Developmental Changes in Release Properties of the CA3-CA1 Glutamate Synapse in Rat Hippocampus

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INTRODUCTION

Following their formation, synapses undergo various collective changes, commonly referred to as synaptic maturation. One salient example of such maturation is a developmental decrease in release probability to a single action potential (Pᵣ). This decrease in Pᵣ has been documented among glutamate synapses in several regions, including the developing neocortex (Kumar and Huguenard 2001; Reyes and Sakmann 1999), calyx of Held (Iwasaki and Takahashi 2001; Taschenberger et al. 2002), striatum (Choi and Lovinger 1997), and hippocampus (Bolshakov and Siegelbaum 1995; but see Hsia et al. 1998; Muller et al. 2002), striatum (Choi and Lovinger 1997), and hippocampus (Bolshakov and Siegelbaum 1995; Hessler et al. 1993; Huang and Stevens 1997). During the first postnatal week, however, the synapse population has been described both to have a uniformly high Pᵣ (Bolshakov and Siegelbaum 1995) and to display, as the mature population, a considerable Pᵣ heterogeneity (Hanse and Gustafsson 2001b). Likewise, conflicting data exist regarding a developmental change in Pᵣ in that both a developmental decrease in Pᵣ (Bolshakov and Siegelbaum 1995; Muller et al. 1989), and the absence of such a decrease (Hsia et al. 1998), have been reported. Whether there is a developmental decrease in Pᵣ among these hippocampal synapses is thus uncertain. Moreover, if there is one, do the synapses change from a uniformly high Pᵣ state to a lower one, or is there a more general shift in Pᵣ within a heterogeneous Pᵣ population?

The aim of this study is to examine to what extent the CA3-CA1 synapse changes its release properties in the early postnatal period, and if so, if this change is homogenous or heterogeneous among the synapses. Moreover, if such a change in Pᵣ occurs, is it explained by a decrease in the number of vesicles that are immediately available for release (Dobrunz and Stevens 1997) and/or by a change in Pᵣ among the vesicles themselves (Hanse and Gustafsson 2001b). Finally, is such a developmental change in Pᵣ affected by an altered neural activity during the critical period?

METHODS

Slice preparation and solutions

Experiments were performed on hippocampal slices from 3- to 27-day-old Wistar rats (n = 142). The animals were killed in accordance with the guidelines of the local ethical committee for animal research. Rats older than 8 days were anesthetized with isoflurane (Abbott) prior to decapitation. The brain was removed and placed in an ice-cold solution containing (in mM) 124 NaCl, 3 KCl, 0.5 CaCl₂, 6 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 D-glucose. In the whole cell experiments, the solution was composed of (in mM) 140 cholineCl, 2.5 KCl, 0.5 CaCl₂, 7 MgCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, 1.3 ascorbic acid, and 7 dextrose. Transverse hippocampal slices (300–400 μm thick) were cut with a vibratome (Campden Instruments; or Slicer HR 2, Sigmann Elektronik) in the same ice-cold solution, and were subsequently stored in artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 0.5 ascorbic acid, 3 myo-inositol, 4 d,L-lactic acid, and 10 D-glucose at 25°C. After ≥30 min of storage, a single slice was transferred to a recording chamber where it was kept submerged in a constant flow (~2 ml/min) at 30–32°C. The perfusion ACSF contained (in mM) 124 NaCl, 3 KCl, 4 CaCl₂, 4 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 D-glucose. Bicuculline methiodide (20 μM)
was always present in the perfusion ACSF to block GABA_A receptor-mediated activity. All solutions were continuously bubbled with 95% O_2–5% CO_2 (pH ∼ 7.4). A surgical cut between the CA1 and CA3 regions and the higher than normal Ca^{2+} and Mg^{2+} concentrations were used to block spontaneous network activity.

Recording and analysis

Electrical stimulation of Schaffer collateral/commissural afferents and recordings of synaptic responses were carried out in the CA1 hippocampal region. Stimuli consisted of 0.2-ms negative or biphasic constant current pulses (15–80 µA) delivered through bipolar tungsten wires (resistance ∼0.1 MΩ, custom-made stimulator) or a glass pipette (resistance ∼0.5 MΩ; STG 1002, Multi Channel Systems, Reutlingen, Germany), in field and whole cell recordings, respectively. Stimulation electrodes were positioned in the stratum radiatum. Inputs received a test stimulus every 10 (field recordings) or 5 s (whole cell recordings). The stimulation intensity was set not to evoke firing in the postsynaptic neurons as evidenced by the absence of a population spike distorting the field excitatory postsynaptic potential (EPSP).

Field EPSP recordings were made by means of a glass micropipette (filled with 1 M NaCl) in s. radiatum. Field EPSPs were amplified with an Axoclamp-2A (Axon Instruments) and filtered at 3 kHz. Data were digitized (10-kHz sampling rate) and collected using a PC computer. Whole cell patch-clamp recordings were performed on visually identified CA1 pyramidal cells, using infrared-differential interference contrast videomicroscopy (CV-M50 IR, JAI Corp.) mounted on a Nikon E600FN microscope (Nikon). The pipette solution contained (in mM) 130 Cs-methanesulfonate, 2 NaCl, 20 HEPES, 0.2 EGTA, 5 QX-314, 4 Mg-ATP, and 0.4 GTP (pH ∼ 7.2 and osmolality 290–300 mOsM). Patch pipette resistances were 3–5 MΩ. Excitatory postsynaptic currents (EPSCs) were recorded at a sampling frequency of 10 kHz and filtered at 1 kHz, using an EPC-9 amplifer (HEKA Elektronik, Lambrecht, Germany). Cells were held in voltage-clamp mode at −70 mV for AMPA EPSC recordings. Series resistance was monitored using a 5-ms 10-mV hyperpolarizing pulse. Series resistance was not allowed to change more than −10% during an experiment; otherwise the experiment was discarded. Evoked responses were analyzed off-line using custom-made IGOR Pro (WaveMetrics, Lake Oswego, OR) software. Field EPSP magnitude was estimated by linear regression over the first 0.8 ms of the initial slope. Field NMDA receptor-mediated responses were measured by the initial 40-ms area. The presynaptic volley was measured as the peak-to-peak amplitude of the initial positive-negative deflection, and it was not allowed to change by >5% during the experiment. AMPA EPSCs were measured as the difference between the baseline level immediately preceding the stimulation artifact and the mean amplitude during a 1-ms time window around the negative peak between 3 and 8 ms after the stimulation artifact. Slope measurements (least-square linear regression from 20 to 80% of the rising phase) of EPSCs confirmed the results obtained with amplitude measurements (data not shown). In Fig. 3, all experiments were performed in 50 µM d-AP5 and 100 µM Ni^{2+} to block induction of long-lasting changes of synaptic strength (Wasling et al. 2002). In experiments in which the sensitivity of the field EPSP and paired-pulse facilitation (PPF) to extracellular [Ca^{2+}] was examined, the sum of [Ca^{2+}] and [Mg^{2+}] was kept at 10 mM to minimize changes in membrane excitability while altering Ca^{2+} ion concentration. We also did not observe any changes in the amplitude of the presynaptic volley while changing the divalent ion concentrations in this manner (data not shown). When using extracellular [Ca^{2+}] above 4 mM, recordings always started at the higher [Ca^{2+}] solution before changing to the lower [Ca^{2+}] solution. When field EPSPs were compared when changing from 4/4 to 4/4 Ca^{2+}/Mg^{2+}, the change in volley was corrected for. In experiments estimating the releasable pool of vesicles, high-frequency (20 and 50 Hz) stimulation was used (Fig. 6). The axons appeared to follow this frequency without failure as judged from the stability of the amplitude of the fiber volley. Thus the ratio between the 10th and 1st volley in a 50-Hz train was 0.96 ± 0.05 in experiments (n = 4) in which the volley was obtained in isolation during blockade of AMPA receptors (10 µM NBQX). To calculate average release probability based on MK-801–dependent decrease in NMDA field EPSP magnitude, the following equation was used: P_r = [1 − exp(−1/τ)]/FB, where FB is the fraction of NMDA receptors blocked by MK-801 after transmitter release at a synapse (Hessler et al. 1993). Data are expressed as means ± SE. Statistical significance for paired and independent samples was evaluated using Student’s t-test.

Tetanus toxin experiments

A previous study from our laboratory (Groc et al. 2003) has shown that injection of tetanus toxin into the hippocampus in vivo (at P1) leads to an ∼80% reduction of spontaneous transmitter release for several days; the reduction still was >50% at P6–P8. The same degree of reduction was found in slices taken close to the injection site as in those taken >600 µm away, indicating that the toxin had spread over a large part of the hippocampus. Nevertheless, in this study, only the two slices closest (on either side) to the injection site were taken from each tetanus toxin-injected hippocampus, to ascertain an as extensive blockade as possible.

To examine the possible effect of neural activity on the developmental change in P_r, tetanus toxin was injected into the hippocampus of rat pups at P1 or at P6–P7, by the same person and using the same procedures as previously (Groc et al. 2003). The rats were anesthetized by inhalation of isoflurane, and their heads were placed in a surgical mask to maintain the skull stable. A constant flux of a mixture isoflurane/air was applied inside the surgical mask. A heat sheet was used to maintain body temperature. A midline incision was made on the head and a hole was drilled in the skull (OD, 0.4 mm). The stereotactic coordinates for injection were (P1/P6) as follows: anterio-posterior, −1.4/−2.0 mm; mediolateral, +2.0/+2.9 mm, coordinates relative to the bregma: dorsoventral, −2.0/−2.9 mm from the cortical surface. Tetanus toxin was dissolved in a phosphate-buffered saline (0.1 M, pH 7.4) to the final concentration of 10 µg/ml. Five nanograms of tetanus toxin was injected into the hippocampus at ∼125 nl/min using a fused silica needle (ID, 75 µm; OD, 150 µm; Skandi-navia Genetec AB). After injection, the needle was left in situ for 5 min to reduce reflux. The incision was chemically sutured (Vet-Scan, B. Braun Med).

Drugs

Chemicals were from Sigma-Aldrich, except for d-AP5, α-conotox VIA, MK-801, NBQX, and QX-314 (Tocris Cookson, Bristol, UK). Cytochrome C (0.2 mg/ml; bovine heart) was included in all α-conotoxin GVIA experiments to block nonspecific peptide binding sites in the perfusion system.
week constitutes a period of substantial decrease in P_r among these synapses.

To examine whether the change in PPF during the second postnatal week is driven by neural activity, tetanus toxin was injected in vivo into the hippocampus of P6–P7 rats (n = 9; see METHODS). When examined in slices taken from rats at P12, PPF averaged 1.48 ± 0.03 (n = 31) compared with 1.55 ± 0.04 (n = 25) in slices from noninjected rats (Fig. 1B). It thus appears that the change in PPF proceeds unabated, despite the considerable activity reduction caused by such an injection (Groc et al. 2003). However, the tetanus toxin injection may, either by a differential action on synapses with different P_r, or by the activity reduction itself, have caused an increase in PPF, thus masking an effect on the second week developmental change in PPF. The effect of tetanus toxin was thus examined prior to the second week when P_r appears to be rather stable (see also Hanse and Gustafsson 2001b). Injection of tetanus toxin and examination of PPF at P5–P6, did, however, not reveal significantly larger PPFs (1.15 ± 0.06, n = 9) than in age-matched controls (1.08 ± 0.04, n = 21; Fig. 1B).

**Release probability changes during the second postnatal week in a nonuniform manner among the synapses**

Since P_r is a major determinant of PPF, with a decrease in P_r leading to an increased PPF, the simplest explanation for the observed change in PPF is a second week decrease in P_r. The neonatal synapse was originally reported to have (uniformly) a very high P_r, approaching 1.0 (Bolshakov and Siegelbaum 1995), and a reduction of this P_r during the second postnatal week would then seem a likely explanation for the increase in PPF. However, more recent studies have shown that P_r in the neonatal synapse, as in the more adult one, is very heterogeneous, with values from close to one to close to zero (Hanse and Gustafsson 2001b). With such a heterogeneous population, it is less clear in what manner the synapses may have changed, uniformly or not, to explain the PPF change. To explore this matter, we examined the MK-801–induced decay of pharmacoologically isolated NMDA receptor–mediated field EPSPs (10 μM NBQX). Figure 2A shows the average decay curves obtained from such experiments on P6 (○, n = 9) and P12 (●, n = 9) rats, respectively. In both cases, the NMDA EPSP decays with a rapid initial phase followed by a slower phase. The decay time course obtained in MK-801 experiments was originally fitted by a double exponential indicating two major categories of CA3-CA1 synapses, a high and a low P_r group (Hessler et al. 1993; Rosenmund et al. 1993). When examined for the individual experiments, the present decay curves were also better fitted by a double than by a single exponential [sum of squares for the single exponential fits were at P6 7.4 ± 2.2 (n = 9) and at P12 7.6 ± 2.35 (n = 9) times larger than for the double exponential fits]. This result is consistent with a similar kind of P_r heterogeneity among the synapses during the first as well as after the second postnatal week (Dobrunz and Stevens 1997; Hanse and Gustafsson 2001b). However, the decay is, on average, substantially slower for the P12 synapses (Fig. 2A, ●) than for the P6 ones (Fig. 2A, ○), suggesting a developmental decrease in P_r. As judged from the parameters of the double exponential fits obtained from the individual experiments, the decrease in P_r was mainly restricted to the high P_r group of synapses. Thus, whereas the time constant of the fast component changed considerably from 3.7 ± 0.2 (n = 9) at P6 to 7.8 ± 0.7 (n = 9) at P12 (P < 0.001), there was no significant difference between the time constants of the slow component: 55.2 ± 1.8 (n = 9, P6) and 63.8 ± 3.6 (n = 9, P12).

Knowing the fraction of NMDA receptors that are blocked at each trial (when glutamate is released), the (average) P_r of the high and low P_r groups of synapses can be calculated (Hessler et al. 1993). Moreover, the proportion of synapses belonging to the high and low P_r groups, respectively, can be obtained from the relative area of the fast and slow exponential components (exponential amplitude × time constant) (Rosenmund et al. 1993). Assuming a value of 0.3 for the fraction of NMDA receptors blocked/trial (Hessler et al. 1993; Huang and Stevens 1997; Rosenmund et al. 1993), the synaptic population at P6 would consist of a high P_r group with an average P_r value of
The question arises whether such a nonuniform change in \( P_{r} \) can alter PPF in the manner observed above. The MK-801 experiments were performed in 4/0 extracellular Ca\(^{2+}/Mg\(^{2+}\)) conditions to examine the relation between PP ratio and \( P_{r} \) for single CA3-CA1 synapses during the first postnatal week (Hanse and Gustafsson 2001a), the PP ratios for high \( P_{r} \) (0.88) and low \( P_{r} \) (0.07) synapses at P6 would be about 0.25 and 2.20, respectively, resulting in an overall PPF of 1.04. Corresponding values for the P12 synapses would be PP ratios of about 0.60 and 2.20, respectively, resulting in an overall PPF of 1.32. The observed change in PPF from P6 to P12 can thus be accounted for by the rather selective decrease in \( P_{r} \) of high \( P_{r} \) synapses indicated from the MK-801 experiments.

Possible complicating factors with the MK-801 experiments

The NMDA EPSP observed when stimulation commenced after a 10-min-long stimulus interruption was substantially larger than the baseline level present before stimulus interruption (Fig. 2B). The NMDA EPSP decay in MK-801 should then include a decay of this enhancement, which is unrelated to the MK-801 block of open channels. However, the decay of this enhancement is blocked by NMDA receptor antagonists (Niu et al. 1999), implying that it will only occur following NMDA receptor activation. Since NMDA receptor activation will lead to block by MK-801, it seems reasonable to believe that the decay of this enhancement will not occur in the presence of MK-801. Another possible complication is that the MK-801–induced decay is not only a function of the \( P_{r} \) of a synapse, but also of the open probability of the NMDA receptor channels. The NMDA EPSPs observed in the P12 synapses were found to decay faster than those in P6 synapses (P6, \( \tau = 66 \pm 5 \) ms, \( n = 9 \); P12, \( \tau = 42 \pm 4 \) ms, \( n = 9 \); \( P < 0.05 \); Fig. 2C), possibly reflecting a developmental shift in NMDA receptor subunit composition to an increasing proportion of NR2A subunits (Kirson et al. 1999). However, such a shift from NR2B to NR2A subunits should not be expected to affect the total open probability of the NMDA receptor channels during an EPSP, since the reduced duration is compensated for by a higher peak open channel probability (Chen et al. 1999).

It should also be noted that the good double exponential fit to the MK-801 data not necessarily implies the existence of...
two separate homogeneous groups of synapses, but may also be compatible with more continuous \( P_r \) distributions (Huang and Stevens 1997). The high and low \( P_r \) groups of synapses may thus represent the upper and lower parts of a continuous distribution rather than two distinct groups.

Does the sensitivity of \( P_r \) and PPF to \( \text{Ca}^{2+}/\text{Mg}^{2+} \) change during the second postnatal week?

The change in \( P_r \) indicated by the MK-801 experiments can be explained by a change in \( P_{\text{ves}} \) as well as in the number of vesicles that are immediately available for release. To explore whether a \( P_{\text{ves}} \) change is involved, the effect of changes in the extracellular \( \text{Ca}^{2+}/\text{Mg}^{2+} \) concentration on field EPSPs was examined at both P6 and P12. To minimize changes in axonal excitability, divalent ion concentration was kept constant at 10 mM, and \( \text{Ca}^{2+}/\text{Mg}^{2+} \) concentrations used were from 1/9 \( \approx \) 10/0. Field EPSP values obtained at these different concentrations, normalized to the value obtained at 4/6 \( \text{Ca}^{2+}/\text{Mg}^{2+} \), show that P6 and P12 synapses respond differently to these changes in \( \text{Ca}^{2+} \) influx (Fig. 3A). Thus, while reductions in \( \text{Ca}^{2+} \) influx decrease the EPSPs of P6 and P12 synapses in a rather similar manner, an increase in \( \text{Ca}^{2+} \) influx causes a considerably larger increase of the EPSP at P12 than at P6. This result suggests that P6 synapses are closer to saturation of their release machinery to \( \text{Ca}^{2+} \) ions, indicative of either a larger influx of \( \text{Ca}^{2+} \) ions or of a shift in the \( \text{Ca}^{2+} \) sensitivity curve. Nonetheless, this result seems compatible with a reduction in \( P_{\text{ves}} \) as underlying the change in \( P_r \) from P6 to P12.

These synapses also responded differently with respect to PPF to changes in the \( \text{Ca}^{2+}/\text{Mg}^{2+} \) relation (Fig. 3B). Thus, while a larger \( \text{Ca}^{2+} \) influx resulted in a substantial PPF decrease in P12 synapses, there was little change at P6. On the other hand, a decrease in \( \text{Ca}^{2+} \) influx caused a large PPF increase at P6 and a small one at P12. These results are also compatible with the release machinery being closer to saturation by \( \text{Ca}^{2+} \) ions at P6 than at P12.

Can a reduced \( \text{Ca}^{2+} \) influx quantitatively account for both the decrease in \( P_r \) and increase in PPF? The plot in Fig. 3C between (relative) field EPSP amplitude and PPF shows that the field EPSP (at P6) has to decrease to 40% of control for PPF to increase from its control value (1.11 ± 0.04, \( n = 12 \)) to the value found at P12 (1.55 ± 0.04, \( n = 16 \)). As indicated from the MK-801 experiments, overall \( P_r \) at P12 would be 85% of that at P6. However, these experiments were performed at 4/0 \( \text{Ca}^{2+}/\text{Mg}^{2+} \). A change from 4/0 to 4/4 \( \text{Ca}^{2+}/\text{Mg}^{2+} \) decreased the field EPSP on average to 67 (\( n = 12 \)) and 52% (\( n = 6 \)) (after volley correction, see METHODS) for P6 and P12 synapses, respectively. This would indicate that, at 4/4 \( \text{Ca}^{2+}/\text{Mg}^{2+} \), \( P_r \) at P12 should be 66% (85% \( \times \) 52/67) of that at P6. This value (66%) differs substantially from the decrease to 40% expected from the data in Fig. 3C. Thus a reduction in \( \text{Ca}^{2+} \) influx (by altered \( \text{Ca}^{2+}/\text{Mg}^{2+} \) ratio) does not replicate the developmental change in release properties. A close match may, however, not be expected since the developmental change in release properties only affects high \( P_r \) synapses. Moreover, the alteration of the \( \text{Ca}^{2+}/\text{Mg}^{2+} \) ratio may have effects on the
release machinery unrelated to the change in Ca\(^{2+}\) influx per se (such as changes in resting intracellular Ca\(^{2+}\) levels).

**Is the expression of presynaptic voltage-gated Ca\(^{2+}\) channels altered?**

In interpreting the above results as indicating a developmental decrease in \(P_{\text{ves}}\), possibly related to a decrease in Ca\(^{2+}\) influx, the question arises of what may underlie such a change. One possibility is a developmental change in the type of voltage-gated Ca\(^{2+}\) channels that is expressed in the presynaptic membrane and whose openings underlie the Ca\(^{2+}\) influx leading to release. In fact, at some synapses, including hippocampal glutamate synapses in culture, there is a developmental reduced contribution from N-type voltage-gated channels (see Iwasaki et al. 2000). We thus examined the effect of \(\omega\)-conotoxin GVIA, a blocker of these channels, on field EPSPs from P6 and P12 synapses, respectively. As shown from one such experiment (P6 rat), this toxin had a substantial effect on release, reducing the field EPSP to less than one-half its amplitude (Fig. 4A). On average, the \(\omega\)-conotoxin–sensitive fraction of the EPSP was 55 ± 4% (\(n = 4\)) at P6. This fraction was not significantly different (51 ± 2%, \(n = 6\)) at P12, indicating that there is no major shift in N-type channel expression during this time period (Fig. 4B). This fraction is also similar to what has been reported in 3- to 4-wk-old animals (Wheeler et al. 1994). The reduction in the field EPSP by \(\omega\)-conotoxin was associated with an increase in PPF [from 1.15 ± 0.04 to 1.72 ± 0.02 at P6 (\(n = 4\)) and from 1.62 ± 0.08 to 2.12 ± 0.10 at P12 (\(n = 6\))]. Thus (by linear approximation) the EPSP, i.e., \(P_{r}\), at P6 has to be reduced to 60% to produce a PPF increase corresponding to that at P12. Such a reduction is in line with that calculated above to occur between P6 and P12 (66%), suggesting that a reduction in Ca\(^{2+}\) influx, by blockade of N-type channels, can essentially replicate the developmental change in release properties.

**Variation in action potential duration with age**

During the second postnatal week, there is a substantial change in action potential characteristics, at least as observed from recordings from CA1 pyramidal cell somata and from Calyx of Held (Spigelman et al. 1992; Taschenberger and von Gersdorff 2000). The amplitude is increased, and the duration of the volley observed at the end of the train length (Schneggenburger et al. 1999). The rationale is that when the cumulative amplitude rises in a linear manner, release is no longer coming from vesicles in the preprimed pool but also by the number of vesicles that are immediately available for release, i.e., the preprimed pool (Hanse and Gustafsson 2001b). On one hand, a developmental decrease in pool size could contribute to the developmental decrease in \(P_{r}\) in high \(P_{r}\) synapses. On the other hand, a developmental increase in pool size that parallels the developmental decrease in \(P_{\text{ves}}\) may explain the nonuniform developmental change in \(P_{r}\) indicated by the MK-801 data. This is because an increased pool size will have a larger impact on the \(P_{r}\) of low than of high \(P_{\text{ves}}\) synapses (Hanse and Gustafsson 2001b), and may thus counteract a developmental decrease in \(P_{r}\) in low but not in high \(P_{r}\) synapses.

An estimate of the preprimed pool can be obtained by evoking EPSCs by a brief high-frequency train and plotting the cumulative EPSC amplitude against train length (Schneggenburger et al. 1999). The rationale is that when the cumulative amplitude rises in a linear manner, release is no longer coming from vesicles in the preprimed pool but only from newly recruited ones (that are assumed to be recruited/released at the same rate). By assuming that this recruitment starts at zero time, the intersection of the linear relation with the y-axis will give an estimate of the preprimed pool. This estimate will not be in absolute terms, but will be the relation between the preprimed pool and the invading the presynaptic bouton, one may expect a developmental decrease in Ca\(^{2+}\) influx associated with the reduced duration of the spike. As previously described (Berg-Johnsen and Langmoen 1992; Laerum and Storm 1994), procedures that alter the action potential duration, such as TEA and temperature, have significant effects on the fiber volley duration. Following the total blockade of the NMDA receptor–mediated EPSP by MK-801, the fiber volley could be observed in isolation and thereby be accurately measured with respect to its duration. The volley observed at the end of the first postnatal week averaged 1.59 ± 0.03 (\(n = 24\)) ms compared with 1.32 ± 0.02 (\(n = 42\)) ms for that observed 1 wk later, i.e., there was a substantial shortening (Fig. 5). To examine the activity dependence of this shortening, the fiber volley was also measured in slices from P12 rats that had been injected with tetanus toxin at P6. The volley duration in these slices averaged 1.28 ± 0.03 (\(n = 13\)), indicating that the action potential shortening proceeds independent of neural activity.

**Does the immediately releasable pool of vesicles change with age?**

\(P_{r}\) is determined not only by \(P_{\text{ves}}\) but also by the number of vesicles that are immediately available for release, i.e., the preprimed pool (Hanse and Gustafsson 2001b). On one hand, a developmental decrease in pool size could contribute to the developmental decrease in \(P_{r}\) in high \(P_{r}\) synapses. On the other hand, a developmental increase in pool size that parallels the developmental decrease in \(P_{\text{ves}}\) may explain the nonuniform developmental change in \(P_{r}\) indicated by the MK-801 data. This is because an increased pool size will have a larger impact on the \(P_{r}\) of low than of high \(P_{\text{ves}}\) synapses (Hanse and Gustafsson 2001b), and may thus counteract a developmental decrease in \(P_{r}\) in low but not in high \(P_{r}\) synapses.
number of vesicles released by the first stimulus in the train, i.e., it will be a function of $P_r$. Such estimates of the preprimed pool at P6 and P12 were obtained by evoking synaptic activity by 10 impulse trains at 20 and 50 Hz. Since the estimate should be independent of train frequency, the use of both frequencies can be seen as an internal control. In these experiments, synaptic activity was recorded as EPSCs using whole cell recording (rather than field recording) primarily to avoid buildup of depolarization (and subsequent spike activation) during the train. An example of such cumulative EPSC curves, normalized to the peak amplitude of the first EPSC, given by 20- and 50-Hz trains (1.64 and 1.66 at P6; 2.06 and 2.11 at P12), as should be expected. However, compared for P6 and P12 synapses, there was a significant age group difference, the values being $1.64 \pm 0.11$ ($n = 15$) and $2.07 \pm 0.09$ ($n = 13$) for P6 and P12 synapses, respectively (Fig. 6B). Since these estimated pool values are a function of $P_r$, the initial release probability (indicated by the y-axis), they will, however, only be useful if one knows the relation between the $P_r$ values of the P6 and P12 synapses when these pool estimates were performed. It should first be noted that PPF of the synapses involved averaged 1.07 (P6) and 1.65 (P12) (estimated from the 1st of the 30 trains given for each synapse; see following text), indicating that these synapses were representative of P6 and P12 synapses, respectively. As calculated above, at $4/4 \text{Ca}^{2+}/\text{Mg}^{2+}$, $P_r$ at P12 should be 66% of that at P6. However, during the initial stages of the train activation (5–10 1st of the 30 trains given at 0.2 Hz) release conditions were altered in that the EPSC amplitude and/or PPF changed. Thus the pool estimations were performed after a steady state had been reached, during which the EPSC (following the 1st stimulus of the train) of P6 synapses had decreased (on average) to 0.68 of the initial value, whereas that of P12 synapses was less altered (0.93). Thus, under these conditions, $P_r$ of the P12 synapses should be $90\% \times (0.93/0.68)$ of that of the P6 ones. The pool estimates of $1.64 \pm 0.11$ ($n = 15$) and $2.07 \pm 0.09$ ($n = 13$) given above should be translated into $1.64 \pm 0.11$ and $1.86 \pm 0.08$ (90% of 2.07) after correcting for this estimated $P_r$ difference, indicating no significant change in the overall value of preprimed pool during the second postnatal week.

**Discussion**

These results show that the second postnatal week constitutes a period of substantial alterations in the release properties of CA3-CA1 hippocampal synapses manifested as a large increase in paired-pulse plasticity. Hence, these results do not substantiate a previous report that paired-pulse plasticity is unaltered throughout this time period (Hsia et al. 1998), but rather those reports indicating such a change (Bolshakov and Siegelbaum 1995; Muller et al. 1989). However, the results

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**Fig. 6.** Estimation of vesicle pool size. A: example experiment showing cumulative excitatory postsynaptic current (EPSC) amplitudes (normalized with respect to the amplitude of the 1st response) obtained by 20- Hz (■) and 50-Hz (○) stimulation. Dashed lines are extrapolations from linear regression over the last 5 data points. Intersection between dashed lines and the y-axis represents the estimated pool size as a function of initial release probability (indicated by arrow). $R_r$ mean relative pool size at P4–P7, at P12–P15, and at P12–P15, with mean value corrected for lower $P_r$.
also suggest that the alteration takes place in only a subpopulation of the synapses, those with high $P_r$ values, and leaves most of the population largely unaffected. This may explain the failure of Hsia et al. (1998) to observe the developmental change in paired-pulse plasticity, since fewer synapses will be sampled in whole cell than in field recordings. The alteration in paired-pulse plasticity was found to take place despite a profound blockade of synaptic transmission during this time period, by local in vivo tetanus toxin injection, suggesting that normal neural activity during the second postnatal week is not a prerequisite for the alteration in paired-pulse plasticity to occur. Our results suggest that the observed alteration in release properties is explained by a large reduction in the release probability of the individual vesicles ($P_{ves}$) in the affected synapses, possibly mediated by a reduced Ca$^{2+}$ influx.

Since the observed modification in release largely occurs within a few days, it is not likely a consequence of synaptogenesis per se, which occurs continuously throughout the first 4 wk (Steward and Falk 1991).

The CA3-CA1 synapses in the first neonatal week have previously been described as uniformly characterized by a $P_r$ close to one and by paired-pulse depression (Bolshakov and Siegelbaum 1995). This behavior was in contrast to that found in 2- to 3-wk-old animals, where $P_r$ was substantially lower and there was PPF (Bolshakov and Siegelbaum 1995), indicating a switch from high to low $P_r$ synapses in the intervening period. More recently, using minimal stimulation technique, a more heterogeneous first week $P_r$ distribution was described (Hanse and Gustafsson 2001b). The present results support the existence of such first week heterogeneity. As in older animals (Hessler et al. 1993), the NMDA EPSP decayed, following MK-801 application, with an initial rapid and a later slower exponential phase that indicates the presence of both high and low $P_r$ synapses. There is presently no clear understanding of the exact nature of the $P_r$ distribution in older (>2 wk) animals. That is, MK-801 data taken to indicate the existence of two distinct groups of synapses (Hessler et al. 1993; Rosenmund et al. 1993) can equally well be accounted for by more continuous $P_r$ distributions (Huang and Stevens 1997). Nevertheless, these results suggest that the neonatal population consists of synapses with higher and lower $P_r$, the low $P_r$ synapses constituting the majority of the population. Assuming the fraction of NMDA receptors channels to be blocked following vesicular release to be 0.3–0.4 (Hessler et al. 1993; Huang and Stevens 1997; Rosenmund et al. 1993), the high $P_r$ group would have an average $P_{ves}$ of 0.88–0.66 (at 4/0 mM Ca$^{2+}$/Mg$^{2+}$) and the low $P_r$ group would average 0.07–0.05. These deduced $P_r$ values are not incompatible with data from the minimal stimulation experiments (Hanse and Gustafsson 2001b). Although these latter data show a continuous distribution of $P_r$ values, they indicate two peaks, at low (<0.1) $P_r$ and high (0.4–0.6) $P_r$ that suggests the presence of two broad categories of synapses. These peaks may represent the presently deduced high and low $P_r$ groups, considering the uncertain value of the blocking fraction, and the fact that the minimal stimulation data were obtained at 4/4 Ca$^{2+}$/Mg$^{2+}$, and using train stimulation, both procedures that would decrease $P_r$.

A main finding in this study was that the second week synaptic maturation in $P_r$ only affects the high $P_r$ synapses, changing the estimated average $P_r$ from 0.88 to 0.52 (blocking fraction = 0.3). Calculations using PP ratio–$P_r$ data from minimal stimulation experiments (Hanse and Gustafsson 2001a) suggested (see Results) that such a selective $P_r$ change would produce an overall change in PPF consistent with that observed experimentally. However, whether this developmental $P_r$ change is a distinct shift within a homogenous group of synapses with high $P_r$, or a variable shift in $P_r$ among the synapses with the higher $P_r$ values within a continuous distribution is, as indicated above, uncertain. Nevertheless, the maturation is not a switch of release properties from one group to another but an adjustment of the release probability within one group, or population, of synapses. This second week maturation thus differs from that observed in cultured hippocampal neurons where a proportion of the high $P_r$ synapses converts into low $P_r$ ones (Chavis and Westbrook 2001). On the other hand, it agrees with the developmental change within the Calyx of Held synapse (Iwasaki and Takahashi 2001). In this synapse, containing hundreds of release sites, a double-exponential fit of MK-801 data also suggested a shift within a high $P_r$ group of release sites from much the same values and to much the same extent as shown presently for the hippocampal synapses, whereas little change was observed in the low $P_r$ group. Thus, interestingly, the developmental change observed among a population of heterogeneous hippocampal synapses, containing a single release site, is mirrored by that occurring in a heterogeneous population of release sites within a single synapse.

**What underlies the decrease in $P_r$ in the high $P_r$ group?**

Controversy exists to what extent heterogeneity in $P_r$ is determined solely by a variation in the immediately releasable vesicle pool (Dobrunz and Stevens 1997), or in addition, by variation in $P_{ves}$ among the synapses (Hanse and Gustafsson 2001b). According to the first viewpoint, the developmental change in $P_r$ should be explained by a reduced vesicle pool in high $P_r$ synapses. If $P_r$ is decided by the pool alone, the high $P_r$ group would produce the majority of the releasable vesicles, and a reduction of its pool size would be detectable. However, this study found no evidence for a developmental reduction in pool size, estimated using the cumulative EPSC amplitude arising from train stimulation (Schneegansburger et al. 1999). On the other hand, high $P_r$ synapses that differ from the low $P_r$ synapses mainly with respect to $P_{ves}$ will produce only a minority fraction of the releasable pool, and a reduction in this fraction may go undetected in our pool estimations. However, a pool decrease in high $P_r$ synapses would not be expected to lead to a developmental increase in PPF (Hanse and Gustafsson 2001a).

Evidence for the involvement of a $P_{ves}$ change, rather than a pool change, in the developmental $P_r$ reduction is the differential effect of changes in Ca$^{2+}$/Mg$^{2+}$ ratio on the EPSPs evoked from P6 and P12 synapses, respectively. Specifically, high Ca$^{2+}$/Mg$^{2+}$ ratios increased the EPSP of P6 synapses considerable less than that of P12 synapses, suggesting that the P6 synapses are closer to saturation with respect to Ca$^{2+}$, i.e., operate higher up on the upper limb of the $P_{ves}$-Ca$^{2+}$ relation. This suggestion seems reasonable with respect to the high $P_r$ group of synapses at P6. However, considering the large number of low $P_r$ synapses in the P6 population, one may have...
expected greater latitude for increased EPSP amplitude with increased Ca^{2+}/Mg^{2+} ratios. Previous MK-801 experiments (Hessler et al. 1993) have, however, indicated a smaller effect of Ca^{2+} influx changes (induced by the addition of Ca^{2+} to the perfusion solution) on the low than on the high P_r synapses. The low P_r group (at the high Ca^{2+}/Mg^{2+} ratios used) may also operate on the upper limb of its P_r–Ca^{2+} relation. That is, the maximal P_r value of these synapses is substantially <1, as has been observed for cortico-geniculate synapses (Granseth and Lindstrom 2003, 2004). The effect of changes in Ca^{2+}/Mg^{2+} ratio on PPF was also consistent with P6 synapses operating higher up on the upper limb of the P_r–Ca^{2+} relation. These data point toward a second week reduction in P_{ves} that should be largely restricted to high P_r synapses, possibly explained by a reduction in the amount of Ca^{2+} reaching the Ca^{2+} acceptors (triggering release). In support of this explanation, a decrease in Ca^{2+} influx induced by a Ca^{2+} channel blocker could quantitatively replicate the combined P_r and PPF shift occurring from P6 to P12.

It should be noted, however, that a restriction of the developmental P_r reduction to the high P_r synapses does not necessarily imply that the P_{ves} reduction is so restricted. Since an increased pool size will have more impact on P_r in low than in high P_{ves} synapses (Hanse and Gustafsson 2001b), a developmental increase in preprimed pool size for all synapses could counteract a decrease in P_{ves} in low but not in high P_{ves} synapses, specifically if, as noted above, P_{ves} in low P_r synapses is less affected by changes in calcium influx than is P_{ves} in high P_r synapses. However, this scenario is not supported by our pool estimations, which did not indicate any significant increase in preprimed pool size.

These results cannot explain why the amount of Ca^{2+} reaching the Ca^{2+} acceptors should decrease with age. A possible explanation is a developmental decrease in action potential duration that results in a reduced Ca^{2+} influx. The action potential in the CA1 neuron somata undergoes a second week maturation characterized by increased amplitude and shortened duration (Spigelman et al. 1992) that likely reflects an increased density of voltage-gated sodium and potassium channels. We also observed a substantial developmental shortening of the fiber volley duration that possibly reflects a shortening of the axonal (and bouton) action potential. In common with PPF, this shortening was not affected by tetanus toxin-induced activity blockade. However, whether such a shortening should lead to a decreased Ca^{2+} influx that selectively affects high P_r synapses is unknown. Another possibility is a developmental shift in the type of voltage-gated Ca^{2+} channel that is involved in the release. N-type voltage-gated Ca^{2+} channels differ from P/Q and R types in their coupling to release (Qian and Noebels 2001; Urbano et al. 2003), and developmental changes in N-type channels have been described for some synapses (Iwasaki et al. 2000). However, we found no evidence for such a developmental shift in that α-conotoxin, a N-type channel blocker, did not differentially affect EPSPs at P6 and P12. This lack of developmental shift in N-type channel contribution agrees what has been reported for another cortical glutamate synapse, a thalamo-cortical one (Iwasaki et al. 2000).

Alternatively, a reduction in the amount of Ca^{2+} reaching the Ca^{2+} acceptors may relate to an increase in presynaptic buffering capacity of Ca^{2+} ions in the high P_r group (Blatow et al. 2003; Oleskevich and Walmsley 2002). However, whereas an increase of slow, as well as fast, buffering would lead to a decreased P_r, it would also lead to a decreased PPF (Rozov et al. 2001), and can thus not explain the developmental change in release. Moreover, a fast buffer, if present, is expected to lead to an increase in PP ratio when extracellular calcium levels are increased (Blatow et al. 2003). It was presently noted (Fig. 3B) that, at the lowest Ca^{2+} levels, P12 synapses did not increase their PPF when Ca^{2+} was lowered, indicative of a small concentration of fast buffers in these synapses. However, since, for P12 synapses, the overall dominating relation between P_r and PP plasticity is an inverse one, fast buffering is likely to have only a minor effect on P_r and PPF in these synapses.

An alternative explanation to a reduced amount of Ca^{2+} reaching the Ca^{2+} acceptors is a developmental change of the distance between Ca^{2+} channels and the Ca^{2+} acceptors or of the affinity of Ca^{2+} acceptors. Such changes would manifest themselves in the same manner as a reduced Ca^{2+} influx, and if occurring selectively in high P_r synapses, may potentially explain the developmental switch in release properties.

Activity dependence of PPF maturation?

We used in vivo injections of tetanus toxin into the hippocampal CA1 area to address the question of to what extent the second postnatal week synaptic maturation depends on neural activity. Such injections have been found to cause a profound decrease, for several days, of synaptic release over a large part of the hippocampus (Groc et al. 2003), and when applied at P1, to impair dendritic growth (Groc et al. 2002). However, tetanus injection at P6 did not affect the second week increase in PPF, indicating that this increase does not require the neural activity that normally exists during the second postnatal week. This result differs from work on cultured neurons in which NMDA receptor blockade prevents the developmental decrease in P_r (Chavis and Westbrook 2001). However, this latter P_r change differs from the present one, in that synapses changed from high P_r to low P_r, rather than shifting within the high P_r group. Moreover, there may be a difference when reducing activity in different manners (AP5 vs. tetanus toxin). At anterovernal cochlear nucleus (AVCN) glutamate synapses, P_r is, at P11–P16, lower in control than in deaf mice, possibly reflecting an activity dependent lowering of P_r (Oleskevich and Walmsley 2002). However, whether these synapses actually start out with a high P_r is not known. Moreover, the synapses differed only with respect to P_r, and not with respect to PPF.

In the striatum, there is a temporal correlation between the developmental increase in PPF and a decreased susceptibility for the induction of an LTD based on P_r reduction (Choi and Lovinger 1997). Neural activity leading to induction of LTD may then cause the developmental increase in PPF. In the hippocampus, LTD may also show age dependence, being more easily induced early on, and be presynaptically expressed (Li et al. 2002; Wasling et al. 2002). Since these LTDs are evoked by rather weak neural activity, it cannot be excluded that tetanus toxin blockade does not interfere with their induction. However, these LTDs do not.
show the temporal profile of the second week PPF increase, in that they can be induced at a later date given proper induction conditions. They do thus not appear likely candidates for a second week activity-dependent change in release properties. This study also showed that tetanus injection at P1 did not affect the value of PPF at P6. The lack of effect of tetanus injection on the second week PPF increase was thus not masked by an effect of this activity blockade by itself on PPF (and $P_r$). Previous work on the effect of activity reduction on $P_r$ has indicated that reduced activity, induced either by TTX application on cultured hippocampal neurons (Murthy et al. 2001), or by overexpression of K$^+$ channels at the neuromuscular junction of Drosophila (Paradis et al. 2001), leads to an increase in $P_r$. This result can thus not be reproduced by tetanus toxin application, possibly either because of a difference in cells involved or of the difference in type of activity blockade.

In conclusion, these results show that the CA3-CA1 synapse undergoes a second postnatal week reduction in $P_r$, restricted, however, to the minority group of high $P_r$ synapses. This developmental $P_r$ reduction (and PPF increase) seems produced by a reduction in the vesicle release probability, possibly explained by a reduced Ca$^{2+}$ influx. Such a reduction in Ca$^{2+}$ influx may be secondary to maturation of the action potential underpinned by paired pulse facilitation in calbindin-$\Delta$2K-containing terminals. $Neuron$ 38: 79–88, 2003.


