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Developmental decrease in synaptic facilitation at the mouse hippocampal mossy fibre synapse

Fumiko Mori-Kawakami, Katsunori Kobayashi and Tomoyuki Takahashi

Department of Neurophysiology, University of Tokyo Graduate School of Medicine, Tokyo 113–0033, Japan

Transmission at the hippocampal mossy fibre (MF)–CA3 pyramidal cell synapse is characterized by prominent activity-dependent facilitation, which is thought to provide a wide dynamic range in hippocampal informational flow. At this synapse in mice the magnitude of paired-pulse facilitation and frequency-dependent facilitation markedly decreased with postnatal development from 3 weeks (3W) to 9 weeks (9W). Throughout this period the mean amplitude and variance of unitary EPSCs stayed constant. By altering extracellular Ca^{2+}/Mg^{2+} concentrations the paired-pulse ratio could be changed to a similar extent as observed during development. However, this was accompanied by an over 30-fold change in EPSC amplitude, suggesting that the developmental change in facilitation ratio cannot simply be explained by a change in release probability. With paired-pulse stimulation the Ca^{2+} transients at MF terminals, monitored using mag-fura-5, showed a small facilitation, but its magnitude remained similar between 3W and 9W mice. Pharmacological tests using CNQX, adenosine, LY341495, H-7 or KN-62 suggested that neither presynaptic receptors (kainate, adenosine and metabotropic glutamate) nor protein kinases are responsible for the developmental change in facilitation. Nevertheless, loading the membrane-permeable form of BAPTA attenuated the paired-pulse facilitation in 3W mice to a much greater extent than in 9W mice, resulting in a marked reduction in age difference. These results suggest that the developmental decrease in the MF synaptic facilitation arises from a change associated with residual Ca^{2+}, a decrease in residual Ca^{2+} itself or a change in Ca^{2+}-binding sites involved in the facilitation. A developmental decline in facilitation ratio reduces the dynamic range of MF transmission, possibly contributing to the stabilization of hippocampal circuitry.

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Corresponding author T. Takahashi : Department of Neurophysiology, University of Tokyo Graduate School of Medicine, Hongo 7–3-1, Tokyo 113–0033, Japan. Email: ttakahas-tky@umin.ac.jp

During postnatal development, synapses in the CNS undergo morphological, molecular and physiological transformations. With respect to short-term synaptic plasticity, the paired pulse ratio (PPR; the relative amplitude of the second of a pair of synaptic responses) increases with development during the first postnatal month at various CNS synapses in rodents (Bolshakov & Siegelbaum, 1995; Choi & Lovinger, 1997; Pouzat & Hestrin, 1997; Taschenberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001; but see Hsia *et al.* 1998). In general, PPR is larger when the basal transmitter release probability *P* is lower (Zucker & Regehr, 2002). At several synapses, the developmental change in PPR has been attributed to a change in *P* (Bolshakov & Siegelbaum, 1995; Taschenberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001).

Among CNS synapses, those formed between dentate granule cell axons and pyramidal cells in the hippocampal CA3 region (MF–CA3 synapses) are characterized by a prominent synaptic facilitation on repetitive stimulation (Kobayashi *et al.* 1996; Salin *et al.* 1996). This facilitation is thought to contribute to the ability of single granule cells *in vivo* to evoke action potentials in postsynaptic CA3 pyramidal cells when firing in bursts (Henze *et al.* 2002). Thus MF synaptic facilitation may lead to enhanced hippocampal network activity. However, the mechanism underlying this large facilitation is not fully understood. In addition to the classic residual calcium hypothesis (Katz & Miledi, 1968), several specific mechanisms have been proposed for MF synaptic facilitation. These include activity-dependent broadening of presynaptic action potentials and an ensuing increase in presynaptic Ca^{2+} influx (Geiger & Jonas, 2000), presynaptic depolarization mediated by kainate autoreceptors (Schmitz *et al.* 2001; Kamiya *et al.* 2002), and an increase in transmitter release via activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; Salin *et al.* 1996). These previous results suggest that MF synaptic facilitation can be regulated dynamically without alteration in basal *P*.

In the present study, we used hippocampal slices from mice to gain further insight into the dynamic aspect of synaptic facilitation at the MF–CA3 synapse. We

examined whether the synaptic facilitation exhibits any developmental change after 3W, when the synapse becomes morphologically mature (Amaral & Dent, 1981). We found that the magnitude of facilitation decreases with development from 3W to 9W, and that this change in facilitation is not caused by a change in *P*. Given various factors potentially involved in synaptic facilitation, we examined further possible mechanisms underlying the developmental decrease in facilitation at the MF–CA3 synapse.

METHODS

Slice preparation

CD-1 (ICR) mice (21–69 days old) were decapitated under halothane anaesthesia according to the guidelines of the Physiological Society of Japan. After quickly isolating hippocampi, transverse slices (400 μm thick) were cut using a tissue slicer (Vibratome, Lancer, USA or Leica VT1000, Nussloch, Germany) in an ice-cold artificial cerebrospinal fluid (ACSF, see below for composition) bubbled with 95 % O_2 and 5 % CO_2. Slices were then incubated for > 1 h in a humidified interface holding chamber at room temperature (22–28 °C). After incubation a slice was submerged in a recording chamber, held by a platinum grid attached with nylon strings, and superfused (at 2 ml min^{-1}) with the ACSF solution composed of (mM): NaCl 119, KCl 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, glucose 11, $CaCl_2$ 2.5 and $MgCl_2$ 1.3 (pH 7.3–7.4 equilibrated with 95 % O_2 and 5 % CO_2) unless specified otherwise.

Recording and data analysis

Synaptic responses were evoked using a bipolar tungsten electrode placed in the dentate gyrus (DG) granule cell layer at the basal stimulus frequency of 0.05 Hz unless stated otherwise. Field (f) EPSPs w ere recorded from the stratum lucidum in the CA3 region using a glass pipette filled with 2 M NaCl. In whole-cell recordings, both Ca^{2+} and Mg^{2+} concentrations in the ACSF were raised to 4 mM to gain stability in recordings unless stated otherwise. EPSCs were recorded from CA3 pyramidal cells at holding potentials of –60 mV using the blind-patch whole-cell technique. In some experiments, to evoke unitary EPSCs (Fig. 3), a glass pipette filled with ACSF instead of the tungsten electrode was used for stimulation. NMDA-EPSCs were recorded at a holding potential of +40 mV in the presence of 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 10 μM; Tocris, Bristol, UK) and picrotoxin (100 μM). Responses due to MF input were distinguished from those due to the activation of axon collaterals of CA3 pyramidal cells by a greater than 90 % block of synaptic responses by the mGluR2/3 agonist (2*S*, 1′*R*, 2′*R*)-2-(2, 3-dicarboxycyclopropyl)-glycine (DCG-IV, 1 μM; Tocris, Bristol, UK). Whole-cell pipettes were pulled from borosilicate glass capillaries (o.d. 1.5 mm) and had a resistance of 3–5 MΩ when filled with an internal solution composed of (mM): CsOH 138, D-gluconate 133, Hepes 20, EGTA 5, NaCl 8, MgATP 2 and Na_3GTP 0.3, supplemented with a chloride channel blocker, 4, 4′-disothiicynatostilbene-2, 2′-disulphonic acid disodium salt (DIDS, 1 mM) (Nelson *et al.* 1994). In these conditions, EPSCs recorded at a holding potential of –60 mV were almost completely abolished by CNQX (10 μM). For recording EPSCs in the ACSF with modified Ca^{2+}/Mg^{2+} concentrations, *N*-(2, 6-Dimethylphenyl c arbamoylmethyl) triethylammonium bromide (QX314, 5 mM; Alomone Labs, Jerusalem, Israel) was included in the internal solution to block action potential generation. EPSCs and fEPSP were recorded using an Axopatch-1D amplifier (Axon Instruments, USA). The access resistance (range 20–30 MΩ) was not compensated for, but it was monitored continuously, and when it changed by more than 20 %, data were discarded. Records were filtered at 1–2 kHz and fed online into a personal computer via an interface (digitized at 2.5–10 kHz). To calculate PPR, the amplitude of the first and second fEPSP or EPSC was measured from the onset of each event in averaged traces. Statistical significance was evaluated by MANOVA and ANOVA with Tukey and Scheffé tests, unless stated otherwise. All experiments were carried out at 25–27 °C.

Ca^{2+} measurements

Ca^{2+} transients in MF terminals were monitored with a photomultiplier (Olympus, Nagano, Japan) using the membrane-permeable low-affinity Ca^{2+} indicator dye mag-fura-5 AM (Molecular Probes, OR, USA). We used the low-affinity Ca^{2+} indicator to minimize disturbances in intracellular Ca^{2+} dynamics. Mag-fura-5 AM (50 μM) was applied for 20 min to the stratum lucidum by local perfusion near the border between the DG and CA3 regions (Atluri & Regehr, 1996). After incubating for 2–3 h, to allow the Ca^{2+} indicator to reach distant MF terminals, fluorescent Ca^{2+} signals and fEPSPs evoked by the tungsten bipolar stimulating electrode were simultaneously recorded at the stratum lucidum 1.0–1.2 mm away from the stimulating electrode. Fluorescent Ca^{2+} signals and fEPSPs were simultaneously recorded at the stratum lucidum 1.0–1.2 mm away from the stimulating electrode. Since we applied the dye by local perfusion and measured the Ca^{2+} signal at the site away from the application site, the signal probably arises from presynaptic fibres rather than glial or postsynaptic cells. Indeed, in the presence of CNQX (10 μM) and D-2-amino-5-phosphonovaleric acid (D-APV, 25 μM), essentially the same results were obtained as those without these antagonists (see Results). In these experiments, because of relatively small signals obtained from the low-affinity Ca^{2+} indicator, MFs had to be stimulated near the site of dye loading to gain the signal-to-noise ratio. Compared with stimulation of the dentate granule cell layer, stimulation of the stratum lucidum can activate axon collaterals of pyramidal cells more easily for anatomical reasons. However, the following evidence suggests that our Ca^{2+} signal originated mainly from MFs. Firstly, the fluorescence of the indicator dye was detectable only in the stratum lucidum. Secondly, the pyramidal cell collaterals were not clearly stained even when the loading pipette was placed in the stratum radiatum, where pyramidal cell axon collaterals predominate. Therefore, in our loading conditions, MFs are likely to be preferentially stained.

RESULTS

Developmental decrease in paired-pulse facilitation and frequency facilitation

The MF–CA3 synapse shows a marked facilitation in response to paired-pulse or repetitive stimulation (Kobayashi *et al.* 1996; Salin *et al.* 1996). The PPF of fEPSPs was clearly discerned at interstimulus intervals (ISI) shorter than 1 s, with its magnitude being larger for shorter ISI (Fig. 1*Ab*). In 3W mice, at 50 ms ISI, PPR was 5.03 ± 0.21 (n = 6 slices, Fig. 1*Ab*). As mice matured, at 9W, PPR became gradually smaller (Fig. 1*Ac*) and reached a significantly lower level (2.95 ± 0.15, n = 7, $P < 0.01$). Consistent with the field recordings, developmental decrease of PPF was observed for MF-EPSCs recorded from voltage-clamped CA3

pyramidal cells (Fig. 1*B*). At 3W PPR was 5.90 ± 0.61 (50 ms ISI, $n = 14$ cells in 14 slices), but it declined to 3.45 ± 0.28 at 9W ($n = 13$, $P < 0.01$, Fig. 1*Bb*). PPF at the 200 ms ISI also decreased with development (Fig. 1*Ab* and *Bb*).

We next examined frequency-dependent facilitation (FF) induced by repetitive stimulation. When the stimulus frequency was raised from 0.033 to 1 Hz, fEPSPs rapidly increased in amplitude and reached a maximal level within 30 s (Fig. 2*A*). The magnitude of FF measured as the ratio (FFR) of the maximal amplitude to the baseline amplitude depended upon stimulus frequency, and was higher at higher frequencies (Fig. 2*B*). Similar to PPR, FFR significantly decreased as mice matured. In 4W (28- to 34-day-old) mice, FFR at 1 Hz stimulation was 8.69 ± 0.62 ($n = 6$), whereas it was 5.46 ± 0.40 ($n = 4$) in 9W mice ($P < 0.01$, unpaired *t* test).

Unitary EPSC remains unchanged between 3W and 9W

At other CNS synapses a developmental decrease in PPR arises from a developmental increase in *P* (Bolshakov & Siegelbaum, 1995; Iwasaki & Takahashi, 2001). Therefore a developmental increase in PPR and PPF at MF synapses might conversely arise from an increase in *P*. To examine this possibility, we analysed unitary EPSCs recorded from voltage-clamped CA3 pyramidal cells (Jonas *et al.* 1993).

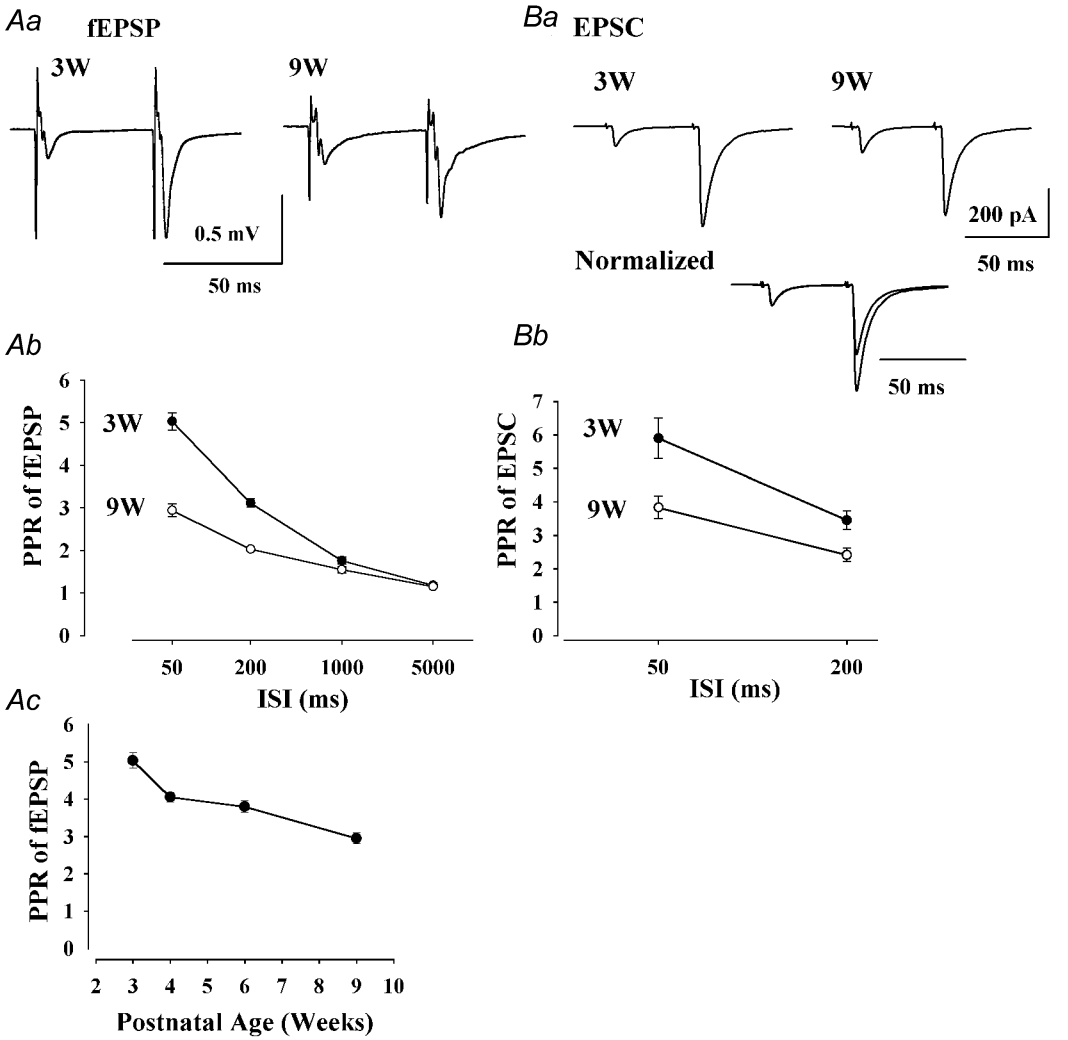

Figure 1. Developmental change in PPR

Aa, sample traces of fEPSP evoked by paired-pulse stimulations at 50 ms ISI in 3W and 9W mice. Traces are averaged fEPSPs from 40 records. *Ab*, summarized data indicating PPRs of fEPSPs at different ISIs in 3W and 9W mice. Data points and error bars indicate means and S.E.M. derived from 5 slices. The PPRs in 9W mice were significantly smaller than those in 3W mice ($P < 0.01$) at ISIs of 50 ms ($n = 6$–7) and 200 ms ($n = 6$–7). *Ac*, developmental decrease in PPR of fEPSPs (ISI, 50 ms). *Ba*, sample traces of EPSCs evoked at 50 ms ISI at 3W and 9W (averaged from 50 records each). Sample records in 3W and 9W mice are normalized at the amplitude of the first EPSCs and superimposed (bottom). *Bb*, summarized data for PPR of EPSCs at different ISIs in 3W and 9W mice. The PPR in 9W mice was significantly smaller than that in 3W mice ($P < 0.01$), at both the ISIs of 50 ms and 200 ms ($n = 7$–14).

As the stimulus intensity was gradually increased, EPSCs were evoked in an all-or-none manner. After confirming the plateau in the EPSC amplitude for a further increase in the stimulus intensity, the intensity was set in the plateau range, at 1.2–1.5 times the minimal intensity. The unitary EPSCs evoked in this way (at 0.05 Hz) had a fast rise time (20–80 %, 1.28 ± 0.21 ms at 3W, $n = 5$; 1.04 ± 0.30 ms at 9W, $n = 6$) and an exponential decay (time constant 15.4 ± 3.12 ms at 3W, 12.8 ± 4.15 ms at 9W). These values are comparable to those reported for EPSCs at this synapse in juvenile rats (16–25 days old, Toth *et al.* 2000). At both ages, EPSCs showed stochastic fluctuations in amplitude, including occasional failures (Fig. 3*A*). The mean amplitude of unitary EPSCs in 3W mice (47.5 ± 6.5 pA, $n = 7$) was similar to that in 9W mice (41.8 ± 7.1 pA, $n = 8$, Fig. 3*B*). For the Poisson model of quantal release (Del Castillo & Katz, 1954), the coefficient of variation (CV = standard deviation/mean) of evoked EPSCs is inversely related to the square root of mean quantal content (average number of quanta released at a time), and changes in CV generally reflect changes in presynaptic quantal parameters (Forsythe & Clements, 1990). The $1/CV^2$ in 3W mice (1.84 ± 0.41, $n = 7$) was not significantly different from that in 9W mice (1.46 ± 0.24, $n = 8$, $P > 0.3$, unpaired t test, Fig. 3*B*). Another index of the mean quantal content is the rate of failures, which was also similar between 3W (14.1 ± 4.3 %, $n = 7$) and 9W (16.7 ± 2.3 %, $n = 8$, $P > 0.4$, unpaired t test). These results suggest that the mean quantal content remains similar between 3W and 9W.

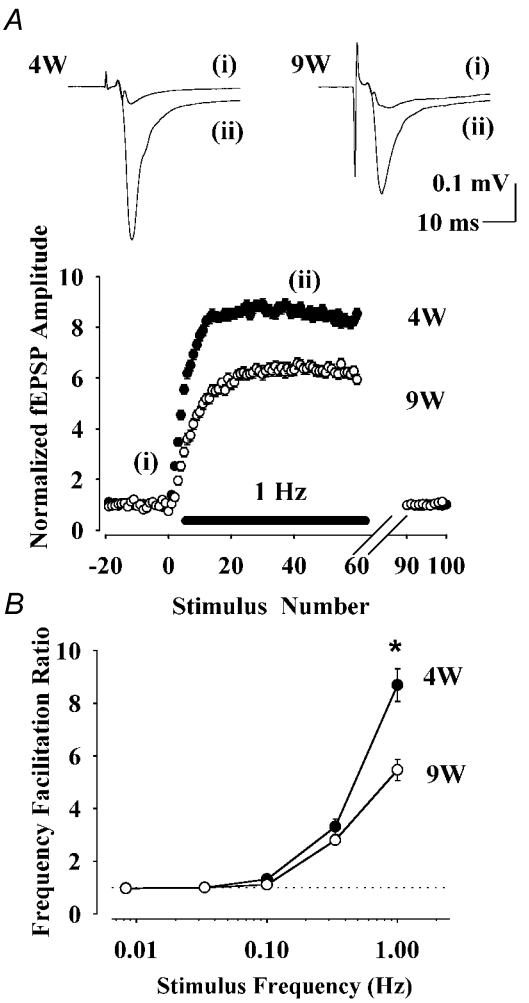

Figure 2. Developmental change in FFR

A, the stimulus frequency to evoke fEPSPs was switched from 0.033 Hz (sample trace i) to 1.0 Hz as indicated by the horizontal bar (sample trace ii superimposed) in 4W (●)and 9W (○) mice. The ordinate indicates the fEPSP amplitude normalized to that evoked at the basal frequency. *B*, the magnitude of FFR at different stimulus frequencies (0.0083, 0.033, 0.1, 0.33 and 1.0 Hz) in 4W and 9W mice. Each data point was derived from 4–6 slices. The FFR magnitude between 4W and 9W at 1 Hz was significantly different ($P < 0.01$).

The EPSC amplitude and the PPR at various extracellular Ca^{2+}/Mg^{2+} ratios

To further assess the effect of varying P on the amplitude of EPSCs and PPRs, we changed extracellular Ca^{2+} and Mg^{2+} concentrations in the ACSF for a wide range of Ca^{2+}/Mg^{2+} ratios (between 0.6 and 16). Over this range of Ca^{2+}/Mg^{2+} ratios the EPSC amplitude changed about 34-fold in 3W and 52-fold in 9W mice (not significantly different, $P > 0.3$) (Fig. 4*A*). The PPR (ISI, 50 ms) in 3W mice was consistently greater than that in 9W mice, irrespective of Ca^{2+}/Mg^{2+} ratio, and in contrast to what is reported at the MF–CA3 synapse in younger mice (14 days old, Blatow *et al.* 2003), was inversely related to the Ca^{2+}/Mg^{2+} ratio at both ages (Fig. 4*B*). Essentially the same results were obtained for fEPSPs (data not shown). However, the

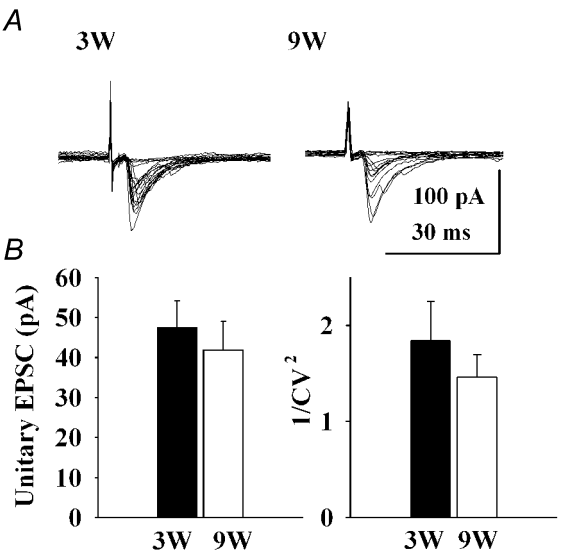

Figure 3. Unitary EPSCs evoked by minimal stimulation in developing mice

A, unitary EPSCs at 3W and 9W showing stochastic fluctuations in amplitude; 40 records are superimposed in each. *B*, the mean amplitude of EPSCs (left) and CV^{-2} (right) compared between 3W and 9W mice. There was no significant difference between 3W and 9W ($P > 0.3$).

dependence of PPR on the Ca^{2+}/Mg^{2+} ratio was relatively weak at both 3W and 9W. For example, in 3W mice, an increase in Ca^{2+}/Mg^{2+} ratio from 0.65 to 8.0 decreased PPR by 40 % (Fig. 4*B*), similar in magnitude to developmental decrease. This manipulation increased the EPSC amplitude over 17-fold (Fig. 4*A*). Given the constant mean amplitude, CV and failure rate of unitary EPSCs between 3W and 9W, it is unlikely that a change in P is responsible for the developmental decrease in synaptic facilitation at the MF–CA3 synapse.

Presynaptic Ca^{2+} transients at the developing MF–CA3 synapse

Direct recordings from MF terminals have shown that presynaptic Ca^{2+} influx is facilitated during repetitive stimulation because of action potential broadening (Geiger & Jonas, 2000). The presynaptic Ca^{2+} influx during repetitive stimulation might be differentially modulated between ages, thereby causing the difference in facilitation. To examine this possibility, we recorded Ca^{2+} transients in MFs using the low-affinity Ca^{2+} indicator mag-fura-5 simultaneously with fEPSPs. In the paired-pulse protocol at 50 ms ISI, the first stimulus caused a Ca^{2+} transient, which rose to a peak within 5 ms followed by a slow decay, on which the second Ca^{2+} transient was superimposed (Fig. 5). At the onset of the second Ca^{2+} transient, the first Ca^{2+} transient declined to 36.9 ± 2.9 % (n = 10) of its peak amplitude in 3W mice, and to 44.9 ± 5.2 % (n = 8) in 9W mice (not significantly different, $P > 0.1$), suggesting that the decay time kinetics of the Ca^{2+} transients is similar between 3W and 9W mice. The Ca^{2+} transients showed small PPF (1.1 ± 0.03, n = 10 at 3W and 1.1 ± 0.04, n = 8 at 9W), whereas the amplitude of simultaneously recorded fibre volleys remained constant between the first and second stimuli (1.0 ± 0.01 in 3W and 1.0 ± 0.01 in 9W). A similar magnitude of Ca^{2+} transient facilitation was observed in the presence of CNQX and D-APV (Fig. 5*B* and *C*; 1.09 ± 0.04, n = 6 at 3W, and 1.09 ± 0.03, n = 6 at 9W), confirming that Ca^{2+} signals arise from presynaptic fibres. Using the high-affinity Ca^{2+} indicator rhod-2 AM, Kamiya *et al.* (2002) have recently reported a similar but larger Ca^{2+} transient facilitation in MF terminals in younger mice (14–20 days old), which can be attenuated by blocking kainate receptors (see below for discussion). Although Ca^{2+} transient facilitation may contribute to synaptic facilitation, it does not account for the developmental decrease in synaptic facilitation.

Possible involvement of protein kinases in the developmental decrease in synaptic facilitation

CaMKII is thought to be involved in FF because its inhibitor KN-62 reduces FFR at the MF–CA3 synapse in guinea-pig hippocampus (Salin *et al.* 1996). We examined whether CaMKII is involved in the developmental change of FFR and PPR by first testing the broad-spectrum kinase inhibitor H-7 (100 μM) on PPR. H-7 had no effect on the PPR either in 3W (107 ± 7 %, n = 7; control, 4.19) or in 9W mice (99 ± 2 %, n = 6; control, 2.30). We next tested the CaMKII inhibitor KN-62 (3.5 μM) on FFR and PPR. It had no significant effect on FFR in either 3W (109 ± 8 %, n = 6; control, 7.08) or 9W mice (101 ± 8 %, n = 6; control, 5.56). It also had no effect on PPR in 3W (106 ± 5 %, n = 7; control, 4.30) or in 9W mice (109 ± 5 %, n = 9; control, 3.05). Given that these blockers effectively attenuate long-term depression at this synapse (Kobayashi *et al.* 1999), these results suggest that CaMKII is not involved in the mechanism of synaptic facilitation, at least in our experimental conditions. The different results for the effect of KN-62 on FFR might arise from the difference in species (guinea-pigs *vs* mice) or in the age of animals (5–15 days old; Salin *et al.* 1996).

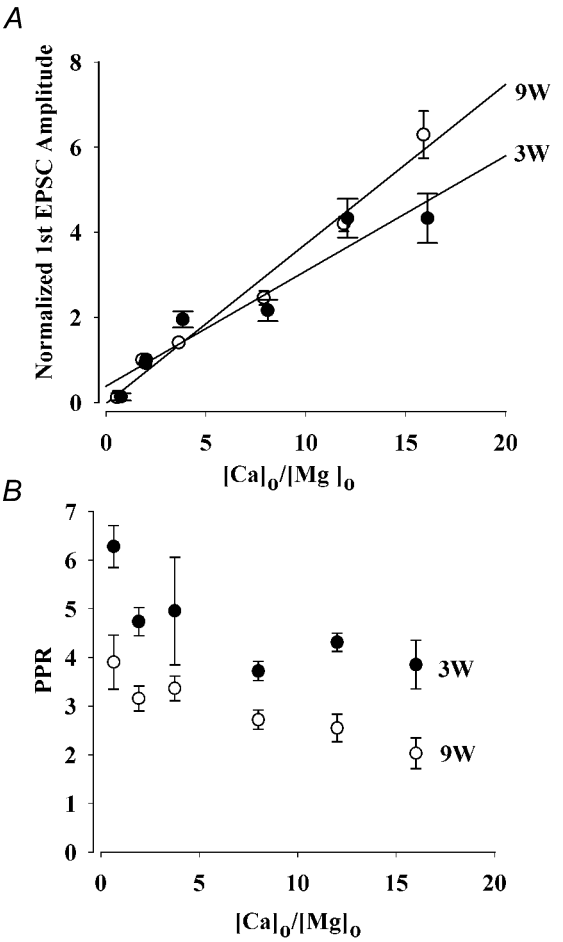

Figure 4. Dependence of the EPSC amplitude and PPR on the ratio of extracellular Ca^{2+} and Mg^{2+} concentrations

A, the relationships between the relative EPSC amplitude and the Ca^{2+}/Mg^{2+} ratio (0.65–16) in 3W and 9W mice. The EPSC amplitudes were normalized to that at 2.5 mM $[Ca^{2+}]_o$ and 1.25 mM $[Mg^{2}]_o$ (Ca^{2+}/Mg^{2+} ratio = 2.0). Ca^{2+}/Mg^{2+} concentrations at each data point are 1.5/2.3, 2.5/1.25, 3/0.8, 4/0.5, 6/0.5 and 8/0.5, respectively. Each data point derives from 4–8 cells. The lowest two points (at Ca^{2+}/Mg^{2+}, 1.5/2.3) are 0.13 (3W) and 0.12 (9W), respectively. *B*, the relationship between PPR and Ca^{2+}/Mg^{2+} ratio in 3W and 9W mice.

Possible involvement of presynaptic receptors in developmental changes in synaptic facilitation

During repetitive stimulation, endogenous ligands released from nerve terminals can activate presynaptic autoreceptors, thereby modulating subsequent synaptic responses. Any developmental change in this autoreceptor function could in principle affect PPR. MF terminals are thought to express kainate receptors, and exogenously applied kainate either facilitates or depresses the synaptic transmission depending upon the concentration used (Kamiya & Ozawa, 2000; Schmitz *et al.* 2001). To determine whether presynaptic kainate receptors are involved in the developmental change in PPR, we compared the PPR of NMDA-EPSCs (at +40 mV holding potential) between 3W and 9W mice after blocking AMPA/kainate receptors by CNQX (10 μM, Fig. 6*A*). In both 3W and 9W mice, the magnitudes of PPR of NMDA-EPSCs at the 50 ms ISI (4.3 ± 0.3, $n = 4$, 3W; 2.8 ± 0.4, $n = 5$, 9W) were significantly smaller than that of AMPA-EPSCs (5.4 ± 0.9, 3W; 3.5 ± 0.4, $n = 5$, 9W, $P < 0.05$, *F*-test, Fig. 6*A*). These results are consistent with the facilitatory role of kainate autoreceptors in the paired-pulse stimulation protocol (Schmitz *et al.* 2001; Kamiya *et al.* 2002). However, our results do not exclude a possible contribution of saturation or desensitisation of NMDA receptors to the smaller PPR. Similar to AMPA-EPSCs, PPR of NMDA-EPSCs in 9W mice was significantly less than that in 3W mice ($P < 0.03$). The developmental decrease in PPR of NMDA-EPSCs (by 35 %) was comparable to that of AMPA-EPSCs (by 42 %). These results suggest that kainate autoreceptors are not crucially involved in the developmental decrease in PPR.

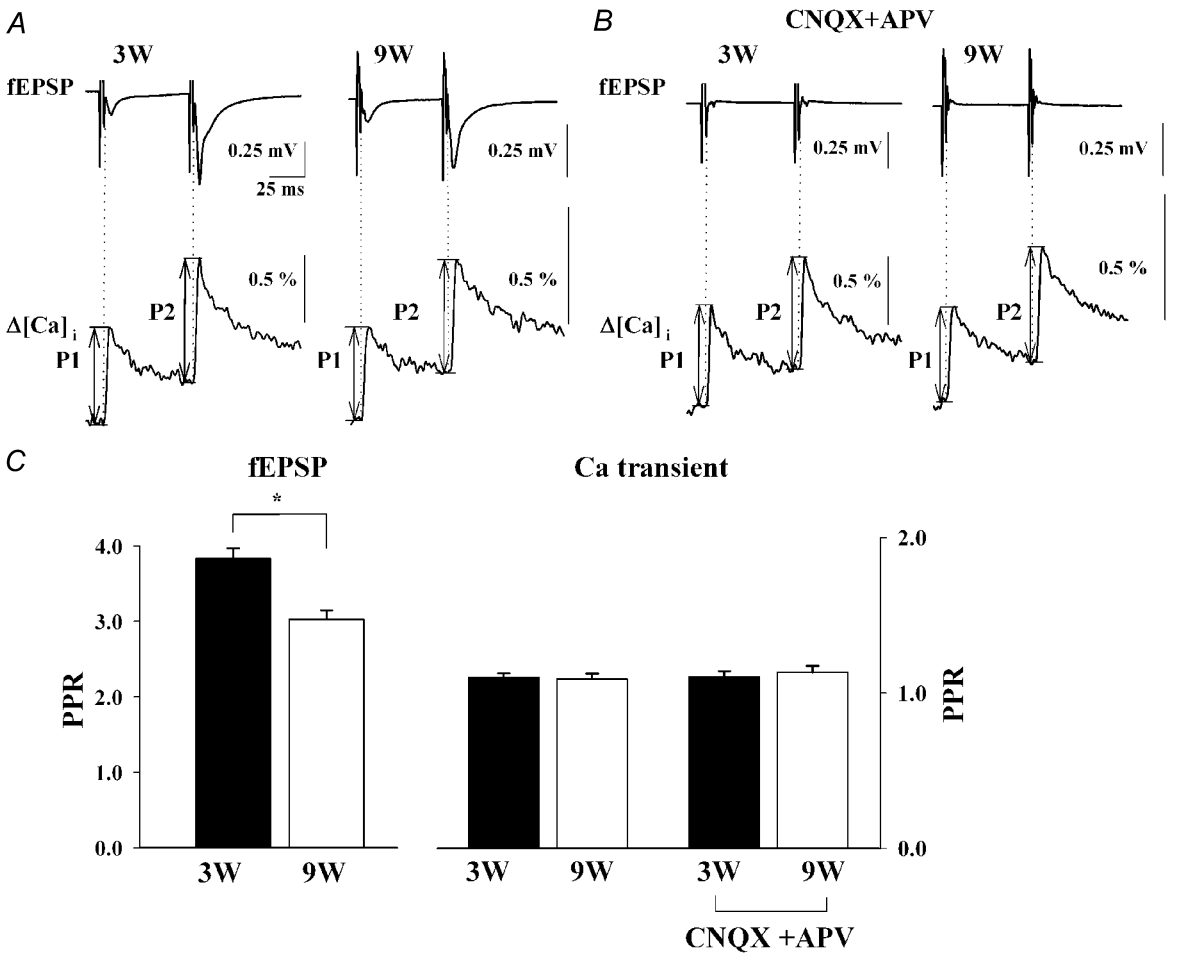

Figure 5. Ca^{2+} transients in MF terminals evoked by paired-pulse stimulations (ISI, 50 ms, 0.05 Hz) and monitored using mag-fura-5

A, field potentials (top traces) were simultaneously recorded with Ca^{2+} transients (bottom traces) in 3W (left) and 9W (right) mice. *B*, field potentials and Ca^{2+} transients after blocking excitatory synaptic transmission with CNQX (10 μM) and APV (25 μM) in 3W and 9W mice; recorded from the same slice as in *A*. *C*, summary of PPR of fEPSPs and Ca^{2+} transients. There was no significant difference in the amplitude ratio of Ca^{2+} transients (P2/P1) between 3W and 9W mice in the presence or absence of CNQX and APV, whereas the PPR of fEPSPs in 3W mice (3.8 ± 0.1, $n = 10$) was significantly larger than that in 9W mice (3.0 ± 0.1, $n = 8$, $P < 0.05$) in these experiments. The magnitude of fibre volley did not change in response to paired-pulse stimulation at both ages.

ATP released from nerve terminals (Silinsky & Hubbard, 1973; Jo & Schlichter, 1999) is rapidly converted to adenosine (Dunwiddie *et al.* 1997) and activates presynaptic adenosine receptors. We next examined whether PPR is modulated by presynaptic adenosine receptors. Bath application of adenosine at a high concentration (100 μM) reduced fEPSP amplitude by 44.0 ± 6.4 % ($n = 5$) in 3W mice and by 54.2 ± 4.6 % ($n = 4$) in 9W mice (Fig. 6*B*). However, adenosine had no effect on PPR in both 3W (96.5 ± 2.5 % of control) and 9W mice (97.2 ± 2.4 % of control, normalized records in Fig. 6*B*), suggesting that presynaptic adenosine receptors are not involved in PPR or its developmental change. The lack of change in PPR despite a large decrease in the fEPSP amplitude after adenosine application is consistent with weak dependence of PPR on the release probability at this synapse (Fig. 4).

The group II metabotropic glutamate receptors (mGluR2/3) are expressed at the preterminal axonal region of MF (Yokoi *et al.* 1996), and are involved in the induction of long-term depression of MF–CA3 synaptic transmission (Kobayahsi *et al.* 1996; Yokoi *et al.* 1996). We examined whether mGluR2/3 might be involved in PPR using (2*S*)-2-amino-2-[(1*S*, 2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495, 100 nM; Tocris, Bristol, UK). LY341495 at this concentration specifically antagonizes mGluR2/3 (Johnson *et al.* 1999). LY341495 blocked the inhibitory effect of DCG-IV on the MF–CA3 EPSPs (data not shown), but had no effect on the amplitude of fEPSP (0.95 ± 0.06, $n = 5$ at 3W; 1.03 ± 0.07, $n = 5$ at 9W) or PPR (1.02 ± 0.05 at 3W, 1.12 ± 0.11 at 9W, Fig. 6*C*). These results are consistent with the report that a broad-spectrum mGluR antagonist has no significant effect on the second response to paired-pulse stimulation at this synapse (Scanziani *et al.* 1997). Thus mGluR2/3 is unlikely to be involved in PPR or its developmental change.

Differential effects of BAPTA-AM on PPF

Our results do not support an involvement of basal release probability or facilitation of Ca^{2+} influx in the developmental change of synaptic facilitation. Also, neither CaMKII nor developmental changes in autoreceptors can explain the age-dependent difference in synaptic facilitation. What mechanism might then underlie this developmental change? The residual Ca^{2+} remaining after the first Ca^{2+} entry is thought to induce synaptic facilitation (Katz & Miledi, 1968; Zucker & Regehr, 2002). Any change associated with the residual Ca^{2+} would in principle affect synaptic facilitation. To test this possibility, we examined the effect of reducing intra-terminal Ca^{2+} concentration

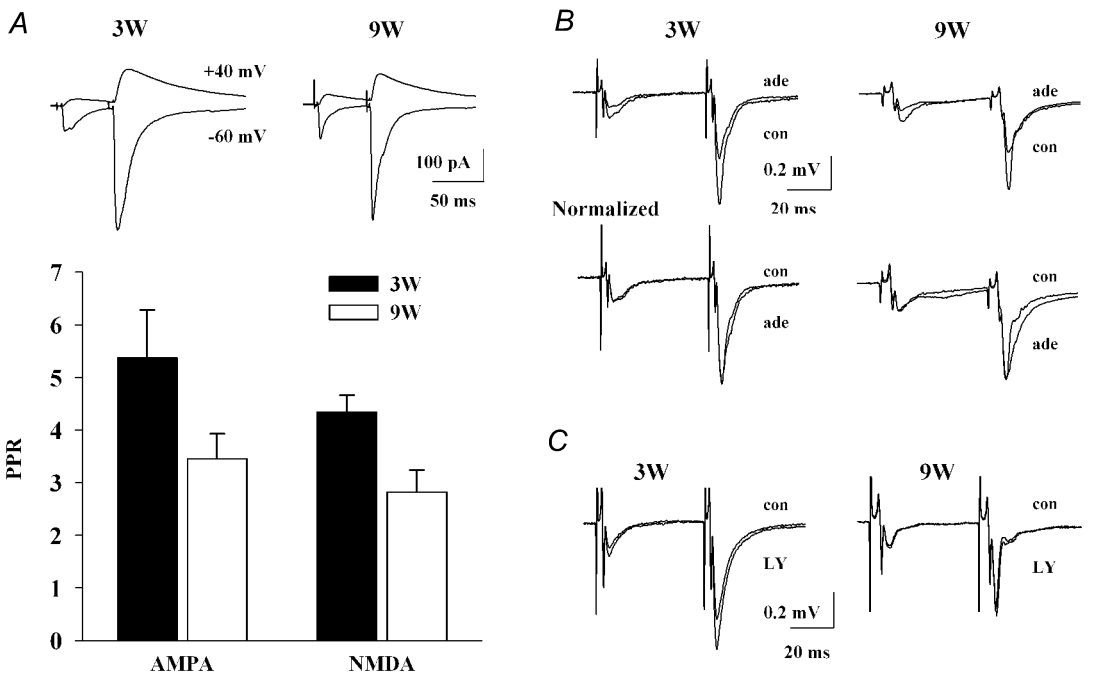

Figure 6. Lack of involvement of presynaptic receptors in the developmental decrease of synaptic facilitation

A, AMPA-EPSCs and NMDA-EPSCs evoked by the paired-pulse protocol in 3W and 9W mice (ISI, 50 ms). NMDA-EPSCs (superimposed with AMPA-EPSCs) were recorded after blocking AMPA/kainate receptors with CNQX at a holding potential of +40 mV. The PPR of NMDA-EPSCs in 3W mice was significantly larger than that in 9W mice. *B*, adenosine (ade, 100 μM) attenuated fEPSPs (fEPSPs at 3W and 9W before and after adenosine application are superimposed in top column), but did not affect PPR at both 3W and 9W (the two records are normalized to the first amplitude and superimposed). *C*, LY341495 (LY, 100 nM) had no effect on fEPSPs or PPR at both 3W and 9W. Field EPSPs evoked by the paired-pulse protocol, before and after LY341495 application, are superimposed both in 3W and 9W mice.

using the membrane-permeable Ca^{2+} chelator BAPTA-AM (50 μM, Molecular Probes, OR, USA). After bath application of BAPTA-AM for 25 min, fEPSP amplitude decreased by 36.2 ± 5.9 % (n = 11) in 3W mice (Fig. 7*A*) and by 27.3 ± 3.8 % (n = 10) in 9W mice (Fig. 7*B*), suggesting that BAPTA is effectively loaded into the MF terminal at both ages. A clear difference was observed between the effect of BAPTA-AM on PPR in 3W and 9W mice. After BAPTA-AM application, PPR decreased by 29.6 ± 3.8 % in 3W mice, whereas it decreased by only 7.5 ± 3.6 % in 9W mice (Fig. 7*C*). Thus BAPTA-AM markedly reduced the age-dependent difference in PPR.

Because attenuation of EPSPs by BAPTA-AM in 3W mice was slightly stronger on average than in 9W mice, it might be argued that the differential effect of BAPTA-AM is caused by a difference in the BAPTA concentration reached in the MF terminal because of possible age differences in the diffusion barrier in slices. Given that the attenuating effect of BAPTA-AM on the first EPSP amplitude varied widely (3.4–60 % in 3W and 9.4–48 % in 9W), we selected those data having the effect falling into 15–50 % from both age groups to match the effect of BAPTA on the first EPSP (3W, reduction by 30.9 ± 4.2 %, n = 6; 9W, by 31.2 ± 3.8 %, n = 8). In these sets of data, the magnitude of PPR reduction by BAPTA was still significantly larger in 3W mice (28.4 ± 5.3 %) than in 9W mice (8.1 ± 4.9 %) ($P < 0.01$). Thus the differential effect of BAPTA cannot simply be attributed to differences in the concentration of BAPTA in the MF terminals.

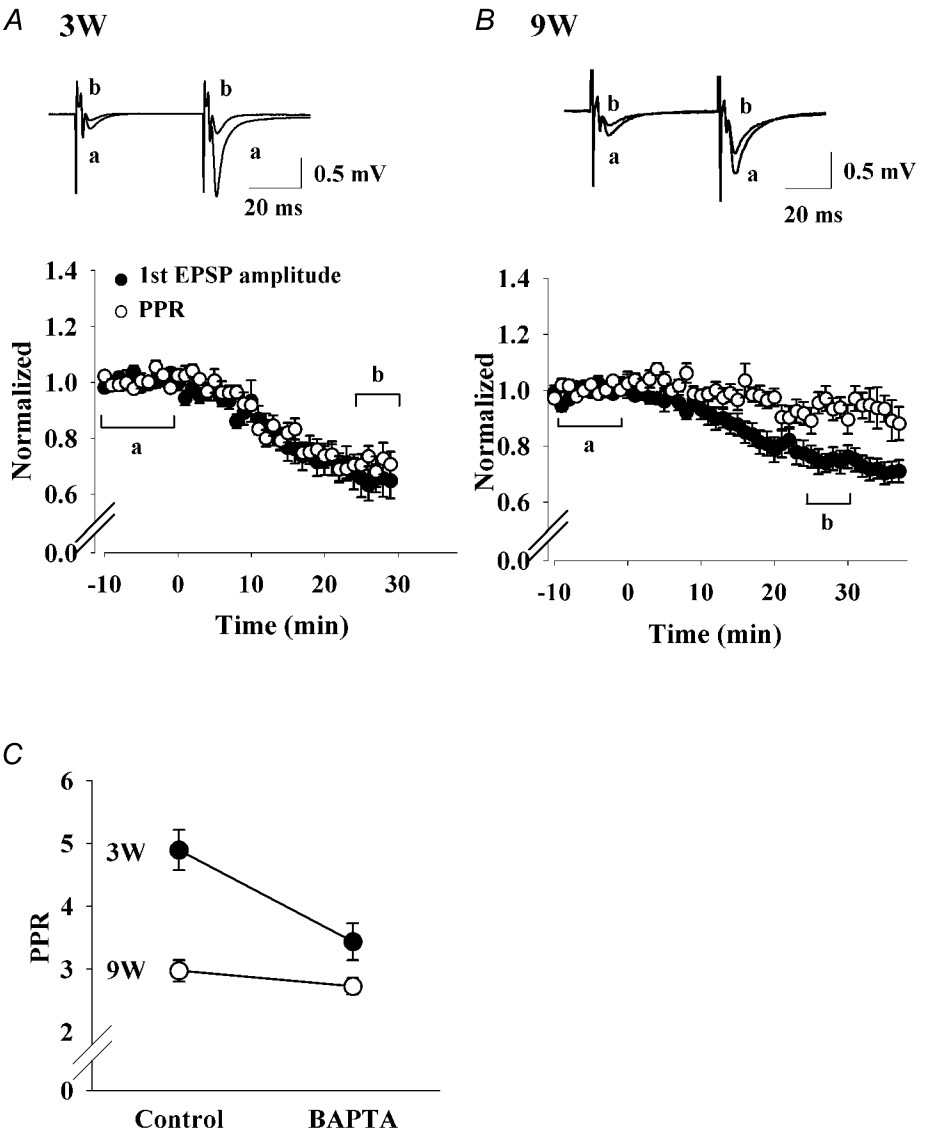

Figure 7. Differential effects of BAPTA-AM on PPR in 3W and 9W mice

A and *B*, time plots of the first fEPSP amplitude (●) and PPR(○) (both normalized to control) after bath application of BAPTA-AM (50 μM) in 3W mice (n = 10, *A*) and in 9W mice (n = 11, *B*). The upper panels show fEPSPs before and after BAPTA application (superimposed) at 3W (*A*) and 9W (*B*). *C*, aummary data of the mean PPR before and after BAPTA application in 3W and 9W mice. All values are calculated from the entire data set.

DISCUSSION

We have demonstrated that PPRs of both fEPSPs and EPSCs decrease with postnatal development from 3W to 9W at the MF–CA3 synapse in mice. This contrasts with the developmental increase in PPR of EPSCs during earlier postnatal periods at other CNS synapses (Bolshakov & Siegelbaum, 1995; Choi & Lovinger, 1997; Pouzat & Hestrin, 1997; Taschenberger & von Gersdorff, 2000; Iwasaki & Takahashi, 2001). Whereas the developmental changes in PPR have been commonly ascribed to the change in *P*, our results at the MF synapse cannot be explained by such a mechanism.

Mechanism underlying developmental changes in PPR

The PPR is widely used as an index for *P* (Manabe *et al.* 1993), because PPR is generally higher when *P* is lower and vice versa (Otsuka *et al.* 1962; Katz & Miledi, 1968). This can be explained by the greater depletion of releasable synaptic vesicles at higher *P* (Betz, 1970; Weis *et al.* 1999). The marked facilitation at the MF–CA3 synapse suggests a very low *P* at this synapse. At the MF–CA3 synapse, stimulation at 100 Hz causes a 20- to 40-fold facilitation of EPSCs (Langdon *et al.* 1995). Similarly, application of caffeine (5 mM) in combination with a 2.5-fold increase in the Ca^{2+}/Mg^{2+} ratio causes a 30-fold increase in fEPSP amplitude (K. Kobayashi, unpublished observation). These data suggest that *P* is less than 0.03 at this synapse. Consistently, unitary EPSCs in the present study showed relatively large fluctuations in amplitude including occasional failures, despite the fact that a single MF bouton makes up to 37 synaptic contacts with a single CA3 pyramidal cell (Chicurel & Harris, 1992). This characteristic behaviour clearly distinguishes the MF–CA3 synapse from other CNS synapses. For example, *P* estimated for the calyx of Held synapse is 0.25–0.4 (in a 8- to 10-day-old rat; Meyer *et al.* 2001) and that for the CA3–CA1 synapse is 0.5 (in 2- to 3-week-old rats; Bolshakov & Siegelbaum, 1995). At the MF–CA3 synapse, because of the low *P* and a large pool size of releasable vesicles (Hallermann *et al.* 2003), the releasable vesicles cannot be easily depleted by repetitive stimulation. This explains the relatively weak dependence of PPR on *P* at this synapse upon changes in the Ca^{2+}/Mg^{2+} ratio or after adenosine application. A large reduction in PPR, despite the unchanged unitary EPSC amplitude during development, suggests that a change in *P* cannot be the primary mechanism underlying the developmental decrease in MF synaptic facilitation.

Residual Ca^{2+} is thought to underlie synaptic facilitation (Katz & Miledi, 1968; Zucker & Regehr, 2002). The residual free Ca^{2+} summates with Ca^{2+} that enters at the second impulse and facilitates the second synaptic response (Katz & Miledi, 1968), being amplified by the non-linear relationship between Ca^{2+} and transmitter release (Dodge & Rahamimoff, 1967). In the present study, the Ca^{2+} chelator BAPTA-AM reduced the PPR more markedly in 3W than in 9W mice, for the entire data set as well as for the selected data after matching the effect of BAPTA-AM on the first EPSP. Assuming no age difference in the sensitivity of the first EPSP to BAPTA, the simplest explanation for these results would be that the residual Ca^{2+} concentration decreases with development because of the strengthened effect of endogenous Ca^{2+} buffers. If the occupancy of endogenous Ca^{2+} buffer by the first Ca^{2+} entry is greater at 3W than 9W, PPR can be larger at 3W because of Ca^{2+} buffer saturation (Blatow *et al.* 2003; Felmy *et al.* 2003), and intraterminal loading of exogenous Ca^{2+} buffer will reduce PPR at 3W more efficiently than at 9W. The MF terminal highly expresses the fast Ca^{2+} binding protein calbindin-D_{28K} (Celio, 1990). A developmental increase in the expression of calbindin-D_{28K} in MF might underlie the developmental decrease in synaptic facilitation.

Whereas a change in the strength of Ca^{2+} buffer would be expected to alter the time course of Ca^{2+} transients, our results showed no such difference between 3W and 9W mice. Even if the overall strength of Ca^{2+} buffer does not change much with development, an increase in the proportion of the fast buffer to slow buffers will affect the Ca^{2+} dynamics at the Ca^{2+} channel domain near the release site, thereby possibly affecting the PPR. The strengthening of fast Ca^{2+} buffers can reduce *P* as well as PPF. We did not see an age difference in the quantal content. However, this does not necessarily indicate that *P* was constant because developmental reduction in *P* can be compensated for by a concomitant increase in the number of releasable quanta or the number of release sites as at the calyx of Held (Iwasaki & Takahashi, 2001). If *P* decreases with development, however, it would contribute to an increase in the magnitude of synaptic facilitation, in an opposite direction to that observed in the present study.

Recently Blatow *et al.* (2003) have reported, in contrast to our results, that the magnitude of PPF is positively correlated with the extracellular Ca^{2+}/Mg^{2+} concentration ratio at the MF synapse. Whereas the reason for this discrepancy is unclear, our experimental conditions are different from theirs in the following respects: (i) in our study mice were 3–9 weeks old, whereas mice in their study were 2 weeks old. The PPF caused by Ca^{2+} buffer saturation requires optimal Ca^{2+} buffer strength (Blatow *et al.* 2003), which might be attained only at a restricted developmental period. (ii) Their data are based entirely upon whole-cell recordings, whereas ours are based upon both whole-cell and field recordings. Changing Ca^{2+}/Mg^{2+} concentration ratio can alter the excitability and the number of input fibres, and thereby potentially affect the EPSC amplitude. This effect will be greater on EPSCs than fEPSPs because of the fewer number of input fibres involved. (iii) In our study, synaptic responses were evoked by stimulation of the dentate gyrus, whereas in theirs the stratum lucidum

was stimulated. Because of the higher number of synapses in the stratum lucidum, stimulation of this region may induce release of transmitters around the stimulation site, which can affect the excitability of MFs via presynaptic receptors. (iv) Inhibitory synaptic transmission was intact in our study, whereas it was blocked in theirs. This makes a difference in the excitability of the cells in the slice, thereby possibly leading to different results.

The residual Ca^{2+} may bind with a putative high-affinity site, thereby increasing the Ca^{2+} sensitivity of transmitter release (Atluri & Regehr, 1996; Bertram *et al.* 1996; Tang *et al.* 2000). If the amount or Ca^{2+} sensitivity of such a 'facilitation site' decreases with development, the PPR and its BAPTA sensitivity would be reduced without changes in Ca^{2+} buffers or residual Ca^{2+} concentrations. Although the molecular identity of the 'facilitation site' remains open, CaMKII is one of the candidates. In heterozygous mice with their α-CaMKII genetically ablated, PPR at the hippocampal CA1 synapse is reduced with no change in basal synaptic efficacy (Chapman *et al.* 1995). Also, at the immature MF–CA3 synapse in the guinea-pig hippocampus, the CaMKII inhibitor KN-62 reduces FFR (Salin *et al.* 1996). However, our present results disagree with this report because KN-62 had no effect on synaptic facilitation in mice.

Our results also indicate that PPF remains after application of BAPTA-AM. Similar BAPTA-resistant PPF reported at the crayfish neuromuscular junction has been attributed to the effect of residual bound Ca^{2+} (Winslow *et al.* 1994). Presynaptic Ca^{2+} transients in the MF terminals, when repeated at a 50 ms interval, are facilitated by 10 % (this study) or 20 % (Kamiya *et al.* 2002). Assuming a fourth power relationship between Ca^{2+} concentration and transmitter release (Dodge & Rahamimoff, 1967), this corresponds to a synaptic facilitation of 46–107 %. Kamiya *et al.* (2002) have also shown that the facilitation of Ca^{2+} transients at the MF terminal is attenuated by blocking kainate receptors with CNQX, and they concluded that this facilitation was in part mediated by kainate autoreceptors. In our present study, in contrast, CNQX had no effect on the magnitude of facilitation of the Ca^{2+} transients, suggesting that kainate receptors are not involved in the facilitation of the Ca^{2+} transient. This discrepancy might arise from the difference in the experimental conditions (e.g. mag-fura-5 *vs.* rhod-2 for Ca^{2+} indicators) or the age of mice used (> 21 days old in our study *vs.* 14–20 days old in their study). The facilitation of the Ca^{2+} transient observed in the present study may be mediated by the activity-dependent broadening of presynaptic action potentials (Geiger & Jonas, 2000), or by the Ca^{2+} current facilitation, which may be dependent on neuronal calcium sensor-1 (NCS-1, Tsujimoto *et al.* 2002) or calmodulin (DeMaria *et al.* 2001). Whatever the mechanism, given no age difference in the facilitation of Ca^{2+} transients at the MF terminal, it does not account for the developmental change in synaptic facilitation.

There are other mechanisms that can potentially affect PPR. These include desensitization of postsynaptic receptors (Trussell *et al.* 1993; Rozov *et al.* 2001), and feedback modulation via presynaptic autoreceptors (von Gersdorff *et al.* 1997; Schmitz *et al.* 2001). At the MF–CA3 synapse, PPR of NMDA-EPSCs underwent a developmental decrease similar to that of AMPA-EPSCs. This parallel change in AMPA- and NMDA-EPSCs suggests that postsynaptic factors such as desensitisation of AMPA receptors may not contribute significantly to the developmental change in PPR. Our results also suggest that presynaptic receptors such as kainate receptors, adenosine receptors or mGluRs do not play a crucial role in the developmental change in PPR.

Physiological role of synaptic facilitation and its developmental change

The MF terminal is one of the largest nerve terminals in the CNS. Despite its large size, unitary EPSPs at a low firing frequency would not exceed the action potential threshold of the postsynaptic CA3 pyramidal cell. However, this synapse possesses a wide dynamic range of synaptic efficacy because of its prominent facilitation. Occasional bursts of action potentials in a single dentate granule cell give rise to suprathreshold EPSPs in CA3 pyramidal cells (Henze *et al.* 2002), thereby enabling the informational flow from granule cells to CA1 pyramidal cells. Furthermore, burst activation of a single MF input can provide postsynaptic activity sufficient for the induction of long-term potentiation at converging associational/ commissural inputs (Kobayashi & Poo, 2002). Our present results indicate a developmental decrease in this dynamic range of transmission at the MF–CA3 synapse. This indicates a decrease in the ability of MF to affect the CA3 circuitry with development, which may potentially contribute to a decrease in the capacity of memory formation and/or recall. However, because dentate granule cells are continuously generated during postnatal development and their survival is regulated by environment and behaviour (Gould *et al.* 1999), newly formed synapses may compensate for the developmental decrease in the dynamic range of facilitation of MF synapses.

Epilepsy is more common in infants than adults (Wong & Yamada, 2001) and temporal lobe epilepsy is related to changes in the excitability of CA3 pyramidal cells associated with MF sproutings (Anderson *et al.* 1999). Experimentally induced MF sproutings lead to epileptic seizures in animals (Anderson *et al.* 1999). In these respects, a developmental decrease in the gain of facilitation at the MF–CA3 synapse may protect CA3 pyramidal cells from over-excitation, thereby contributing to the stability of the hippocampal neuronal network.

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