

Synaptic strength and postsynaptically silent synapses through advanced aging in rat hippocampal CA1 pyramidal neurons

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Abstract

Synaptic dysfunction is thought to contribute to age-related learning impairments. Detailed information regarding the presence of silent synapses and the strength of functional ones through advanced aging, however, is lacking. Here we used paired-pulse minimal stimulation techniques in CA1 stratum radiatum to determine whether the amplitude of spontaneous and evoked miniature excitatory postsynaptic currents (sEPSCs and eEPSCs, respectively) changes over the lifespan of rats in hippocampal CA1 pyramidal neurons, and whether silent synapses are present in adult and aged rats. The amplitudes of both sEPSCs and eEPSCs at resting membrane potential (i.e., clamped at -65 mV) initially increased between 2 weeks and 3 months, but then remained constant through 36 months of age. The potency of the eEPSCs at depolarized membrane potentials (i.e., clamped at $+40$ mV), however, was highest among 36-month old rats. Additionally, presynaptically silent synapses in CA1 stratum radiatum disappeared between 2 weeks and 3 months, but postsynaptically silent synapses were present through advanced aging. The similarity of silent and functional synapses in CA1 hippocampus at resting membrane potentials throughout adulthood in rats may indicate that impairments in the mechanisms of synaptic plasticity and its subsequent stabilization, rather than deficient synaptic transmission, underlie age-related cognitive decline. Such a notion is consistent with the increased amplitude of synaptic currents at depolarized potentials, perhaps suggesting an upregulation in the expression of synaptic NMDA receptors once rats reach advanced age.

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1. Introduction

Synaptic transmission is the primary means of communication between neurons and is thought to be targeted during normal aging. One of the most-studied regions of the brain with regard to synapses is the hippocampal CA1 region (Harris and Kater, 1994; Geinisman, 2000; Spruston and McBain, 2007). The detailed information regarding the functional and structural properties of synapses in this region have proven critical for guiding research aimed at identifying the cellular substrates of individual variability in aged animals (Toescu et al., 2004; Burke and Barnes, 2006; Disterhoft and Oh, 2006; Wilson et al., 2006; Foster, 2007). A major question is why some aged animals show severe impairments

in hippocampus-dependent behavioral tasks, whereas others the same age learn as well as young adults. Multiple processes probably collude to disrupt neuronal function (e.g., synaptic transmission) and plasticity (e.g., activity-dependent changes in synaptic strength or intrinsic excitability), but it is likely that age-related changes in one parameter have a feed-forward effect on other parameters.

For example, both intrinsic and synaptic plasticity in CA1 pyramidal neurons are impaired in aged rats (reviewed in Toescu et al., 2004; Burke and Barnes, 2006; Disterhoft and Oh, 2006; Wilson et al., 2006; Foster, 2007). These forms of plasticity depend on postsynaptic depolarization and could therefore be a consequence of reductions in synapse number or synapse strength. Indeed, previous work using electron microscopy has found evidence that some synapses may be weaker in aged rats with impaired hippocampus-dependent memory (Nicholson et al., 2004; but see Barnes et al., 1992),

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even though synapse number does not predict cognitive status (Geinisman et al., 2004; Calhoun et al., 2007). Therefore, the possibility remains that age-related changes in behavioral, synaptic and intrinsic plasticity derive from a dysregulation of cellular processes responsible for maintaining or altering synaptic strength.

One working model that could account for many of these findings posits that the dyshomeostasis of intracellular Ca^{2+} changes the rules that govern cellular plasticity (Khachaturian, 1987; Landfield, 1987; Toescu et al., 2004; Foster, 2007; Toescu and Verkhratsky, 2007). Though not traditionally considered a form of plasticity, the maintenance of synaptic strength is in fact an active process that involves Ca^{2+} -mediated cascades (Malinow and Malenka, 2002; Nicoll, 2003; Kennedy and Ehlers, 2006). Probing synapses at steady state could thus provide snapshots of the integrity of synapses throughout the lifespan of rats, and reveal crucial insight into what is and is not changing as animals transition from young adults with functional hippocampal neurons to aged animals with dysfunctional ones. For instance, determining the strength of individual synapses throughout the lifespan of rats could help clarify whether synapses are weakened throughout the aging process (e.g., Barnes et al., 1992; Hsia et al., 1998; Nicholson et al., 2004). If such evidence is found, one could posit that synaptic potentials in aged rats result in depolarizations that are unable to recruit the processes that support physiological forms of plasticity like long-term potentiation (LTP) or increases in neuronal excitability. Additionally, determining whether synapses that are especially sensitive to LTP-induction protocols are absent in very old animals is important, as such a loss would make it more difficult to induce plasticity at aged synapses.

All excitatory synapses on hippocampal CA1 pyramidal neurons contain NMDA receptors, but a subpopulation of these synapses lacks AMPA receptor immunoreactivity (Nusser et al., 1998; Petralia et al., 1999; Takumi et al., 1999; Racca et al., 2000; Ganeshina et al., 2004; Nicholson et al., 2006; Nicholson and Geinisman, *in press*). These electron microscopic findings corroborate previous descriptions of “postsynaptically silent” synapses that lack AMPA receptors, contain NMDA receptors, and are rendered functional only by the voltage-dependent removal of Mg^{2+} from their NMDA receptor channel pore (Liao et al., 1995; Isaac et al., 1995; Durand et al., 1996). These postsynaptically silent synapses, which are small and located on small thin spines (Takumi et al., 1999; Nicholson et al., 2006; Nicholson and Geinisman, *in press*), are thought to be especially sensitive to LTP-induction protocols (Kasai et al., 2003; Bourne and Harris, 2007). One possibility is that such synapses are absent or relatively infrequent in aged rats, which has the consequence of reducing the occurrence of plasticity by virtue of the unavailability of postsynaptically silent synapses. Importantly, there also exist “presynaptically silent” synapses whose transmission failures derive from the absence of neurotransmitter release, or from the release of neurotransmitter in an amount that is sufficient to activate only high-affinity NMDA recep-

tors (Kullmann and Asztely, 1998; Gasparini et al., 2000; Voronin et al., 2004; Voronin and Cherubini, 2004).

Previous studies have postulated that postsynaptically silent synapses may be involved in activity-dependent synaptic plasticity, such as that involved in the early development of neuronal microcircuits (Durand et al., 1996; Isaac et al., 1997; Petralia et al., 1999). This notion is consistent with studies in infant rat tissue (<3 weeks old) showing that LTP converts postsynaptically silent synapses into functional ones via the insertion of AMPA receptors into their postsynaptic membrane (Liao et al., 1995; Isaac et al., 1995; Durand et al., 1996; Malinow and Malenka, 2002; Nicoll, 2003). If silent synapses in the hippocampus are present primarily during early development, they should disappear or decrease in frequency with age. If, however, silent synapses are a constant substrate upon which mechanisms of activity-dependent plasticity act (Malinow and Malenka, 2002; Nicoll, 2003), then they should mirror the persistence of LTP and other forms of synaptic plasticity in the hippocampus throughout life in rats (Burke and Barnes, 2006; Disterhoft and Oh, 2006; Gallagher et al., 2006; Foster, 2007).

Here we combine whole-cell patch-clamp recordings from CA1 pyramidal neurons with paired-pulse minimal stimulation techniques (McNaughton et al., 1981; Dumas and Foster, 1995; Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998) and show that postsynaptically silent synapses and the strength of functional ones at resting membrane potentials persist through life in rats, even in rats too old to learn hippocampus-dependent tasks. However, when synapse strength is probed at depolarized potentials (+40 mV), synapses in the oldest rats are strongest. Such voltage-dependent differences in synaptic strength may indicate that advanced aging is associated with an increased expression of synaptic NMDA receptors, which may increase intracellular Ca^{2+} , and possibly exacerbate Ca^{2+} dyshomeostasis following synaptic activation that is strong enough to remove the Mg^{2+} -blockade of NMDA receptor channel pores.

2. Methods

All experiments were performed in accordance with the animal care and handling guidelines set forth by Northwestern University.

2.1. Slice preparation and recordings

Transverse hippocampal slices (300 μm) were prepared in ice-cold oxygenated artificial cerebral spinal fluid (aCSF) from 10 to 16 day, 3 month, 16 month and 32–36 month old Fisher 344 \times BN F1 rats. Slices were quickly transferred to an oxygenated holding chamber and incubated for 25 min at 35 °C, after which they were held at room temperature until recordings were made. The recording chamber was continuously superfused with solution heated to 32–34 °C and saturated with 95% O_2 /5% CO_2 . The standard extra-

cellular aCSF solution contained 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4 , 2.0 mM CaCl_2 , 1.3 mM MgCl_2 , 26 mM NaHCO_3 , 10 mM glucose. Picrotoxin (0.1 mM) was added to suppress GABAergic synaptic inhibitory currents during recording. The concentration of MgCl_2 was raised to 2.0 mM and that of CaCl_2 decreased to 1.3 mM in the slicing solution. Experiment 2 used bath application of the NMDA receptor antagonist AP5 at 0.05 mM.

Somatic whole-cell recordings were obtained from CA1 pyramidal neurons under visual guidance using infrared differential interference contrast microscopy. Internal solution for the patch pipettes contained: Cs methane sulphonate 120 mM, CsCl 10 mM, NaCl 5 mM, HEPES 10 mM, EGTA 0.2 mM or 10 mM, TEA-Cl 5 mM, Mg-ATP 4 mM, GTP 0.3 mM, QX-314 5 mM, pH 7.3–7.4 adjusted with CsOH, osmolality 290 ± 10 mOsm.

After achieving whole-cell configuration, activation of unitary ‘minimal’ EPSCs in CA1 pyramidal cells was achieved with a stimulus intensity just above the EPSC threshold, which produced detectable postsynaptic responses that alternated with failures. Evoked EPSCs (eEPSCs) were considered to be minimal if they appeared abruptly in response to gradually increasing stimulus intensity and alternated with transmission failures. Synaptic transmission was elicited with a 20 μs pulse at 0.0625 Hz using a monopolar glass electrode filled with external solution and positioned in stratum radiatum $\sim 100 \mu\text{m}$ away from the cell being recorded. Stimulation at this low frequency is necessary to avoid frequency-dependent synaptic depression (Saviane et al., 2002; Voronin and Cherubini, 2004).

The experiments in the present study utilized a paired-pulse facilitation (PPF) paradigm, consisting of two stimuli with the same intensity delivered at a 50 ms interval at a holding potential of -65 mV and $+40$ mV. We used the percentage of successful transmission events in response to the paired-pulses as an index of the probability of neurotransmitter release (P_T). To address whether age-related differences are present in the voltage-dependence of PPF, we calculated the P_T ratio as the P_T to the second stimulus in the pair divided by the P_T to the first stimulus at both -65 mV and $+40$ mV. Analyses of spontaneous EPSCs (sEPSCs) were conducted on a 900 ms epoch from trial records at -65 mV from Experiment 1 during and after stimulus calibration trials (a 200 ms baseline, and the 700 ms epoch starting 50 ms after the second pulse of the paired-pulse). Experiment 1 was performed on 56 CA1 pyramidal neurons (infant: 14 neurons from 5 rats; young: 13 neurons from 7 rats; adult: 15 neurons from 8 rats; aged: 14 neurons from 9 rats). Experiment 2 was performed on 26 CA1 pyramidal neurons (infant: 8 neurons from 3 rats; young: 4 neurons from 2 rats; adult: 9 neurons from 4 rats; aged: 5 neurons from 2 rats).

Experiment 1 examined both eEPSC amplitude and response potency to control for possible changes in P_T throughout life in rats. eEPSC amplitude was measured as the peak current amplitude within 7 ms of each pulse on all trials. Response potency was determined as the peak current ampli-

tude within 7 ms of each pulse but only on visually confirmed successful transmission trials. Though minimal stimulation can activate single synapses, some axons near the stimulating electrode may have similar thresholds of activation such that our minimal stimuli excite more than one axon. This notion was explored for each cell by dividing the potency in response to the first pulse by the potency in response to the second pulse (i.e., response potency ratio). If our minimal stimulation was activating the same single synapse, then the response potency ratio should be near 1 (Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998). We considered recordings with response potency ratios within 20% of unity to be from the same single synapse (range: 0.87–1.18). Because this latter group of recordings could reasonably be argued to derive from single-synapse recordings, we analyzed these records separately for age-related trends.

Voltage-clamp recordings were performed using an Axopatch-200B amplifier (Axon Instruments), and series resistance was monitored continuously. The recorded signal was low-pass filtered at 5 kHz, digitized at 10 kHz with a PCI-MIO-16E-4 board (National Instruments). All data were stored on a PC computer with custom software using C++ Builder 5.0 (Borland) and a NI DAQ 6.5 driver (National Instruments). Transmission successes and failures were assayed visually, and post-recording analyses were done using Clampfit 9 (Axon Instruments), MiniAnalysis 6.0.3 (Synaptosoft, Inc.), or custom-written software. All statistical values were evaluated with Statistica (StatSoft). Values are presented as mean \pm S.E.M. Statistical differences were established at $P \leq 0.05$ using one-way and factorial ANOVA. Fisher's least significant difference (LSD) was used to establish post hoc differences at $P \leq 0.05$.

3. Results

We used the method of minimal stimulation to probe synaptic transmission at single synapses (McNaughton et al., 1981; Dumas and Foster, 1995; Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998) on CA1 pyramidal neurons in infant (2–3 weeks), young (3 months old), adult (16 months old), and aged (32–36 months old) rats. In Experiment 1, stimulus intensity was gradually lowered until successful transmission events in response to the first pulse of the paired-pulse were interspersed with transmission failures at resting membrane potential (-65 mV). In Experiment 2, stimulus intensity was lowered until the first pulse failed to evoke an EPSC on all trials.

3.1. Experiment 1

3.1.1. Spontaneous EPSC amplitude

Over two thousand events from each age group were analyzed (infant: 2120; young: 2720; adult: 2446; and aged: 3218). χ^2 analyses revealed that the distribution of sEPSC

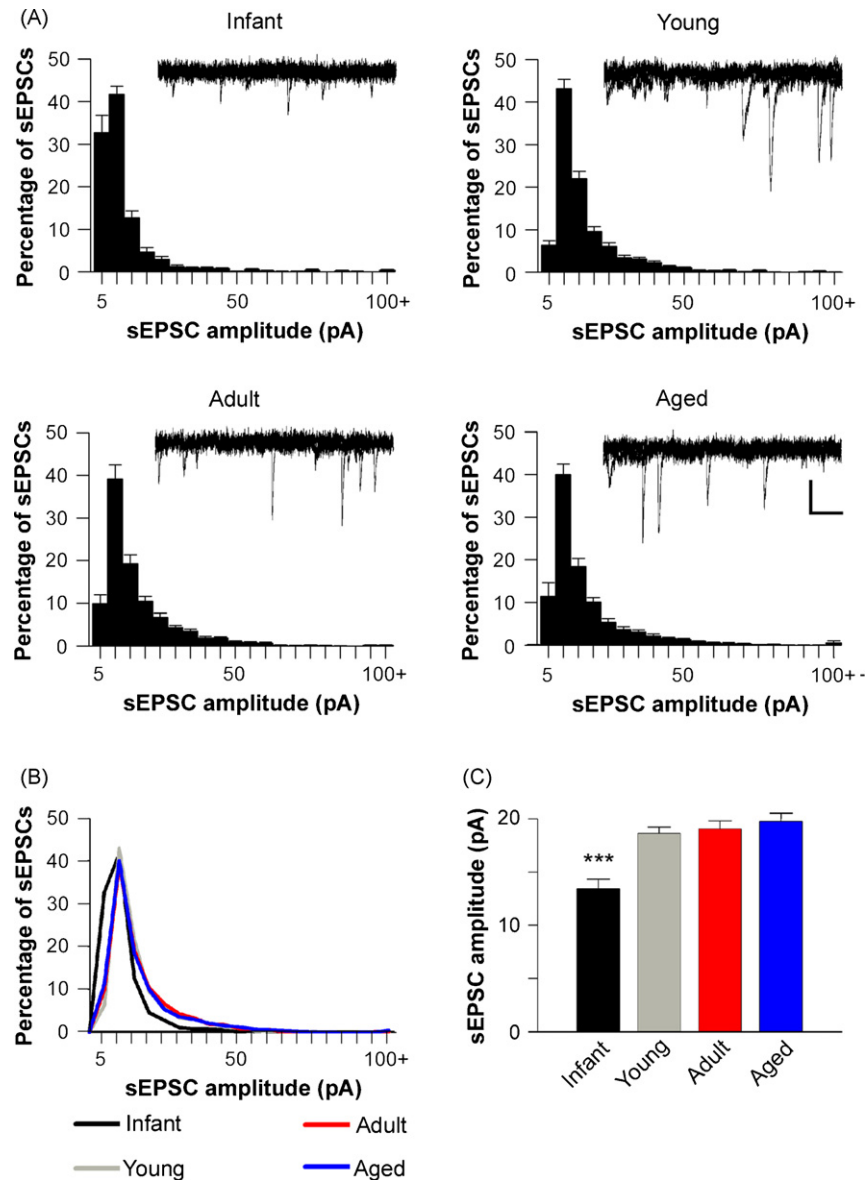


Fig. 1. The amplitude of spontaneous EPSCs through advanced aging in rat CA1 pyramidal neurons. (A) Percentage of spontaneous EPSCs (sEPSCs) with a given amplitude (pA) for the four age groups. Accompanying each histogram are five sweeps of raw data. Scale bar = 2 pA; 100 ms. (B) Line plots of the histograms superimposed. (C) Average sEPSC amplitude (\pm S.E.M.). Asterisks indicate that sEPSC amplitude from infant tissue was lower than all other ages. (B and C) infant: black; young: grey; adult: red; aged: blue.

amplitudes from infant tissue differed significantly from the other ages (versus young: $\chi^2 = 122.864$; adult: $\chi^2 = 67.194$; and aged $\chi^2 = 52.592$, all $df = 10$), but that the distributions from young, adult, and aged rats did not differ from each other (Fig. 1A and B). Similarly, the mean sEPSC amplitude increased initially between infant and young ages, but then remained constant through advanced aging ($F_{(3,52)} = 9.855$; Fig. 1C). Neither the decay time constant (infant: $5.05 \text{ ms} \pm 0.089$ (S.E.M.); young: $4.83 \text{ ms} \pm 0.48$; adult: $4.45 \text{ ms} \pm 0.33$; aged: $4.11 \text{ ms} \pm 0.49$) nor the 10–90% rise time (infant: $2.48 \text{ ms} \pm 0.09$; young: $2.35 \text{ ms} \pm 0.06$; adult: $2.31 \text{ ms} \pm 0.11$; aged: $2.17 \text{ ms} \pm 0.33$) of the individual events differed among the groups.

3.1.2. Transmission failures

In Experiment 1, paired-pulse eEPSCs were recorded from synapses with a range of failure rates to the first pulse (5–80% failure rate) to determine whether aging was associated with changes in (1) the voltage-dependence of failure rate; (2) eEPSC amplitude and response potency (Stevens and Wang, 1995; Isaac et al., 1996); and (3) whether membrane potential affects P_r ratio, as would be expected for presynaptically silent synapses.

The eEPSCs on successful transmission trials closely resembled sEPSCs (Fig. 2A), suggesting that many of the transmission events were unitary. We constrained the failure rates analyses to the first pulse to avoid any possible con-

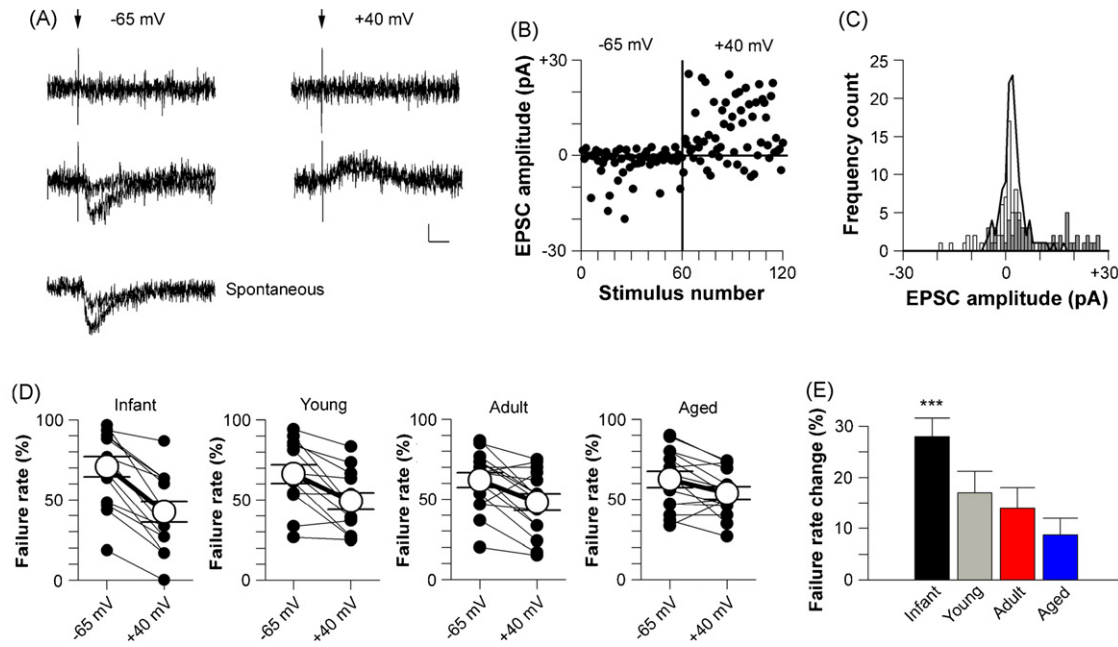


Fig. 2. Analysis of transmission failure rate after postsynaptic depolarization. (A) Left, two superimposed transmission failures (top) and successes (middle) at $V_m = -65$ mV. Lower traces are two spontaneous EPSCs aligned on the rising phase. Right, two superimposed transmission failures (top) and successes (bottom) at $V_m = +40$ mV. Arrows denote onset of stimulus pulse. Scale bar = 15 pA; 15 ms. (B) EPSC amplitude at $V_m = -65$ mV and $+40$ mV. Vertical line denotes change in V_m . (C) Distribution of EPSC amplitude at -65 mV (white bars) and $+40$ mV (grey bars), and the noise distribution (black line). (D) Transmission failure rate at holding potentials of -65 mV and $+40$ mV for neurons from infant, young, adult, and aged rats. Black symbols are data from individual experiments; white symbols represent the group mean (\pm S.E.M.). (E) Average failure rate change (\pm S.E.M.) after depolarizing holding potential to $+40$ mV. Asterisks indicate main effect of age, attributable to the failure change being highest among recordings from infant tissue (black). No other age differences were statistically significant. All statistical comparisons were at $P < 0.05$.

found of paired-pulse facilitation. At all ages, the number of transmission failures was lower at depolarized potentials ($+40$ mV), indicating the silent synapses persist throughout life in rats (Fig. 2B–E). This difference, however, was largest in recordings from infant rats (Fig. 2E). These results suggest that, regardless of whether or not our minimal stimuli were indeed repeatedly activating the same, single synapse, failure rate decreased when the membrane holding potential was depolarized. Such a pattern would be expected if our stimuli were activating synapses with only NMDA receptors, and were therefore silent at resting membrane potentials (i.e., -65 mV), but functional at depolarized potentials (i.e., $+40$ mV). Thus, our data are consistent with the hypothesis that silent synapses are present on rat hippocampal CA1 pyramidal neurons at all ages.

3.1.3. eEPSC amplitude

eEPSC amplitude was measured as the peak current amplitude within 7 ms of each stimulus pulse on all trials, whereas response potency was measured as the peak current amplitude in this same time window, but only on visually confirmed successful transmission trials (Stevens and Wang, 1995; Isaac et al., 1996). This approach allowed us to probe synaptic strength under conditions that are dependent on and independent of P_r (eEPSC amplitude and response potency, respectively; Stevens and Wang, 1995). Specifically, a synapse with a high P_r may have a large eEPSC

amplitude simply because successful transmission events are more common. Response potency, on the other hand, is not affected by P_r since it is only measured on trials that are visually confirmed to be successful transmission events. As a result, provided P_r is less than 100%, eEPSC amplitudes are always smaller than response potencies, a pattern we found at all ages and at both holding potentials (Fig. 3).

Our use of the paired-pulse minimal stimulation technique allowed us to calculate the response potency ratios of each recording as the potency of responses to the second pulse (eEPSC2) divided by the potency of the response to the first pulse (eEPSC1) of the paired-pulse (Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998). When individual events have potency ratios near 1, it is reasonable to attribute them to the minimal stimulation of the same single synapse (Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998). Using these ratios, we categorized our recordings into those which we could assume were from the same, single synapse (i.e., a response potency ratio below 1.2) and those for which we could not safely make that assumption (response potency ratio above 1.2). Additionally, we assessed both eEPSC amplitude and response potency at hyperpolarized (i.e., -65 mV) and depolarized (i.e., $+40$ mV) membrane potentials. Our presumption is that the synaptic responses at hyperpolarized potentials are predominantly generated by AMPA receptor-mediated conductances, whereas those

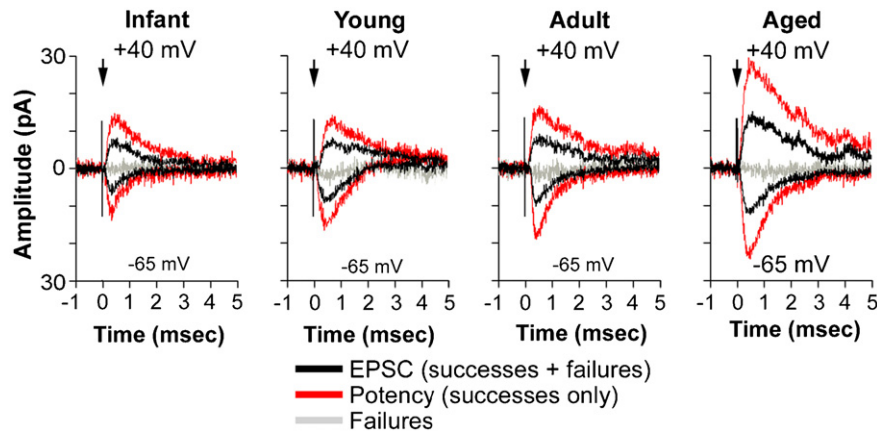


Fig. 3. Evoked EPSCs, transmission successes, and transmission failures in CA1 pyramidal neurons of rats of all ages at both hyperpolarized and depolarized potentials. In response to minimal stimulation (arrows), failures were interspersed with successful transmission events. After visual inspection of the records, transmission failures were identified and removed from the analysis, such that we could analyze response potency (i.e., the strength of responses on visually confirmed successful transmission trials). The end result of this is that failure trials show no average waveform (grey), and response potencies (red) are larger than the averaged evoked EPSC waveforms (black) at both hyperpolarized (-65 mV) and depolarized ($+40$ mV) holding potentials.

at depolarized potentials are mediated by both AMPA and NMDA receptor-mediated conductances.

eEPSC amplitudes in response to the first pulse are plotted as a function of the response potency ratio for the four age groups in Fig. 4A. An ANOVA revealed that eEPSC amplitude at resting membrane potentials increased between infancy and adulthood, but then remained stable through advanced aging ($F_{(3,52)} = 5.0266$; Fig. 4B.). There were no age-related changes in eEPSC amplitude at depolarized potentials between adult and aged rats (Fig. 4B). This same pattern was found when eEPSC amplitudes from only those recordings with response potency ratios below 1.2, and therefore the records of individual synapses, were examined ($F_{(3,18)} = 3.162$; Fig. 4C). Importantly, response potency ratios for these latter single-synapse records were nearly identical at hyperpolarized and depolarized holding potentials, supporting the claim that these records derive from single synapses. These data indicate that AMPA receptor mediated conductances change early in life, but then remain stable through advanced aging. Similarly, the eEPSC data indicate that NMDA receptor-mediated conductances, or at least those active at depolarized potentials, show little change over the lifespan of rats (see also Hsia et al., 1998).

3.1.4. Response potency

eEPSC amplitudes are vulnerable to the P_r of the axon making the synapse. Therefore, changes in P_r could either artificially produce age-related differences in synaptic strength, or mask them. For example, if all synapses had the same strength, but P_r increased with development, one would see an age-related increase in eEPSC amplitude. Such a pattern would, however, be attributable not to changes in synaptic strength, but primarily to increases in the probability that a synapse is activated and thus contributes more successes to the mean eEPSC amplitude values. Conversely,

an age-related decrease in synaptic strength might be masked by a higher P_r at adult and aged synapses. To avoid potential confounds originating from age-related changes in P_r , we analyzed response potency for all synapses at both hyperpolarized and depolarized membrane potentials. Additionally, we separately analyzed the data from those recordings with potency ratios near 1, which we presume were records of unitary events from the same single synapse (Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998).

When all synapses regardless of their response potency ratios were analyzed, response potency at hyperpolarized membrane potentials, similar to sEPSC amplitudes at rest (Fig. 1A–C), increased initially between infancy and young adulthood, but then remained stable through advanced aging ($F_{(3,52)} = 6.151$; Fig. 5A and B). Response potency at depolarized membrane potentials also increased early in life, but then showed stability through advanced age ($F_{(3,52)} = 2.95$; Fig. 5A and B). These data, particularly those at hyperpolarized membrane potentials, show that synaptic strength after infancy is constant through advanced aging, and are consistent with the analyses of sEPSCs (Fig. 1A–C) and eEPSC amplitudes (Fig. 4A–C). Note that the results from the potency analyses show unequivocally that synaptic strength at resting membrane potential, like sEPSC amplitude at resting membrane potential, does not change appreciably between 3 months and 36 months.

Indeed, the potencies in response to the first pulse were similar to the amplitude of the sEPSCs (compare Fig. 5B with Fig. 1C). A factorial ANOVA revealed that eEPSCs were smaller than spontaneous ones ($F_{(1,104)} = 47.237$), but that regardless of whether they were evoked or spontaneous, EPSCs from infant tissue were smaller than those from the older ages ($F_{(3,104)} = 13.640$). However, it is important to note that the response potencies were within the range of amplitudes for sEPSCs, so it is likely that many of our recordings were from single synapses.

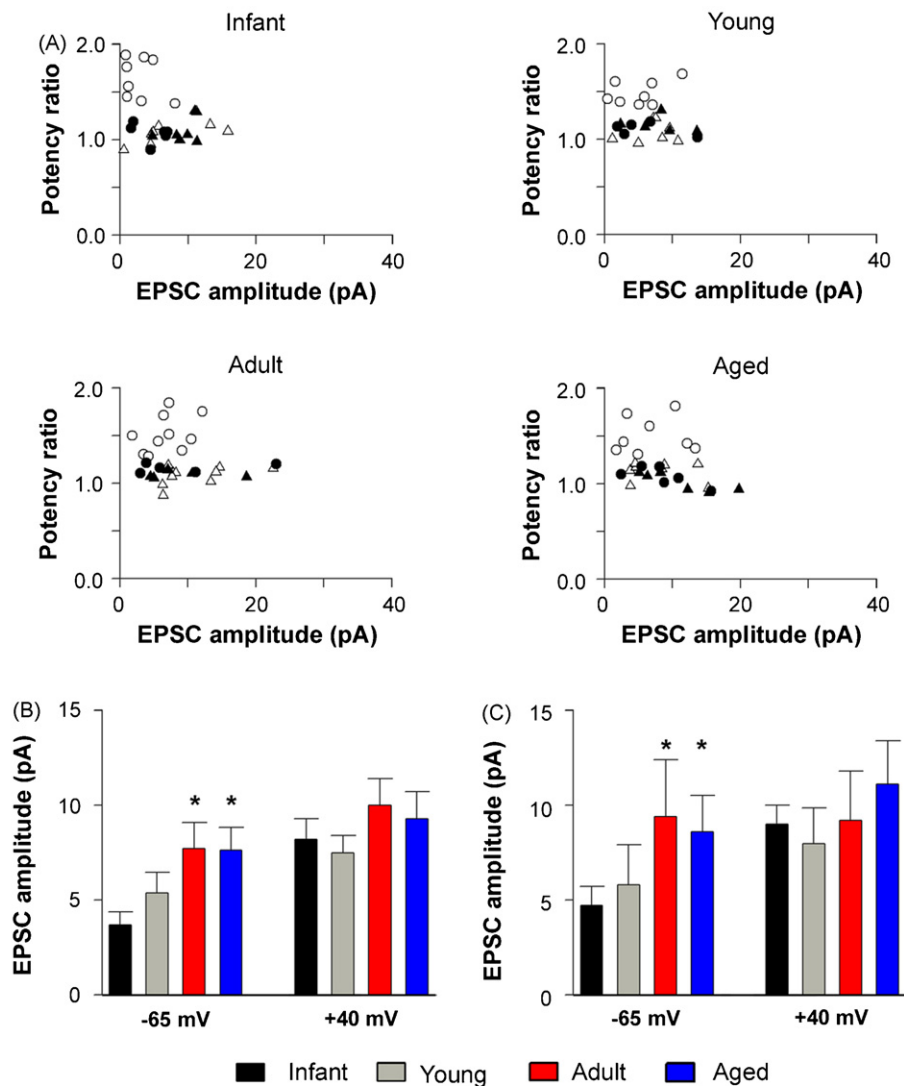


Fig. 4. Paired-pulse minimal stimulation analysis of evoked EPSC amplitude at hyperpolarized and depolarized membrane potentials. (A) EPSC amplitude plotted as a function of potency ratio for all recordings in the present study. Circles are the individual mean EPSC amplitudes at -65 mV; triangles are the individual mean EPSC amplitudes at $+40$ mV. Black objects are data from those records with potency ratios at -65 mV of <1.2 . White objects are all others. (B) EPSC amplitude for all recordings from infant (black), young (grey), adult (red), and aged (blue) neurons. EPSC amplitudes from the adult and aged groups were significantly higher than those from infant rats at -65 mV, but not at $+40$ mV (asterisks). (C) EPSC amplitude for those recordings with a potency ratio at -65 mV of <1.2 . The overall pattern is similar to that from all recordings: age-related increase in EPSC amplitude that stabilizes after 3 months of age.

To explore this notion more directly, we separately analyzed those records with potency ratios below 1.2, and were thus likely from the same single synapse. On average, our overall potency ratios were low (infant: 1.49 ± 0.11 ; young: 1.38 ± 0.06 ; adult: 1.39 ± 0.06 ; aged: 1.34 ± 0.07 ; n.s.), but single-synapse strength is most reasonably assayed in records whose potency ratio is near 1 (Stevens and Wang, 1995; Isaac et al., 1996; Hsia et al., 1998). Accordingly, we separately analyzed the data from synapses with potency ratios at resting membrane potentials near 1 (infant: $n=6$, mean = 1.07 ± 0.04 ; young: $n=5$, mean = 1.1 ± 0.05 ; adult: $n=5$, mean = 1.12 ± 0.02 ; aged: $n=6$, mean = 1.07 ± 0.04 ; potency ratios were nearly identical at depolarized membrane potentials). Consistent with the analysis of all synapses, response potency at hyperpolarized membrane potentials

increased between infant and young ages, and then remained constant through advanced aging ($F_{(3,18)} = 3.171$; Fig. 5A and C). Interestingly, when we analyzed the response potency of single synapses at depolarized membrane potentials, we found that the oldest rats had the highest potencies, whereas those from all other ages did not differ ($F_{(3,18)} = 4.330$; Fig. 5A and C).

Because the NMDA receptor channel pore is blocked at hyperpolarized membrane potentials, synaptic currents at -65 mV reflect primarily the AMPA receptor-mediated conductance, whereas those at $+40$ mV reflect both AMPA and NMDA receptor-mediated currents. Though we did not test this presumption directly with blockade of AMPA and/or NMDA receptors, the ratio of response potencies to the first pulse at these two membrane potentials here are almost iden-

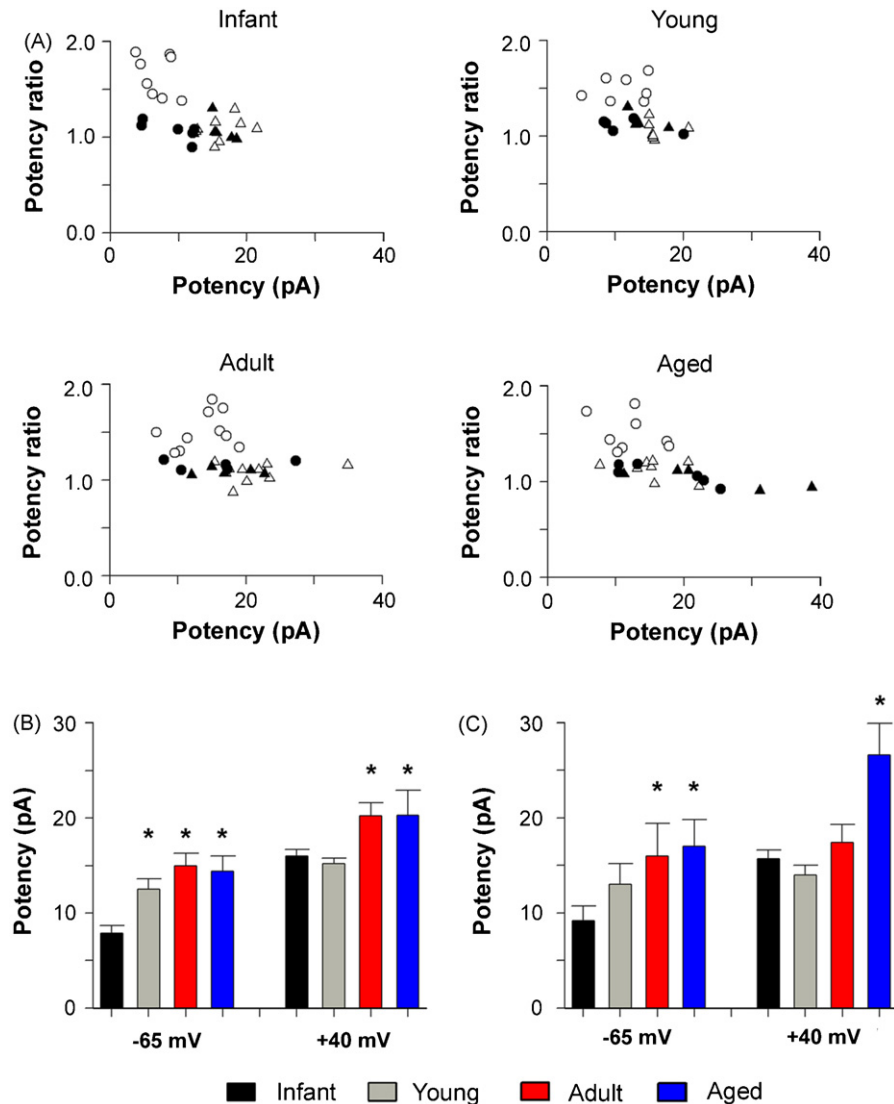


Fig. 5. Paired-pulse minimal stimulation analysis of evoked EPSC response potency at hyperpolarized and depolarized membrane potentials. (A) Response potency plotted as a function of potency ratio for all recordings in the present study. Circles are the individual mean response potencies at -65 mV; triangles are the individual mean response potencies at $+40$ mV. Black objects are data from those records with potency ratios at -65 mV of <1.2 . White objects are all others. (B) Response potencies for all recordings from infant (black), young (grey), adult (red), and aged (blue) neurons. Response potencies from the young, adult and aged groups were significantly higher than those from infant rats at -65 mV. Response potencies at $+40$ mV from adult and aged neurons were higher than the infant and young tissue (asterisks). (C) Response potency for those recordings with a potency ratio at -65 mV of <1.2 . The overall pattern is similar to that from all recordings: age-related increase in response potency at -65 mV that stabilizes after 3 months of age (asterisks). Additionally, only the single-synapse response from aged neurons showed an increase in response potency at $+40$ mV.

tical to the AMPA-to-NMDA ratio found in a previous study that pharmacologically dissected these two components (Hsia et al., 1998; infant: 0.49 ± 0.05 ; young: 0.74 ± 0.06 ; adult: 0.75 ± 0.07 ; and aged: 0.74 ± 0.05). Therefore, we feel it is reasonable to assume that the response potency at $+40$ mV reflects both the AMPA and NMDA receptor-mediated conductances. Consistent with the notion that NMDA receptors contribute more to synaptic conductances in aged rats, this ratio showed a trend toward age-dependent changes among the records from the single-synapse recordings, though these differences are not statistically significant ($F_{(3,18)} = 2.34$, $P = 0.1$; ratio of potency at -65 mV to the potency at $+40$ mV

from those records with response potency ratios below 1.2; infant: 0.58 ± 0.06 ; young: 0.84 ± 0.05 ; adult: 0.89 ± 0.07 ; and aged: 0.68 ± 0.04).

Additionally, the decay time of the responses at $+40$ mV, which would be expected to be larger in the recordings from aged rats if there was a larger NMDA receptor-mediated component (Spruston et al., 1995), showed a significant increase in aged rats (decay time in ms for the response to the first pulse from those recordings with potency ratios below 1.2; $F_{(3,18)} = 3.40$, $P < 0.05$; infant: 8.20 ± 1.58 ; young: 9.62 ± 1.46 ; adult: 9.65 ± 1.78 ; and aged: 16.60 ± 2.97). Though we did not pharmacologically dissect the AMPA and

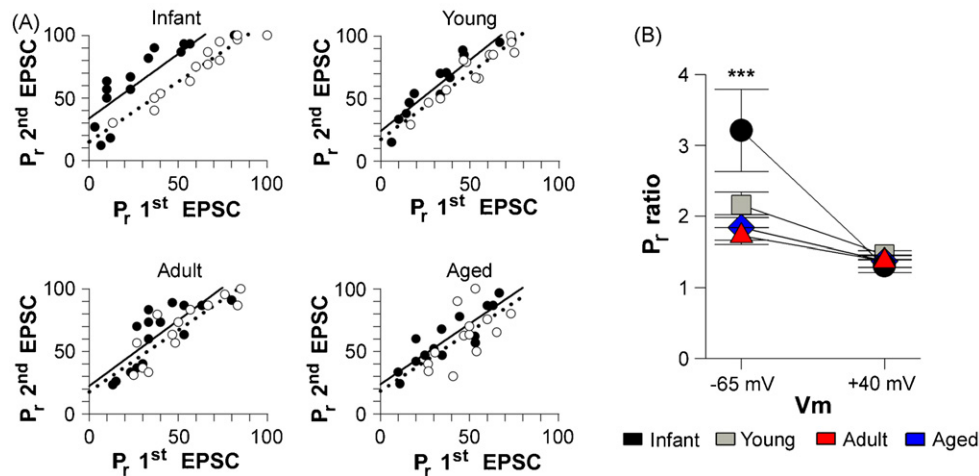


Fig. 6. Age-related change in the voltage-dependence of P_r . (A) Probability of neurotransmitter release to the second pulse of the paired-pulse as a function of the probability of neurotransmitter release to the first pulse at holding potential of -65 mV (black symbols) and $+40$ mV (white symbols) for recordings from infant, young, adult, and aged neurons. Lines are linear fits of the data at -65 mV (solid line) and $+40$ mV (dotted line). (B) P_r ratio for the different age groups at holding potentials of -65 mV (black circles) and $+40$ mV (white circles). Triple asterisks indicate that only the neurons from infant slices showed a voltage-dependent decrease in P_r ratio. Data in (B) are presented \pm S.E.M.

NMDA receptor-mediated components of the responses, both of these trends are consistent with our suggestion that the contribution of NMDA receptors to synaptic current is higher in aged rats than in younger ones, even though their presumed AMPA receptor-mediated conductances are similar (Fig. 5B and C at -65 mV; Barnes et al., 1992; but see Barnes et al., 1997).

3.1.5. Age-dependence and voltage-dependence of P_r

The paired-pulse protocol increases P_r to the second pulse, provided the interpulse interval exceeds ~ 10 ms (Stevens and Wang, 1995). Consequently, this protocol allowed us to examine the probability that a successful transmission event occurs in response to each pulse (i.e., P_r). For presynaptically silent synapses, P_r depends on the postsynaptic membrane potential (Gasparini et al., 2000; Voronin et al., 2004; Voronin and Cherubini, 2004). At -65 mV, P_r to the first pulse of the paired-pulse did not differ (infant: 0.301 ± 0.06 ; young: 0.362 ± 0.05 ; adult: 0.381 ± 0.05 ; aged: 0.376 ± 0.05). However, synapses from infant rats showed a decrease in their P_r ratio when the membrane potential was held at $+40$ mV, whereas the P_r ratio of synapses from the other age groups was not voltage-dependent ($F_{(3,52)} = 5.115$; Fig. 6A and B).

3.2. Experiment 2

3.2.1. Pre- and postsynaptically silent synapses

That the P_r ratio was voltage-dependent in infant tissue suggests that some of the silent synapses at this age may have been presynaptically silent, whereas all of the silent synapses from the other ages were postsynaptically silent. Using single stimulus pulses to examine silent synapses does not allow postsynaptically silent synapses to be distinguished unequivocally from presynaptically silent ones. This distinction is

particularly important because presynaptic neurotransmitter release can be enhanced by depolarizing the postsynaptic membrane potential (Gasparini et al., 2000; Voronin et al., 2004; Voronin and Cherubini, 2004). If a synapse is presynaptically silent, the increase in neurotransmitter release induced by postsynaptic depolarization may cause successful release of transmitter and the subsequent evoked EPSC at $+40$ mV. With single stimuli, the erroneous conclusion could be made that the EPSCs were caused by activation of NMDA receptor-only (and postsynaptically silent) synapses, rather than being caused by voltage-dependent increases in presynaptic neurotransmitter release.

In Experiment 2, we sought to determine whether presynaptically silent synapses do indeed disappear between infancy and young ages, even though postsynaptically silent synapses are present at all ages (Fig. 2). We stimulated synapses until we recorded clear eEPSCs to either pulse and then gradually lowered the stimulus intensity until we achieved 100% failures to the first pulse. Using this approach, we found two major subtypes of silent synapses. The first subtype is the presynaptically silent synapse, which showed 100% failures to the first stimulus at -65 mV, but occasional successful transmission events after the second stimulus (Fig. 7A). Depolarization enhanced P_r at these synapses, which was detected as a decrease in the number of failures to the first stimulus, and by a reduction in the P_r ratio (P_r to the second stimulus divided by P_r to the first stimulus; Fig. 7B). Importantly, the conductances at $+40$ mV were not blocked by bath application of the NMDA receptor antagonist AP5 (Fig. 7C and D), and thus were presumably mediated by AMPA receptors that were able to bind glutamate under conditions of enhanced P_r (summarized in Fig. 7D). Interestingly, these presynaptically silent synapses were found only in slices from younger rats (infant: 3/8 recordings; young:

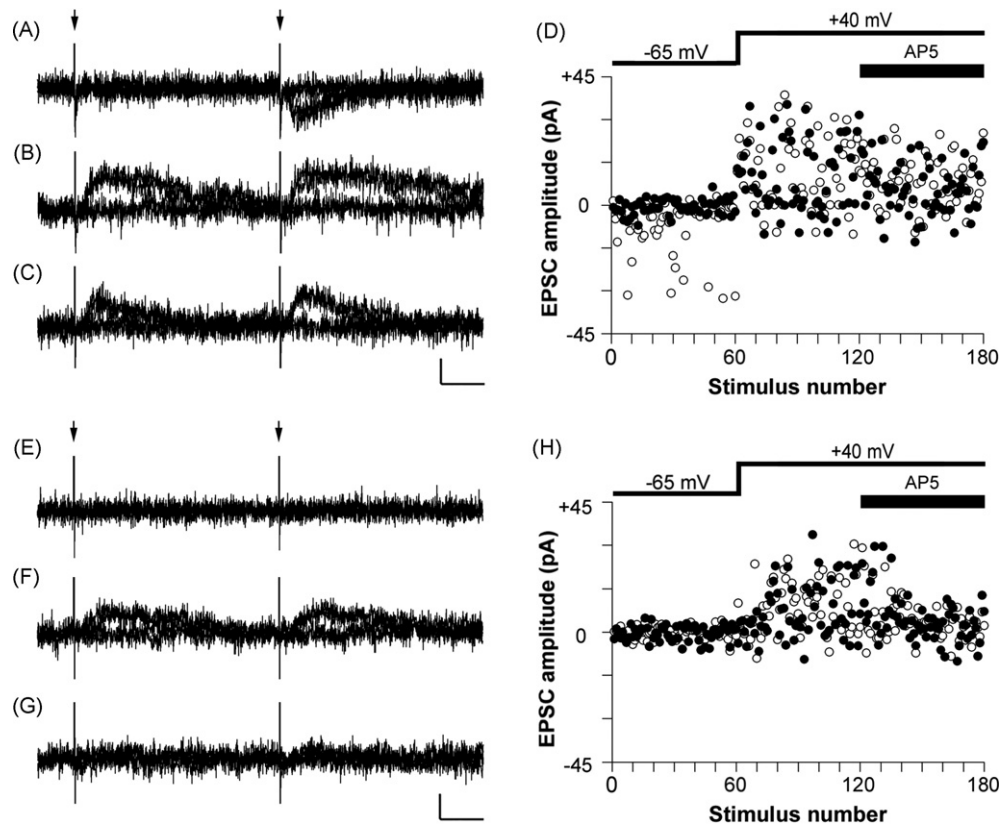


Fig. 7. Pre- and postsynaptically silent synapses on CA1 pyramidal neurons. (A–D) A presynaptically silent synapse, which is conductive only with paired-pulse stimulation (A) or at depolarized membrane potentials (B). Responses at depolarized holding potentials were not blocked by bath application of AP5 (C). Arrows in (A) denote onsets of each of the paired-pulses. (D) Summary of experiment showing amplitude of EPSCs in response to first (EPSC1; black symbols) and second (EPSC2; white symbols) stimuli of the paired-pulse at -65 mV, $+40$ mV, and $+40$ mV after bath application of AP5. (E–H) A postsynaptically silent synapse that showed 100% failures in response to both stimuli of the paired-pulse at resting membrane potential (E), but a substantial reduction in failure rate after membrane depolarization (F). Bath application of AP5 blocked both EPSC1 and EPSC2 at $+40$ mV (G). (H) Summary of experiment showing amplitude for EPSC1 (black symbols) and EPSC2 (white symbols) at -65 mV, $+40$ mV, and $+40$ mV after bath application of AP5. Scale bars in (C) and (G) are 20 pA and 20 ms. Each panel is comprised of two failures and two successes, except panel (E), which shows two failures. Panel (G) also shows two failures, but each trial in this experiment failed to evoke a response because of the blockade of NMDA receptors with AP5.

1/4; adult: 0/9; aged: 0/5). In other words, when the first pulse yielded 100% failures, the second one did so too but only in tissue from older rats.

The other silent synapse subtype showed a 100% failure rate to both stimuli of the paired-pulse at -65 mV (Fig. 7E), but generated responses to both stimuli at $+40$ mV (Fig. 7F). The successful events in response to the first and second pulses were both blocked by bath application of AP5 (Fig. 7G and H), indicating that these synapses contained only NMDA receptors and were thus postsynaptically silent at -65 mV (summarized in Fig. 7H). Such postsynaptically silent synapses were present in slices from infants (1/8 recordings) and older ages (young: 2/4 recordings; adult: 2/9; and aged: 1/5). The remaining recordings showed 100% failures at both holding potentials.

Taken together, the results of the present study indicate that synaptic strength initially increases after the end of infancy, but then stays remarkably constant between 3 months and 36 months of age. Though this strength is constant at resting membrane potentials, our data suggest that the NMDA receptor-mediated component of the synaptic conductance is

highest in 36-month old rats. Importantly, however, this pattern was only noticeable in the analyses of response potencies after substantial depolarization (i.e., to $+40$ mV) and only in those records that derived from the activation of the same single synapse. Additionally, the results of Experiment 2 indicate that presynaptically silent synapses disappear around the third month of life, whereas postsynaptically silent synapses are present through advanced aging in rats.

4. Discussion

The experiments in this study show that both presynaptic and postsynaptic parameters continue to develop throughout the first 3 months of life in rats, but then remain relatively constant throughout their lifespan. Furthermore, the age-related changes in the P_r ratio coupled with the age-dependent differences in eEPSC amplitude and potency indicates that the reliability and strength of information transfer at synapses in CA1 stratum radiatum are not fully mature until ~ 3 months, but then remain stable even in advanced age. Despite the

remarkable stability of synaptic parameters throughout life in rats, our analyses of response potencies and kinetics at depolarized holding potentials suggest that NMDA receptor-mediated synaptic transmission may be enhanced in very old rats.

4.1. Synaptic strength in hippocampal CA1

Our data show that the amplitude of sEPSCs, eEPSCs, and response potencies increase initially after infancy, but then remain stable through advanced age, even though all 36-month old rats show severely impaired hippocampus-dependent learning (Knuttinen et al., 2001). The absence of major changes in synapse strength after ~1 month of age is consistent with previous reports (Dumas and Foster, 1995; Hsia et al., 1998). Hsia et al. (1998) provided evidence that tetrodotoxin-sensitive (TTX) miniature EPSCs (mEPSCs) increased between infancy and young adulthood, whereas TTX-insensitive mEPSCs remained stable. These authors explained the age-related increase in the amplitude of TTX-sensitive mEPSCs as being produced by an initial increase after infancy in the number of synapses onto the recorded neuron that an individual presynaptic axon makes, followed by stability in synapse number. Our sEPSC amplitudes are similar to the amplitudes of their TTX-insensitive mEPSCs, and the stability in their amplitude after infancy is consistent with the lack of age-related synapse loss in CA1 stratum radiatum (Geinisman et al., 2004), though not necessarily consistent with the idea that that aged learning-impaired rats have weaker synapses than aged learning-unimpaired ones (Nicholson et al., 2004). The eEPSC data in the present study show nearly the same exact pattern, albeit with smaller amplitudes.

Importantly, presynaptic axons do occasionally make multiple contacts with the same postsynaptic neuron, though these types of connection are rare (Sorra and Harris, 1993; Yankova et al., 2001; Nicholson and Geinisman, in press). Therefore, we cannot be certain that any of our recordings with potency ratios significantly above 1 were from the same, single synapse. Rather, they could have been recording the activation of multiple synapses between the same axon and the recorded neuron; or they could be records from multiple synapses that are made from adjacent axons with similar activation thresholds. However, the overall pattern of results was similar to that found in the analyses of those records that are presumably from the same, single synapse (i.e., those with potency ratios near 1; Fig. 4A and C; Fig. 5A and C).

One unexpected finding was that response potency at depolarized membrane potentials was highest in the aged rats only for those records deriving from single synapses (Fig. 5A and C). A reasonable explanation for this is that the contribution of NMDA receptors to synaptic current is highest in aged rats, relative to their younger counterparts, but that this would only occur after synaptic activation/depolarization intense enough to remove the Mg^{2+} -blockade of the NMDA

receptor channel pore. This observation may appear to contrast with a previous report, which provided evidence that the NMDA receptor-mediated component of the field excitatory postsynaptic potential (EPSP) is decreased in behaviorally characterized aged rats (Barnes et al., 1997), but these authors were unable to assess the voltage-dependence of their observation. Additionally, the increased contribution of the NMDA receptors to synaptic current in our study was found only at a subset of synapses, which may have been masked by the field EPSP in the Barnes et al. (1997) study. To help clarify this issue, it will be necessary to pharmacologically isolate the different components of the eEPSCs, as has been done in younger rats (e.g., Hsia et al., 1998). Moreover, postembedding immunogold electron microscopy studies for AMPA and NMDA receptors in CA1 hippocampus of behaviorally characterized aged rats are ongoing in this laboratory.

Though the results of our study are consistent with the idea that synapse strength (at resting membrane potentials) and number remain remarkably constant through advanced age in rats, it is important to underscore that we used minimal stimulation techniques, so we do not know the approximate location of the activated synapses. In future studies, it will be necessary to activate synapses locally using focal application of high-osmolar solution (e.g., Magee and Cook, 2000) to determine whether synaptic strength in all regions of CA1 pyramidal neuron dendrites is preserved through advanced aging. Such an experiment is particularly important because perforated synapses are selectively reduced in size in aged rats with impaired spatial learning (Nicholson et al., 2004), and it is this synaptic subtype that increases in strength and number with distance from the soma in CA1 pyramidal neurons in young adult rats (Nicholson et al., 2006; Nicholson and Geinisman, in press).

It is also important to note that the recuperation of hippocampal tissue from slice-induced damage was not tested in the present study, and slicing-induced recuperative synaptogenesis (Kirov et al., 2004; Bourne et al., 2007) may have masked age-related changes in synaptic strength that might otherwise be detected in vivo or in perfusion fixed tissue. Though we did increase the concentration of $MgCl_2^+$ and decrease the concentration of $CaCl_2^+$ in our slicing solution, this is an important issue to address in future studies since the most motile spines are also those most likely to be bearing postsynaptically silent synapses (discussed in Nicholson and Geinisman, in press). The ranges of spine and synapse sizes from the Kirov et al. (2004) and Bourne et al. (2007) studies were not reported for the different conditions, but the mean data from Kirov et al. (2004) are consistent with the idea that only these smaller spines and synapses participate in recuperative synaptogenesis. It is unknown whether tissue prepared at room temperature would be viable for use in whole-cell patch-clamp recording experiments, but these are, in any event, important considerations to take into account in the future.

4.2. Silent synapses in the hippocampus

Previous studies have suggested that silent synapses and their conversion into functional ones via AMPA receptor insertion play a central role in constructing neuronal microcircuits during development (Durand et al., 1996; Isaac et al., 1997; Petralia et al., 1999). The data presented here are the first to describe these silent connections in adult and aged tissue, and they show that the reservoir of postsynaptically silent synapses does not disappear with brain maturation, but rather is maintained as the exclusive subtype of silent synaptic connection. The disappearance of presynaptically silent synapses early in life suggests that the conversion of both presynaptically and postsynaptically silent synapses into functional ones may drive microcircuit formation during development, whereas adding AMPA receptors to those synapses lacking them (i.e., postsynaptically silent synapses) may be the primary form of silent synapse conversion used throughout adult life in rats.

The present study suggests that the pool of postsynaptically silent synapses, and therefore the pool of potential functional synaptic connections, does not decrease with age in rats. Therefore, the substrates upon which activity-dependent AMPA receptor trafficking mechanisms act (Malinow and Malenka, 2002; Brecht and Nicoll, 2003; Nicoll, 2003) are present throughout the lifespan of rats, even though hippocampus-dependent learning is severely impaired beyond 32 months of age (Knuttinen et al., 2001). This enduring presence of postsynaptically silent synapses may provide a mechanism for the enduring ability of synapses in aged tissue to support LTP (Kumar et al., 2007).

4.3. Conclusions

Taken together, this and previous studies suggest that age-related learning impairments are caused not by the unavailability of convertible silent synapses, nor by losses in the strength of functional ones, but rather may be a consequence of synaptic dysregulation. Though future experiments are necessary to explore this notion in detail, it remains possible that age-related cognitive decline arises from dysregulated activity-dependent synaptic plasticity at functional synapses or an inability to insert AMPA receptors into silent ones. Such a notion is consistent with the dyshomeostasis of intracellular calcium in aged rat hippocampal neurons (Toescu et al., 2004; Gant et al., 2006), which may alter behavior by changing the rules or stability of experience-dependent plasticity (Burke and Barnes, 2006; Disterhoft and Oh, 2006; Wilson et al., 2006; Foster, 2007). Furthermore, large depolarizations that are capable of removing the Mg^{2+} -blockade of NMDA receptors in aged rats may actually exacerbate calcium dyshomeostasis by overloading clearance mechanisms with additional Ca^{2+} in flux through NMDA receptors.

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