{\texttau_{\text{decay}}} (1.53) compared to a representative neuron at P11, (B1), CV\textsubscript{\texttau_{\text{decay}}} = 0.36) (bin width 0.5 ms). A2, B2. Median quantal $\tau_{\text{decay}}$ is indicated by the dotted line from cumulative probability distributions of quantal $\tau_{\text{decay}}$ for the individual neurons in A1, B1, respectively. C. Cumulative probability distributions of quantal $\tau_{\text{decay}}$ at P5-7 (cf. A2) were group-averaged and compared to group-averaged distributions at P8-18 (cf. B2). Averaged cumulative probability distributions for all P5-7 (grey circles, n=15) were significantly different (***p=0.0022 K-S test) from all P8-18, (black circles, n=23) (horizontal error bars may be smaller than symbols). D. Median quantal $\tau_{\text{decay}}$s were significantly faster for grouped P5-7 (grey circles, mean of median $\tau_{\text{decay}}$s (dark line) =3.48 ± 0.467 (n = 15)) compared to all grouped P8-18 (black circles,
mean of median $\tau_{\text{decay}}$s (dark line) = 7.03 ± 0.22 (n = 23), **p<0.001, Mann-Whitney Rank Sum test). Median quantal $\tau_{\text{decay}}$s were segregated into two groups at P5-7 (Hartigan’s Dip test, D = 0.097, modal break point (dotted line) = 2.6 ms, n = 15, p < 0.1). E. The distribution of aEPSC quantal $\tau_{\text{decay}}$ from each neuron at P5-7 (grey circles) had a significantly higher CV$_{\text{decay}}$ (mean CV$_{\text{decay}}$ (dark line) = 0.876 ± 0.099, n = 15) compared to neurons at P8-18 (black circles) (mean CV$_{\text{decay}}$ (dark line) = 0.57 ± 0.33, n = 23, *p=0.023, Mann-Whitney Rank Sum).

Figure 6. AMPAR Single-channel conductance ($\gamma$) did not change significantly during early postnatal development (P5-18). A1. Typical AMPA-mediated aEPSCs (arrows) subjected to peak-scaled NSFA at P6 compared to P12. (A2) that follow the blanked stimulus artifact at *. B1,B2. NSFA resulted in near-parabolic relationships between current variance ($\sigma^2$) and associated mean current (I) for the selected ensemble of aEPSCs meeting criteria from each of the individual neurons in A1 and A2, respectively. Single-channel current (i) was calculated from the initial slope of the parabola and was then used to calculate single-channel conductance (B1: $R^2_{\text{sp}} = 0.92$, p ≤ 0.0001; B2: $R^2_{\text{sp}} = 0.99$, p ≤ 0.0001). C, $\gamma$ values when grouped by age were not significantly different between the two groups (P5-7: 9.70 ± 3.48 pS (n=6), P8-18: 7.52 ± 0.38 pS (n = 15); p=0.76, Mann-Whitney Rank Sum) but more variable at P5-7 (Levene’s F=10.59, p < 0.0042). D. $Q_{\text{calc}}$ was not correlated with $\gamma$ (linear regression: n = 21, $r^2 = 2.86$) suggesting $\gamma$ did not directly modulate Q.

Figure 7. AMPAR N*P$_o$ increased to support a greater median Q per neuron. A. N*P$_o$ across early development was not clearly correlated with age and most likely adjusted with $\gamma$ in order to yield Q. B. N*P$_o$ directly correlated with amplitude (Q) (n=21, $r^2=0.74$, p<0.001).

Supplemental Figure 1: AMPAR-mediated Q, $\tau_{\text{rise}}$, and $\tau_{\text{decay}}$ were independent of series resistance. A. Median Qs did not correlate with series resistance (n=38). B. Median $\tau_{\text{rise}}$s were unaffected by series resistances recorded (n=38). C. Median $\tau_{\text{decay}}$s were unaffected by series resistances recorded (n=38).

Supplemental Figure 2: Only AMPAR-mediated $\tau_{\text{rise}}$ was affected by filtering. A. Cell capacitance, an estimate of cell size, increased steadily during the developmental window examined from P5-18 (n=38). B. Similar to cell capacitance, median $\tau_{\text{rise}}$s steadily increased with development (n=38), suggesting that these parameters were related. C. Scatter plot of median $\tau_{\text{decay}}$s across all developmental time points P5-P18 also showed an opposing relationship compared to age and, therefore, cell capacitance, suggesting that median $\tau_{\text{decay}}$s were not influenced by cell capacitance and likely filtering in the same way as median $\tau_{\text{rise}}$s.

Supplemental Figure 3. Correlation of quantal amplitudes (Q) and quantal $\tau_{\text{decay}}$ for individual, pooled events at P5-7 and P8-18. Quantal $\tau_{\text{decay}}$ and Q (732 events) pooled from neurons (n = 15) at P5-7 were plotted (grey circles) and compared to P8-18 (1985 events, n = 23 neurons, black circles) to determine whether these two parameters were related. Q and quantal $\tau_{\text{decay}}$ did not appear correlated at either age based on
regression lines for events at P5-7 (grey line, \( r^2 = 0.0027 \)) and P8-18 (black line, \( r^2 = 0.0015 \)).

Supplemental Figure 4. Modeling sources of variability of CV \( \tau_{\text{decay}} \) to demonstrate that distinct synapses with different kinetics best describe experimental distributions of quantal \( \tau_{\text{decay}} \). AMPARs were modeled with a detailed macroscopic kinetic scheme (67). Rate constants and channel conductances for AMPARs were published except the closing rate (\( \alpha \)) was adjusted to give different rates for mean quantal \( \tau_{\text{decay}} \). In order to simulate the variability of peak glutamate concentration in the synaptic cleft, it was assumed that glutamate was released from vesicles containing 80 mM glutamate with a mean radius of 17nm with a standard deviation of 7.5 nm (which is three times reported values (32) in order to maximize variability) into a synaptic cleft volume of 9 aL. The resulting exponential distribution of glutamate cleft concentrations had a mean of 0.3 mM. Stochastic AMPARs, simulated with the Monte Carlo technique as done previously (2), when activated by a glutamate pulse with a peak time of 2 \( \mu \)s and decay time of 1 ms, resulted in an average response \( \tau_{\text{decay}} \) of 6.1 ms with \( \alpha = 300 \text{s}^{-1} \) and average response \( \tau_{\text{decay}} \) of 2.1 ms with \( \alpha = 900 \text{s}^{-1} \). Total AMPAR numbers were fixed at 25 to result in mean simulated Q (-12 pA at -70 mV) similar to experimental results. Random noise (2 pA rms) was added to ensembles of 250-1000 modeled synaptic AMPAR responses; filtering (Gaussian low-pass of 2 kHz) did not significantly alter results. Modeled CVQ (0.37) was similar to that found experimentally. Empiric Gaussian distributions from non-binned event data were fitted with a maximum likelihood estimator via a simplex method(72). Five different scenarios were tested in order to investigate the source of variability of quantal \( \tau_{\text{decay}} \). Responses were analyzed in a similar fashion to experimental data. A. In the first scenario, all 25 AMPARs were given \( \alpha = 900 \text{s}^{-1} \). This resulted in a CV \( \tau_{\text{decay}} \) of 0.42 and a median quantal \( \tau_{\text{decay}} \) of 2.0 ms (250 trials). B. In the second scenario, all 25 AMPARs were given \( \alpha = 300 \text{s}^{-1} \). This resulted in a CV \( \tau_{\text{decay}} \) of 0.44 and a median quantal \( \tau_{\text{decay}} \) of 6.1 ms (250 trials). C. In the third scenario, 13 AMPARs had \( \alpha = 900 \text{s}^{-1} \) and 12 AMPARs had \( \alpha = 300 \text{s}^{-1} \). This resulted in a CV \( \tau_{\text{decay}} \) of 0.40, a median quantal \( \tau_{\text{decay}} \) of 4.5 ms and no nadir of separation seen (250 trials). D. In the fourth scenario, AMPAR were randomized with a uniform probability distribution to have either \( \alpha = 900 \text{s}^{-1} \) or \( \alpha = 300 \text{s}^{-1} \). As expected, this resulted in a median \( \tau_{\text{decay}} \) between the distributions in (A) and (B) (3.6 ms) and did not show peaks or nadirs; however, CV \( \tau_{\text{decay}} \) was increased to 0.55 (1000 trials). E. In the final scenario, the responses with \( \alpha = 900 \text{s}^{-1} \) (A) and \( \alpha = 300 \text{s}^{-1} \) (B) were pooled to simulate two independent AMPAR synapses (i.e. each activated with equal probability) with different kinetics. Peaks and a nadir appeared in the distribution of quantal \( \tau_{\text{decay}} \) with median \( \tau_{\text{decay}} \) of 4.2 ms and CV \( \tau_{\text{decay}} \) of 0.69, similar to that seen experimentally at P5-7 (c.f. Fig. 5A). However, Hartigan’s Dip test did not identify significant multimodality (\( D = 018, p \approx 0.8 \), modal break (2.7 ms) indicated by dotted line), even though the distribution appeared multimodal and was generated from two distributions. Fitted Gaussian distributions (solid grey lines) segregated 51% faster events with mean of 2.1 ms (\( \sigma = 0.9 \text{ ms} \)) from 49% slower events with mean of 6.3 ms (\( \sigma = 2.5 \text{ ms} \)), consistent with equal contributions from the 2 independent synapses. F. Pooled quantal \( \tau_{\text{decay}} \) events from all neurons at P5-7 suggested 2 distributions but did not have a
significant Dip test ($D = 0.011$, 732 events, $p \approx 0.9$, modal break (2.9 ms) indicated by dotted line). Fitted Gaussian distributions (solid grey lines) segregated 33% faster events with mean of 2.1 ms ($\sigma = 0.8$ ms) from 67% slower events with mean of 7.5 ms ($\sigma = 4.2$ ms). If there are 2 synapse types, "faster" and "slower", with equal release probabilities (c.f. Fig. 2A,B), this suggest that approximately 30% of activated synapses at P5-7 are distinct and "faster".
**Fig. 1** Stubblefield, EA and Benke, TA

A1

P6 inward rectifying

50 pA

25 ms

A2

P6 non-rectifying

50 pA

25 ms

A3

P15

500 pA

25 ms

B

Rectification index

<table>
<thead>
<tr>
<th></th>
<th>all P5-7</th>
<th>P8-18</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

- P8-18
- P5-7 inward
- P5-7 linear

I normalized

mV
Fig. 3  Stubblefield, EA and Benke, TA

A1

A2

Ba

Bb

Bc

Bd

C

Amplitude (pA)

Minutes after break-in
**Fig. 4 Stubblefield, EA and Benke, TA**

A1

![Histogram of Q (pA)](image)

A2

![Cumulative Probability vs. Q (pA)](image)

B1

![Histogram of Q (pA)](image)

B2

![Cumulative Probability vs. Q (pA)](image)

C

![Cumulative Probability vs. Q (pA)](image)

D

![Median Qs vs. Q (pA)](image) **(P5-7 P8-18)**

E

![CVQ vs. Q (pA)](image) **(P5-7 P8-18)**
Fig. 5 Stubblefield, EA and Benke, TA
Fig. 6 Stubblefield, EA and Benke, TA

A1

A2

B1

B2

C

D

\[ I (\text{pA}) \]

\[ \sigma^2 (\text{pA}^2) \]

\[ \gamma (\text{pS}) \]

\[ Q_{\text{calc}} (\text{pA}) \]

\[ i = -0.38 \]

\[ \gamma = 5.4 \text{ pS} \]

\[ i = -0.602 \]

\[ \gamma = 8.6 \text{ pS} \]

P5-7  P8-18

P5-7  P8-18
Fig. 7 Stubblefield, EA and Benke, TA
Supplemental Figure 1; Stubblefield, EA and Benke, TA

A

Rseries (MΩ)

B

Median Qs (-pA)

C

Median τrise (ms)

Median τdecays (ms)