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Late embryonic expression of AMPA receptor function in the CA1 region of the intact hippocampus *in vitro*

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Abstract

Studies in slices suggest that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated synaptic currents are not present in CA1 (*Cornu ammonis*) pyramidal neurons at birth (P0). We have re-examined this issue in the rat intact hippocampal formation (IHF) *in vitro*. Injections of biocytin or carbocyanine show that the temporo-ammonic, commissural and Schaffer collateral pathways are present at birth in the marginal zone of CA1. Electrical stimulation of these pathways evoked field excitatory postsynaptic potentials (fEPSPs) in the marginal zone of CA1 from embryonic day 19 (E19) to postnatal day 9 (P9). These fEPSPs are mediated by synaptic AMPA receptors as they are reduced or completely blocked by: (i) tetrodotoxin; (ii) high divalent cation concentrations; (iii) the adenosine A1 receptor agonist CPA; (iv) anoxic episodes; (v) the selective AMPA receptor antagonist 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine (GYKI-53655) or the mixed AMPA-kainate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX). The amplitude of the fEPSPs is also reduced by D(–)-2-amino-5-phosphonopentanoic acid (D-APV) and its duration is increased by bicuculline suggesting the participation of *N*-methyl-D-aspartate (NMDA) and $GABA_A$ (γ-aminobutyric acid) receptors. Finally, AMPA receptor-mediated fEPSPs are also recorded in P0 slices, but they are smaller and more labile than in the IHF. Our results suggest that in embryonic CA1 neurons, glutamate acting on AMPA receptors already provides a substantial part of the excitatory drive and may play an important role in the activity-dependent development of the hippocampus. Furthermore, the IHF may be a convenient preparation to investigate the properties of the developing hippocampus.

Introduction

The activity-dependent development and differentiation of neuronal networks generally requires an increase of intracellular concentration of calcium ions (Ca^{2+}) that can occur via voltage-dependent calcium channels and/or ionotropic glutamate receptors, in particular *N*-methyl-D-aspartate (NMDA) receptors. Because NMDA receptors are blocked in a voltage-dependent manner by magnesium ions, their activation requires both the presence of glutamate and the depolarization of the membrane. In the adult rat hippocampus, this depolarization is generally provided by the activation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors that are colocalized with NMDA receptors (Kharazia *et al*., 1996; He *et al*., 1996). A different situation seems to prevail during the embryonic and early postnatal period, as recent patch-clamp experiments indicate that functional AMPA receptors cannot be detected in CA1 (Cornu ammonis) pyramidal neurons until P1–2 (Durand *et al*., 1996). However, this observation is surprising as AMPA receptor subunits have been detected by immunochemical methods before P2 in rat CA1 pyramidal cell bodies (Arai *et al*., 1997; Martin *et al*., 1998). Their mRNAs have also been detected in CA1 by *in situ* hybridization (Monyer *et al*., 1991) and Northern analysis (Durand & Zukin, 1993). Whether these receptors are functional already at birth is not known. We have therefore re-investigated this issue using extracellular recordings of field excitatory postsynaptic potentials (fEPSP) that provide a statistical representation of synaptic responses. A similar approach has been used to show the presence, already at E47, of synaptic excitation in the subplate of the cat visual cortex (Friauf & Shatz, 1991). We centred our study on the recently developed intact hippocampal formation (IHF) (Khalilov *et al*., 1997) that allows the use of neonatal interconnected structures that have kept most of their inputs, outputs and neuronal arborization, and that seems to be particularly relevant in immature hippocampi in which there is a small density of synapses.

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Materials and methods

Animals

Wistar rats were raised in our breeding centre and all protocols were designed to minimize animal suffering as well as the number of animals used, according to INSERM guidelines for animals care and use. Female rats were mated overnight, the day of insemination being noted at embryonic day 0 (E0). Birth usually occurred on E22 and was designated as postnatal day 0 (P0). In the present study, P0 animals were killed less than 6 h after birth. E18–21 embryos were removed by Caesarean section after ether anaesthesia of the mother. Brains from E18–P9 animals were rapidly dissected out in ice-cold

artificial cerebrospinal fluid (ASCF) containing (in mM): NaCl, 126; KCl, 3.5; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; $CaCl_2$, 2; $NaHCO_3$, 25; glucose, 11; saturated with 95% O_2 and 5% CO_2, pH 7.4. The dissected IHF were kept at room temperature in oxygenated ACSF until use.

Morphology

Tract-tracing experiments were conducted on P0 IHF. For carbocyanine-tracing experiments, the IHF containing the entorhinal cortex was directly fixed for 1 week in 4% paraformaldehyde. A small crystal of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indolcarbocyanine perchlorate (DiI) was inserted into the region of interest with the aid of a glass micropipette. After 5–7 weeks incubation at 37 °C in the dark, the tissue was embedded in 3% agar, then cut at 60 µm with a vibratome. Sections were counterstained with bis-benzidine, then examined with an epifluorescence microscope. Biocytin injection (5% in ACSF) was applied in the hippocampal pars ventralis (prospective CA3) with a patch-clamp pipette under visual control of the preparation, maintained in perfused ACSF. After a 4–8-h survival period to allow tracer diffusion, the tissue was fixed in 4% paraformaldehyde for 2 days. After cryoprotection in a 30% sucrose solution, 60-µm-thick slices were prepared with a freezing microtome. Biocytin revelation was performed using diaminobenzidine as a chromogen.

Electrophysiology

Dissected IHF were transferred to a fully submerged chamber and perfused with ACSF at a flow rate of 4–6 mL/min at 31 °C. A modified ACSF, obtained by replacing glucose by sucrose and gassed with 95% N_2 and 5% CO_2 (pH 7.4) was perfused during anoxic-aglycaemic episodes. fEPSPs were evoked upon stimulation (0.017 Hz, 30 µs duration, 30–50 V) with a bipolar electrode generally placed in the inner marginal zone of CA1. The inner and outer marginal zones that appear around the second week of gestation give rise to the stratum radiatum and stratum lacunosum-moleculare, respectively. In some experiments, the stimulating electrode was placed in the commissure or the entorhinal cortex. fEPSPs were recorded using a glass microelectrode filled with NaCl (3 M). Conventional transverse hippocampal slices (400 µm) were prepared and allowed to equilibrate in gassed ACSF at room temperature before use.

Drugs

Bicuculline methochloride, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) and D(–)-2-amino-5-phosphonopentanoic acid (D-APV) were obtained from Tocris Cookson (UK), tetrodotoxine (TTX) from Latoxan (France), biocytin, N^6-cyclopentyl adenosine (CPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were provided by Sigma (France). DiI was purchased from Molecular Probes (USA) and 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine (GYKI-53655) was a gift from Dr D. Leander (Lilly Research Laboratories, USA).

Results

Morphological evidence for the presence of CA1 afferent fibres at P0

The marginal zone of CA1 receives three main excitatory afferent pathways: (i) the temporo-ammonic path from the entorhinal cortex; (ii) the commissural path from the contralateral hippocampus; (iii) the Schaffer collaterals from the ipsilateral CA3 region. Carbocyanine-tracing experiments were performed to determine whether, as in the mouse (Super & Soriano, 1994; Super *et al*., 1998), these fibres are present at birth in the rat hippocampus. The IHF preparation is particularly suitable for that purpose as it is possible to inject the dye in intact interconnected limbic structures *in vitro*. Injection of DiI in the entorhinal cortex (both medial and lateral parts) revealed the presence of a densely arborized network of fibres in the CA1 prospective stratum lacunosum moleculare, and to a much lesser extent in the stratum oriens (Fig. 1A). This projection was observed throughout the septo-temporal hippocampal axis, with the stratum oriens-labelled fibres being more frequent in the septal pole. Similar

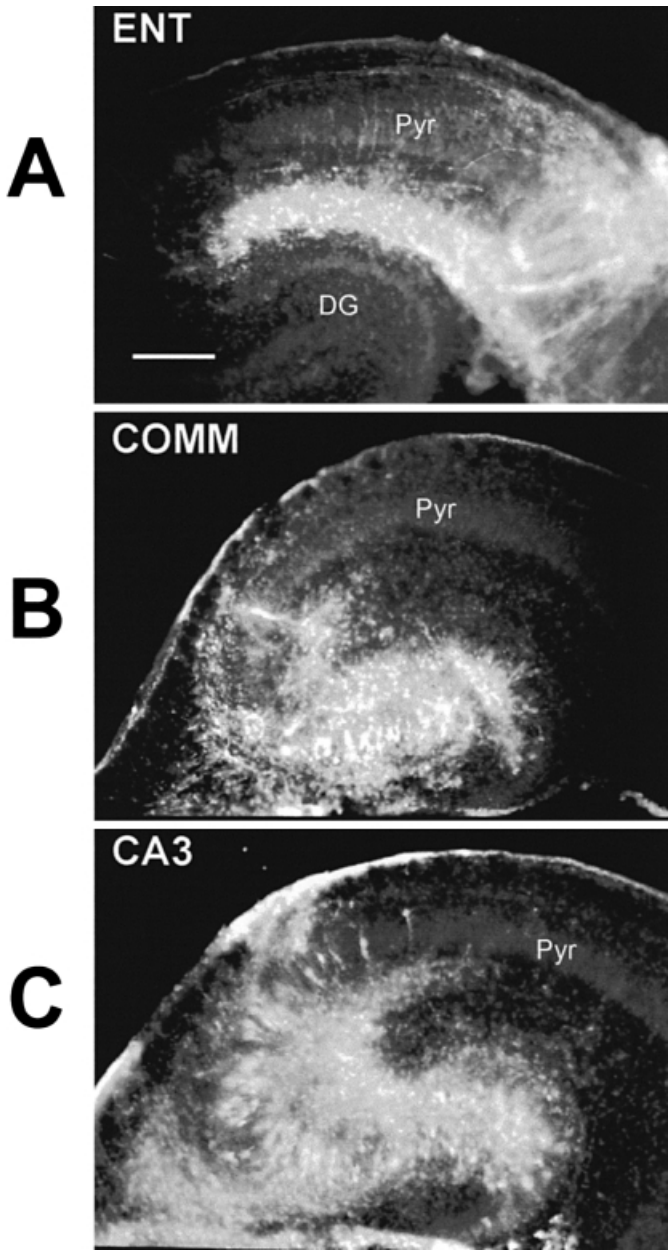

FIG. 1. Carbocyanine experiments demonstrate the presence at P0 of the three major excitatory fibre systems in the CA1 region of the IHF. Double exposure (rhodamine filter for DiI and ultraviolet filter for bis-benzidine) micrographs of hippocampal sections from a P0 rat taken from a similar mid-septo-temporal level after injection of carbocyanine in (A) entorhinal cortex (ENT); (B) commissure (COM); or (C) the CA3 region (CA3). Note that all these fibre tracts are present in CA1. Scale bar, 100 µm.

injections directly in the commissural fibres revealed the presence of retrogradely labelled CA3 pyramidal cells and hilar interneurons (Fig. 1B). The retrogradely labelled CA3 pyramidal cells were located in the lateral region in the septal pole and in the medial region in the temporal pole. Anterogradely labelled fibres penetrate the hippocampus through the ventral part of the fimbria and arborize densely in the stratum radiatum of CA3 and (to a lesser extent) CA1. The commissural fibres in the CA1 stratum radiatum were much more developed in the septal pole than in the temporal pole, where they were nearly absent. Finally, injection of DiI in CA3 revealed one set of fibres coursing in the fimbria toward the contralateral hippocampus (where retrogradely labelled CA3 pyramidal cells were observed) and the septum, and another set travelling in the CA1 inner marginal zone (Fig. 1C). They probably represent both Schaffer collaterals and commissural fibres as carbocyanine injection in CA3 also labelled en-passant commissural fibres. We therefore used biocytin injections to verify the presence of Schaffer collaterals at P0 (Fig. 2). Biocytin-labelled CA3 pyramidal cells and axons were present at the injection site (Fig. 2A) and in the CA1 inner marginal zone (Fig. 2B). Varicosities, suggestive of en-passant synaptic contacts, were also observed (Fig. 2C insert). Reconstruction along the septo-temporal axis revealed that these fibres are present in several rostro-caudal sections (Fig. 2D), indicating that they are not already restricted at this early developmental stage to the plane of CA3 pyramidal somata from which they originate. Therefore, the temporo-ammonic, commissural and Schaffer fibres are present at birth in the marginal zone of CA1 of the rat and are sufficiently developed to establish synaptic contacts.

Evoked fEPSP in the marginal zone of CA1 at P0

We took advantage of the IHF preparation to examine whether the pathways are functional. The two interconnected hippocampi, septal complexes and entorhinal cortices, were placed on the ventral part (Fig. 3A). We first observed that when the stimulating electrode (S1) was placed in the inner marginal zone of CA1, a fEPSP of $517 \pm 13\,\mu V$ ($n = 112$) was always recorded in this area (R1) with a latency to peak of 5.7 ± 0.1 ms ($n = 112$). An afferent volley was also observed when the stimulating electrode was close to the recording

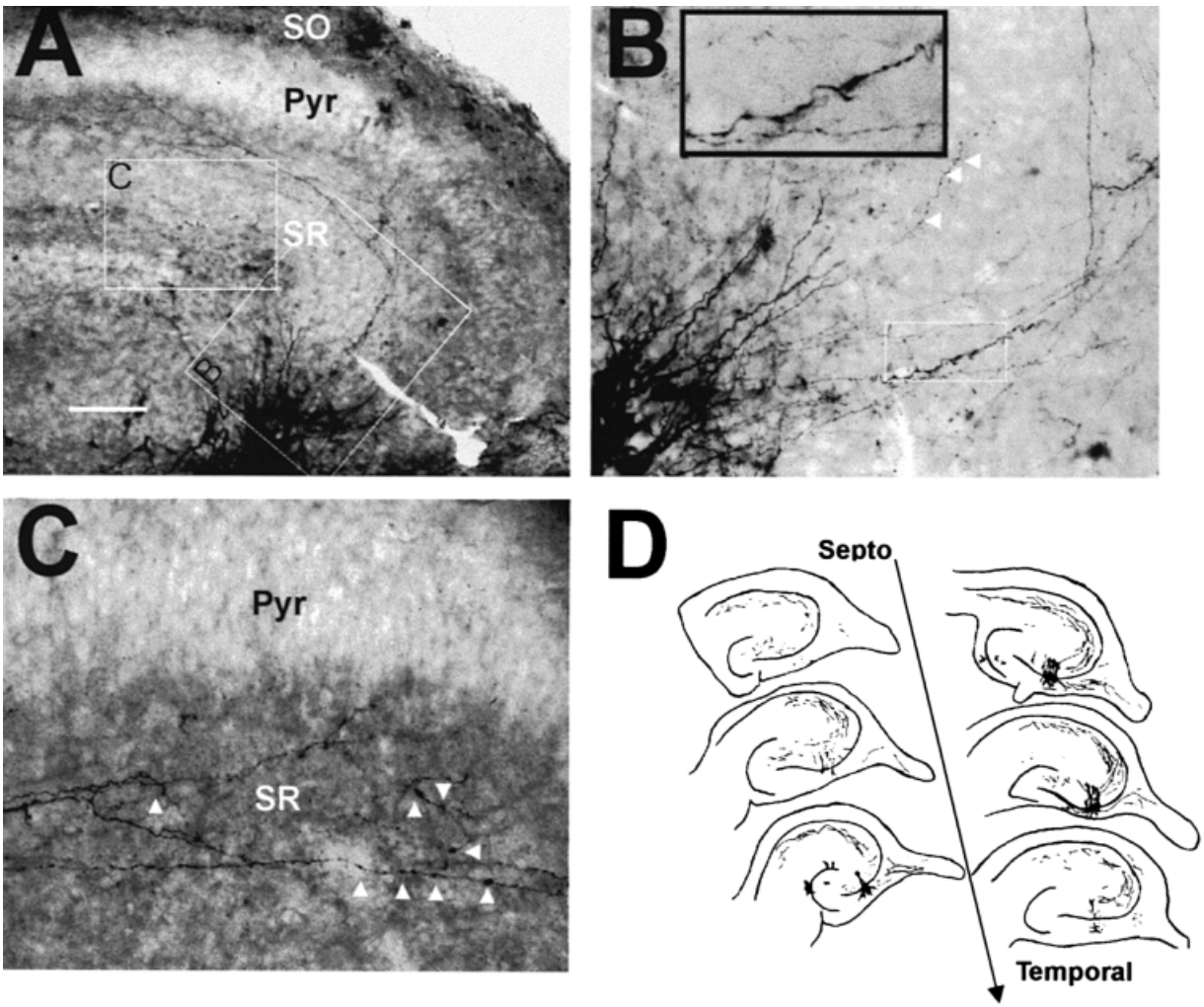

FIG. 2. Biocytin injection in the IHF confirms the presence of Schaffer collaterals at P0. In this experiment, few CA3 pyramidal cells were labelled with biocytin. (A) Schaffer collateral axons originating from labelled CA3 cells are clearly identified in the inner marginal zone of CA1. Scale bar, 100 µm. The contents of the two white rectangles were represented at a higher magnification in (B) (× 2) and (C) (× 4). (B) The departure of collateral axons from CA3 cells is clearly seen. (C) Schaffer collaterals often exhibit varicosities (arrows). (D) Serial drawings (more septal section top left, mode temporal, bottom right) of biocytin-labelled elements after a small injection in CA3 (injection site shown in A). Note the high divergence of Schaffer collaterals that are present in 660-µm sections while the injection site is restricted to three sections.

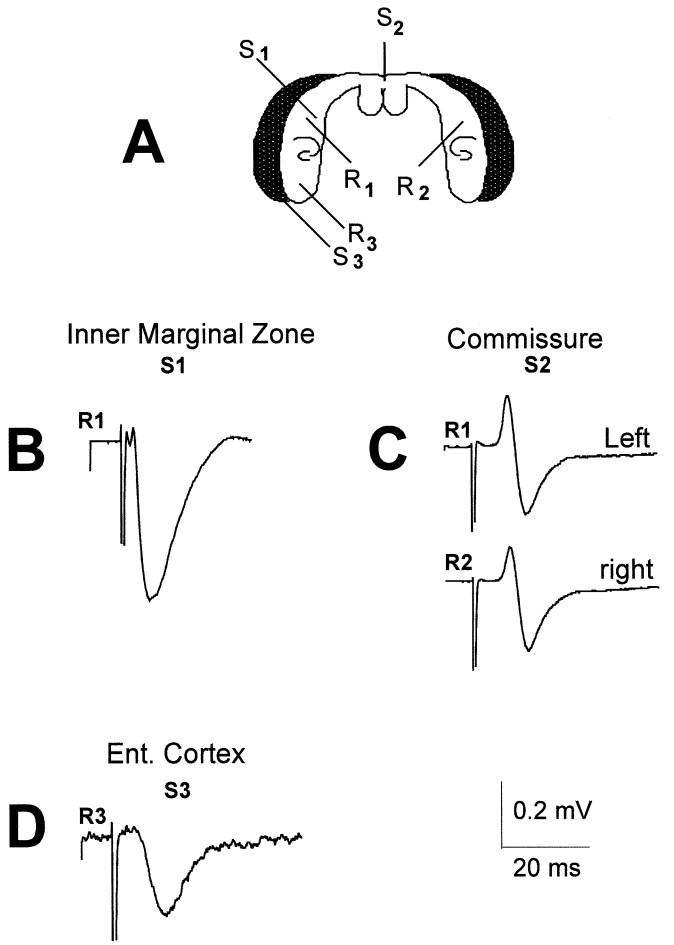

FIG. 3. Electrical stimulation of afferent pathways at P0 evoked fEPSPs in the marginal zone of CA1. (A) Schematic representation of the IHF and localization of the position of the stimulating (S1, S2, S3) and recording electrodes (R1, R2, R3). (B) fEPSP evoked in the marginal zone (R1) following electrical stimulation (S1) of Schaffer collaterals in the same transversal plane, close to R1. Note the presence of an afferent volley. (C) Electrical stimulation of the commissure (S2) evokes a fEPSP in the marginal zone of CA1 in the left (R1) and right (R2) hippocampus. (D) Electrical stimulation of the temporal part of the entorhinal cortex (S3) and recording in the hippocampus (R3).

electrode (Fig. 3B). Because both Schaffer associational and commissural fibres are present in this region, we tried then to specifically evoke fEPSP at commissural fibres. Stimulation directly in the commissure (S2) of two interconnected hippocampi evoked fEPSPs in the CA1 area of each hippocampus (R1) and (R2) (Fig. 3C). The fEPSP amplitude (347 ± 41 μV; $n = 11$) decreased whereas the delay increased with the distance of the recording electrodes from S2 (not shown). Stimulation of the temporo-ammonic pathway in the entorhinal cortex close to the temporal end of the hippocampus (S3) also evoked a fEPSP in the hippocampus (R3) (Fig. 3D) characterized by a small amplitude (185 ± 29 μV; $n = 7$) and a short latency (7.1 ± 0.3 ms). Stimulation at other cortical places either failed to evoke a fEPSP or evoked one with a longer delay (> 15 ms).

Profiles of the fEPSP in the IHF at birth

Because stimulation in the inner marginal zone of CA1 evokes a fEPSP throughout the IHF, its spatio-temporal profile was then determined in three dimensions (Fig. 4A). The stimulating electrode was placed where the maximal response was evoked, generally around 300 μm from the surface in the inner marginal zone of CA1 close to the CA2 border. Along the *z*-axis (depth), the negative fEPSPs peaked between 300 and 350 μm from the surface and then decreased, a positive field being recorded at more superficial sites ($n = 5$, Fig. 4B). Similar profiles were obtained when the stimulating electrode was placed in the commissure ($n = 6$), indicating that the contacts between afferents fibres and CA1 cells occur mainly in the marginal zone of CA1.

The stimulating electrode was then placed at the inner marginal zone of CA1 in the septal part (in A1) and the recording electrode was moved along the septo-temporal axis (*x*-axis) (in B1, C1, D1 or E1) (Fig. 4C, $n = 6$), or along the *y*-axis (in A2 or A3) (Fig. 4D, $n = 4$). A fEPSP was evoked throughout the IHF, highlighting that the connectivity of the network is intact. Its amplitude decreased rapidly and significantly when the recording electrode was moved along the septo-temporal axis or along the *y*-axis (Fig. 4C and D). The delay of the fEPSP can be measured between two points in the IHF (Fig. 4D), and the delay between the septal and temporal poles of the IHF was 16 ± 2 ms.

Properties of the fEPSP in the intact hippocampus at birth

Analysis of the characteristics of the responses evoked at P0 by electrical stimulation of the inner marginal zone of CA1 indicated that they are synaptic responses as the slopes of fEPSPs were: (i) reversibly blocked ($-94 \pm 6\%$, $n = 3$) by application of TTX (1 μM) for 3 min (Fig. 5A); (ii) significantly reduced by high concentrations (4 mM) of magnesium and calcium ions ($-26 \pm 5\%$, $n = 6$, Fig. 4B), conditions known to reduce the release probability of neurotransmitters; (iii) dramatically reduced ($-79 \pm 8\%$, $n = 3$, Fig. 4C) by an anoxic-aglycaemic episode (10 min) that also decreases glutamatergic transmission in slices from immature rats (Cherubini *et al.*, 1989); (iv) reduced by presynaptic selective A_1 adenosine receptor agonist, CPA (100 nM, $-66 \pm 9\%$, $n = 3$). In contrast, the selective A_1 receptor antagonist DPCPX (200 nM) increased the slope of the fEPSP ($+24 \pm 7\%$, $n = 5$, Fig. 5D). These results indicated that the release of glutamate is operative at birth and that adenosine exerts a tonic control on this release through presynaptic A_1 receptors.

Pharmacological characterization of the fEPSP-mediated components at birth

These experiments were performed on the intact hippocampus. The fEPSPs evoked by electrical stimulation of the inner marginal zone of CA1 were inhibited by CNQX, a non-specific AMPA/KA receptor antagonist ($-82 \pm 3\%$, $n = 12$), or by GYKI-53655 (30 μM, $-84 \pm 4\%$, $n = 6$), a relatively selective antagonist of AMPA receptors in cultured hippocampal neurons (Paternain *et al.*, 1995, Fig. 6A), indicating that kainate receptors are probably not involved in this response.

NMDA and γ-aminobutyric acid (GABA) A receptors contribute to the fEPSP, as following blockade of AMPA receptors with CNQX (10 μM) the remaining response was completely blocked by D-APV (50 mM) and bicuculline (10 μM, Fig. 6Ba). Furthermore, D-APV (50 μM) or bicuculline (10 μM) reduced by $12 \pm 2\%$ ($n = 18$, Fig. 6Bb) and $10 \pm 2\%$ ($n = 23$, Fig. 6Bc), respectively, the amplitude of the fEPSP. To examine the contribution of NMDA and $GABA_A$ receptors, AMPA receptors were first blocked with NBQX (3 μM), a selective AMPA receptor antagonist that does not affect NMDA receptors (Parsons *et al.*, 1994, Fig. 6Cb). As with GYKI-53655 or CNQX, ~80% of the response was affected by NBQX ($-83 \pm 5\%$, $n = 3$). In the presence of NBQX (maintained throughout the experiment), the remaining response was increased by lowering the concentration

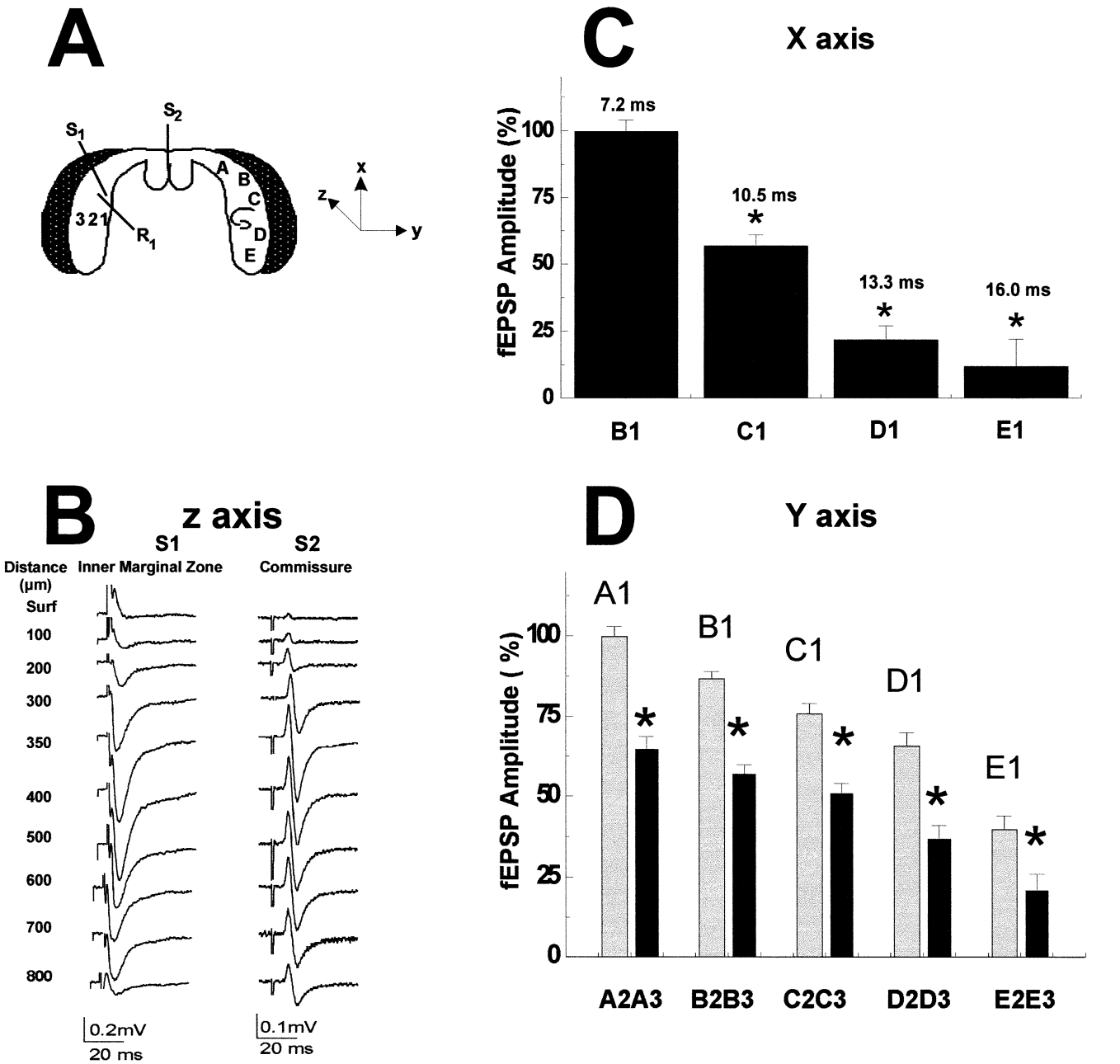

FIG. 4. Spatio-temporal characteristics of the fEPSP at P0 in the marginal zone of CA1. (A) Schematic representation of the IHF and localization of the recording positions along the *x*-, *y*- and *z*-axes. (A)–(E) Registration sites located along the septo-temporal axis (*x*-axis); A corresponds to the more septal position and E to the more temporal one; 1–3 correspond to CA1 sites located on the same transversal line (*y*-axis), 1 is near CA2 and 3 is close to the subiculum. (B) Laminar profiles (along *z*-axis) obtained in the marginal zone of CA1 following electrical stimulation of Schaffer collaterals (S1) and the commissure (S2), respectively. Records were made every 100 µm starting from the alveus (Surf). (C) Along the *x*-axis, the stimulating electrode was positioned in A1 at the septal pole and the recording electrode was successively positioned in B1, C1, D1 and E1. The asterisk denotes a statistically significant (*t*-test; $P < 0.05$, $n = 6$) decrease in the fEPSP from one position to another. One-hundred per cent corresponds to an amplitude of 473 ± 19 µV, $n = 6$. (D) The stimulating electrode was first placed in A1 and records were made in A2 (grey columns) and in A3 (black columns). Similar recordings were performed along the *y*-axis with the stimulating electrode moving along the septo-temporal axis in B1, C1, D1 and E1, respectively. The asterisk denotes a statistically significant (*t*-test; $P < 0.05$, $n = 4$) decrease in the fEPSP from one position to another one along the septo-temporal axis (grey column) or between two positions along the *y*-axis (grey versus black column). One-hundred per cent corresponds to an amplitude of 494 ± 15 µV, $n = 4$ for the grey column and 320 ± 20 µV, $n = 4$ for the black columns.

of Mg^{2+} from 1.3 to 0.1 mM (Fig. 6Cc). The fEPSP that was evoked in these conditions is mediated by GABA and NMDA receptors as it was reduced by D-APV (50 µM) and fully blocked by the addition of bicuculline (10 µM, Fig. 6Cd).

Therefore, fEPSPs evoked in the inner marginal zone of CA1 are mediated primarily by AMPA receptors, although NMDA and $GABA_A$ receptors also participate in their generation to ~ 10% each.

Embryonic and postnatal glutamatergic neurotransmission in the IHF

In order to evaluate the date of expression of glutamatergic fEPSPs, we used embryonic intact hippocampi. At E18, electrical stimulation of the CA1 inner marginal zone evoked a TTX-sensitive afferent volley but generally no fEPSPs (Fig. 7A, $n = 5/6$), suggesting that afferent

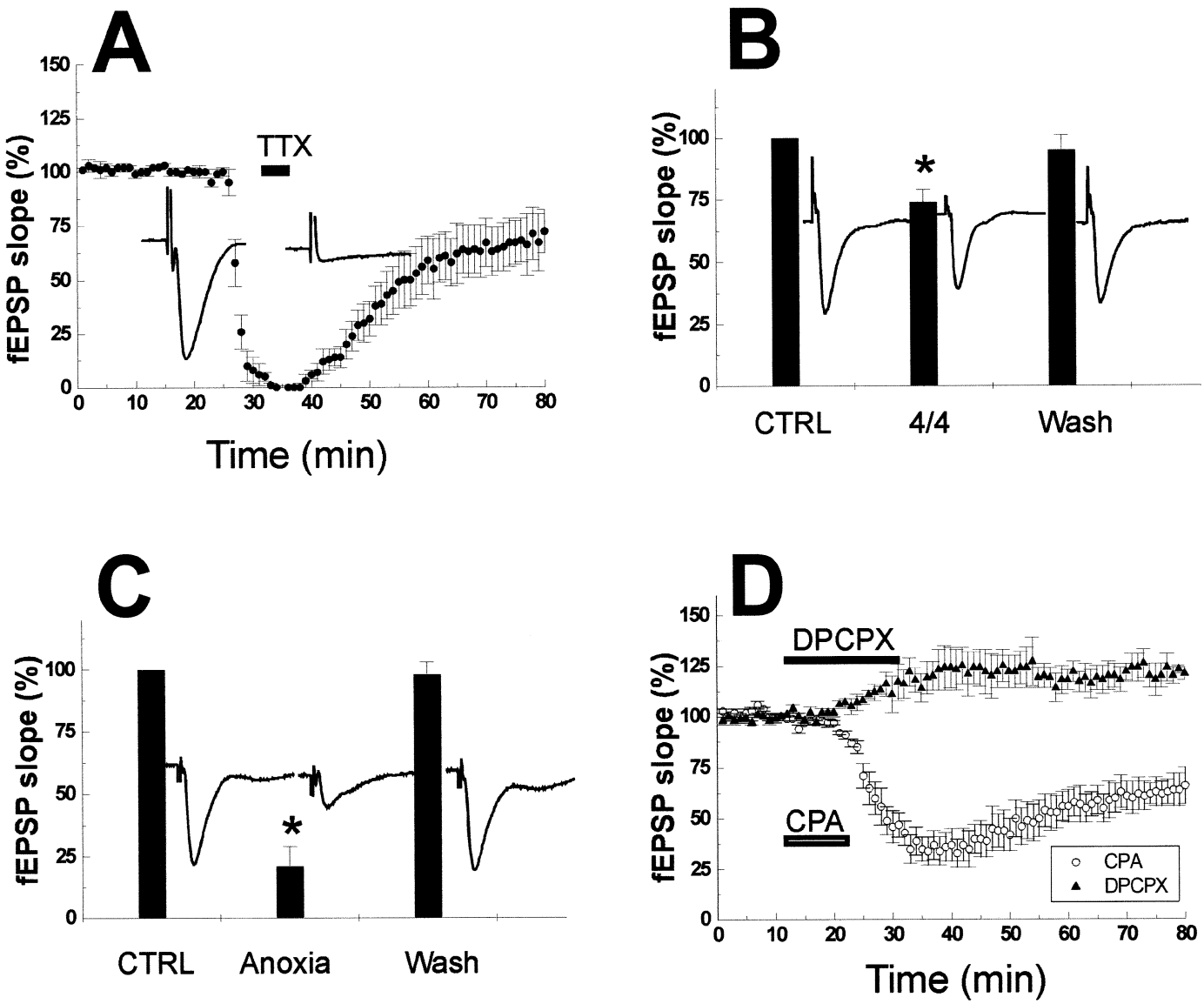

FIG. 5. Presynaptic modulations of the fEPSP evoked at P0 in the marginal zone of CA1. The stimulating electrode was placed in the inner marginal zone of CA1 to stimulate Schaffer collaterals and commissural fibres. Records were performed in the same area at a frequency of 0.017 Hz. (A) Application of TTX (1 μM) for 3 min reversibly inhibits the slope of the evoked fEPSP. (B) The slope and amplitude of the fEPSP were reversibly and significantly (*t*-test; $P < 0.05$) reduced by an increase of Ca^{2+} from 2 to 4 mM and Mg^{2+} from 1.3 to 4 mM, respectively. (C) An anoxic-aglycaemic episode of 10 min was performed by replacing glucose by sucrose in the ACSF and the oxygen by nitrogen in the perfusing buffer. Note that the synaptic transmission was significantly (*t*-test; $P < 0.05$), but not completely inhibited. (D) CPA (100 nM) perfused for 10 min, reduces the slope and the amplitude of the fEPSP. Note the slow recovery of the slope upon washout of CPA. (D) DPCPX (200 nM) was applied for 30 min, inducing an increase in the slope and amplitude of the fEPSP. This effect is long lasting. For both A1 adenosine ligands, $P < 0.05$, 20 min after washout of the drugs (*t*-test).

fibres are present in the inner marginal zone but not in the synaptic complex. fEPSPs were in contrast consistently observed at E19 (136 ± 15 μV; $n = 6$) and E20 (234 ± 19 μV; $n = 3$) throughout the whole hippocampus. Already at E19, fEPSPs are blocked by CNQX ($-85 \pm 6\%$, $n = 3$) and slightly reduced by D-APV ($-8 \pm 3\%$, $n = 3$), indicating that both receptors contribute to this fEPSP. The amplitude of the fEPSP was small at E19 but increased regularly until P9, and for a similar afferent volley, the slope and amplitude of the fEPSP were larger in more mature IHF (Fig. 7B), suggesting that the number of glutamate receptors increases with development, in keeping with recent reports (Dumas & Foster, 1995; Hsia *et al.*, 1998).

fEPSPs in CA1 hippocampal slices at birth

Electrical stimulation of conventional transverse slices in the inner marginal zone of CA1 generally evoked a fEPSP of small amplitude (144 ± 27 μV, $n = 34$ out of 43), and in nine out of 43 slices no response could be evoked. At mid-septo-temporal levels of the hippocampus, fEPSP are readily saturated by increasing the intensity of stimulation (Fig. 8). For a similar afferent volley, the slope and amplitude of fEPSPs evoked in such slices were ~five times smaller than those evoked at a similar level in the IHF (Fig. 8).

Discussion

The principal conclusion of the present study is that AMPA receptor-mediated EPSPs are functional in the intact embryonic or neonatal CA1 area of the rat hippocampus. To the best of our knowledge, this is the first description of AMPA receptor-mediated synaptic response

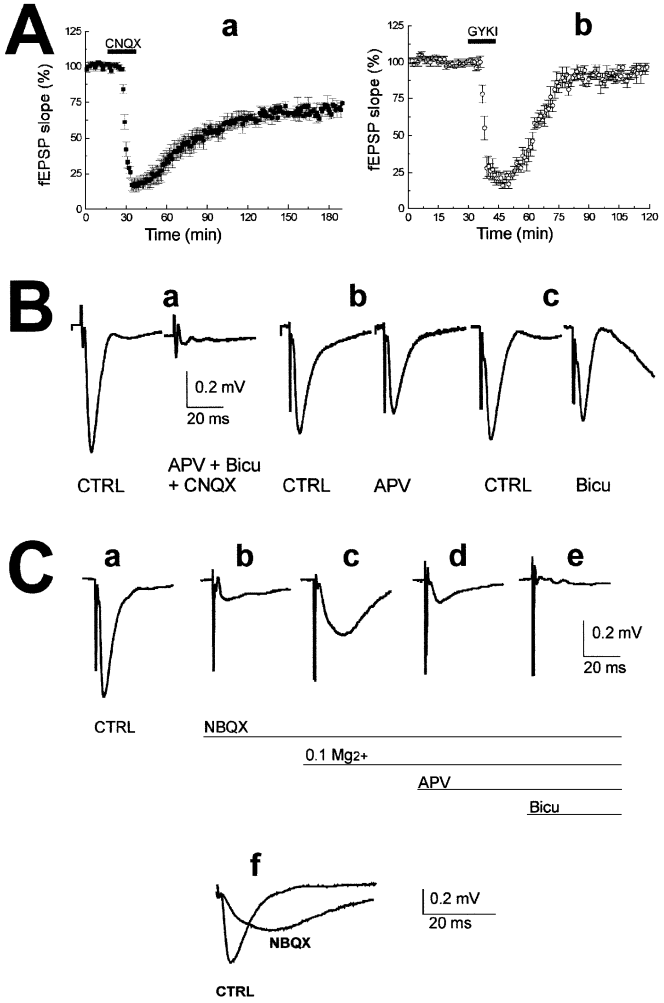

FIG. 6. Pharmacological characterization of the fEPSPs evoked at P0 in the marginal zone of CA1. (Aa) Perfusion of the IHF with CNQX (10 μM) for 10 min strongly blocks the slope and amplitude of the fEPSP. Note the slow recovery of the synaptic response upon washout of CNQX. (Ab) Perfusion of GYKI-53655 (30 μM) also reduces the slope and amplitude of the fEPSP. This effect is nearly fully reversible. (B) Significant blockade of synaptic responses obtained after 10 min application of (a) a mixture of CNQX (10 μM), D-APV (50 μM) and bicuculline (10 μM); (b) only D-APV (50 μM) and (c) only bicuculline (10 μM, t-test; $P < 0.05$). (C) In normal ACSF, the amplitude of control fEPSP (a), is reduced by 10-min application of NBQX (3 μM) (b). NBQX is continuously applied in the rest of the experiment. Decreasing the concentration of Mg^{2+} in ACSF increases the remaining fEPSP (c) and 10-min application of D-APV (50 μM) highly reduces the evoked response (d), further addition of bicuculline (10 μM) completely blocked the evoked response.

in the CA1 region of the hippocampus before birth, which suggests that AMPA receptors may play an early role in the activity-dependent development of the hippocampus.

Tracing studies performed in mice have shown that the three afferent excitatory pathways are present at birth in the marginal zone of the hippocampus (Super & Soriano, 1994; Super *et al*., 1998). In the rat, the situation is less clear except for entorhinal axons that have been detected in the CA1 area of the hippocampus at birth (Loy, 1980). Thus, the earliest tracing of commissural axons was achieved by rhodamine-labelled microspheres at P3 (Buchhalter *et al*., 1990), and no convincing data were available for Schaffer collaterals. Our observations using DiI and biocytin labelling confirmed the presence of the temporo-ammonic fibres in the outer marginal zone and the stratum oriens of the P0 rat. In addition, both commissural and Schaffer collaterals are univocally detected in CA1 inner marginal zone at birth with a higher density of fibres in the septal part than in the temporal part of the hippocampus. Electrical stimulation of Schaffer collaterals, commissural fibres and the temporo-ammonic pathway at birth, elicited TTX-sensitive synaptic responses in the marginal zone of CA1. Mature presynaptic terminals are probably involved in this process as presynaptic modulations of glutamate release by calcium ions and adenosine A1 ligands affect the amplitude and slope of the postsynaptic response. This hypothesis is supported by the synaptophysin-like immunoreactivity detected in the CA1 marginal zone at early prenatal stages (Grabs *et al*., 1994). Furthermore, in agreement with morphological studies, the amplitude of the fEPSP decreases along the septo-temporal axis, suggesting that the development of commissural fibres and Schaffer collaterals is not fully completed at birth.

The fEPSPs are mediated primarily by AMPA receptors as they were blocked by the selective AMPA receptor antagonist GYKI-53655 (Paternain *et al*., 1995) and by the broad spectrum antagonists CNQX and NBQX. They are detected from E19, but not at E18, indicating that glutamatergic synapses already operate in the late embryonic period. These observations are in agreement with the immunochemical detection of GluR1 and GluR2/R3 AMPA receptor subunits in the CA1 region at E20 (Arai *et al*., 1997), and in line with the identification of AMPA receptors at early embryonic stages in other rat brain regions including hypothalamic neurons (E15, Van den Pol *et al*., 1995) and raphe cells (E14, Konig *et al*., 1992). Finally, in addition to AMPA receptors, NMDA receptors also contribute **(~10%)** to the synaptic response, indicating that at least these two glutamate receptors are functional in the late embryonic period.

The presence of a robust AMPA receptor-mediated fEPSP at E19 is apparently unexpected in view of patch-clamp recordings from CA1 pyramidal cells in slices suggesting that functional AMPA receptor-mediated postsynaptic currents (PSCs) are detected at least 4 days later, around P2 (Durand *et al*., 1996). Several factors could, however, underlie this discrepancy including the following.

1 The intensity of stimulation, minimal (Durand *et al*., 1996) and high stimulation in this paper.

2 The difference between IHF and slices preparations. fEPSPs are expected to be larger in intact preparations because of the volume of the tissue and the destruction during the slicing procedure of many inputs and outputs. Because the number of synapses is very small in immature hippocampi, this loss may be particularly detrimental suggesting that IHF preparations are more suitable than slices to study neonatal synapses. In keeping with this, in conventional slices, electrical stimulation of the marginal zone of CA1 evoked a fEPSP of small amplitude, which rapidly saturated and fatigued in contrast to the reliable fEPSPs observed in the IHF.

3 The heterogeneity of pyramidal neurons at early stages of development. Pyramidal neurons are generated during a time window of several days, between E15 and E19, and are subjected to several gradients of maturation (Bayer, 1980; Altman & Bayer, 1990). In addition, there are important differences in their dendritic arborization and morphological properties, suggesting a heterogeneous population with different degrees of maturation (Minkwitz & Holz, 1975; Minkwitz, 1976). These morphological differences may be expressed at early developmental stages in terms of the expression of functional AMPA receptors. Indeed, in preliminary patch-clamp studies combined with morphological reconstruction, we have found that only a small percentage (10%) of CA1 pyramidal neurons, those that have an arborized apical dendrite, have functional AMPA receptor-mediated PSCs at P0 (Gozlan *et al*., 1999).

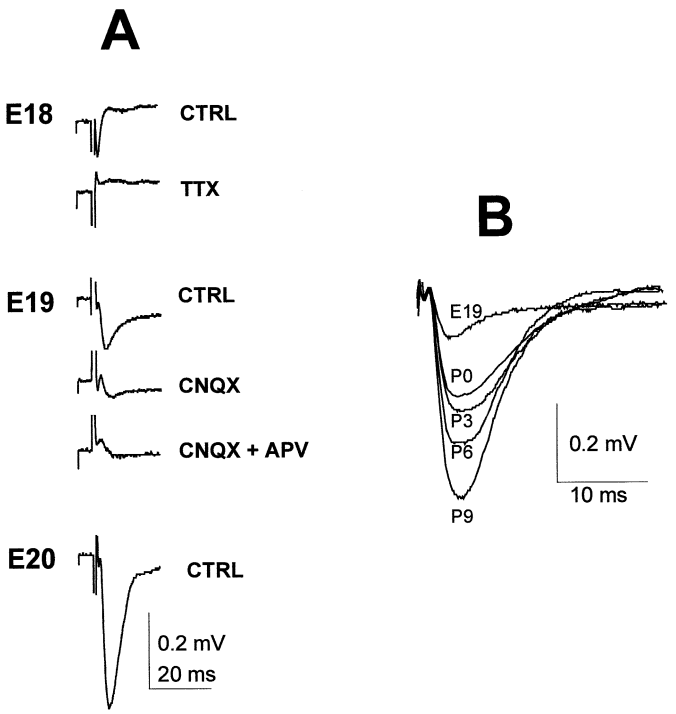

FIG. 7. AMPA receptor-mediated fEPSP from E19 to P9 in the IHF. (A) Embryonic IHF were collected at E18–20. (a) At E18, TTX (1 μM; 10 min) suppresses the afferent volley. (b) At E19, a fEPSP is consistently evoked in the marginal zone of CA1. Application of CNQX (10 μM, 10 min) reduces its amplitude, and further addition of D-APV (50 μM, 10 min), completely blocks the fEPSP. (c) At E20, a fEPSP is evoked in the marginal zone of CA1. (B) For a similar afferent volley, the amplitude and slope of fEPSPs evoked in the stratum radiatum of CA1 regularly increase from E19 to P9.

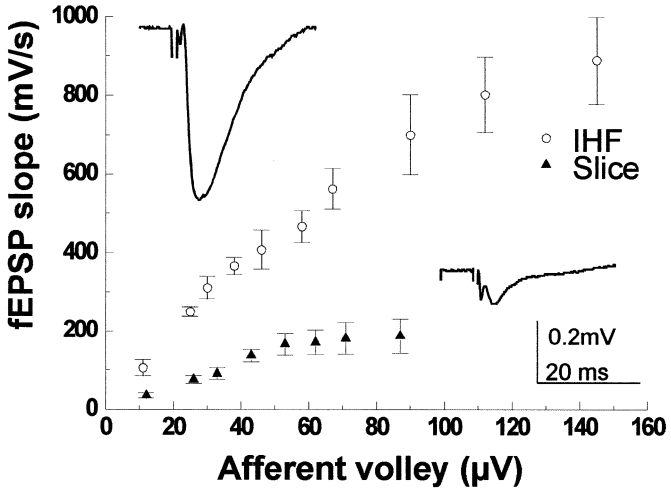

FIG. 8. At P0, the slopes of fEPSP evoked in slices are less important than those evoked in the IHF. fEPSPs were recorded in conventional transverse slices (400 μm) from the mid-temporal part of the hippocampus. Comparison of input–output curves made on IHF and slices from the mid-septo-temporal level. Slices and IHF were taken from the same animal.

Nevertheless, the robust fEPSPs that have been recorded throughout the hippocampus may not be compatible with a low density of synaptic AMPA receptors on pyramidal neurons (Nusser *et al.*, 1998; Petralia *et al.*, 1999), although the minimal amount of receptors required to generate a fEPSP is not known. Therefore, we cannot exclude the possibility that the synaptic activation of AMPA receptors located on interneurons of immature hippocampus would contribute to the generation of a fEPSP.

In favour of this hypothesis, the following points are noted.

1 At E19, most CA1 pyramidal neurons have just finished their formation (Altman & Bayer, 1990) and have not yet developed an apical dendrite (Minkwitz & Holz, 1975; Gozlan *et al.*, 1999) required for the establishment of synaptic contacts in the inner marginal zone (Fletcher *et al.*, 1994).

2 In contrast, GABAergic interneurons that are formed between E15 and E17 are already in place at E19, as attested by the intense calbinding (Super *et al.*, 1998), GABA (Rozenberg *et al.*, 1989) or glutamic acid decarboxylase (GAD, Dupuy & Houser, 1996) immunoreactivity detected in the inner marginal zone.

3 In the immature brain, GABAergic interneurons are densely packed (Dupuy & Houser, 1996) and organized forming a network of cells orientated in the horizontal plane along the pyramidal layer (Rozenberg *et al.*, 1989). Furthermore, the dendrites of a subpopulation of interneurons (calretinin-positive cells) located in the marginal zone were orientated parallel to the hippocampal plate (Jiang & Swann, 1997). This kind of transient organization of GABAergic neurons may be responsible for the fEPSP observed throughout the whole hippocampus.

4 Varicosities that have been detected on Schaffer collaterals and commissural fibres suggest en-passant synapses with the dendrites of these interneurons. Indeed, commissural fibres have been shown to form synapses with GABAergic interneurons in more than 60% of the cases (Super *et al.*, 1998), suggesting that GABAergic interneurons may constitute the initial target of commissural fibres (Super *et al.*, 1998).

5 In agreement with this hypothesis, we have observed in a preliminary patch-clamp study that glutamatergic receptors are expressed and functional on these interneurons at P0 (Hennou *et al.*, 1999).

In conclusion, the combined use of extracellular recordings and the IHF allowed us to show that glutamatergic neurotransmission mediated by both AMPA and NMDA receptors is functional in the late embryonic period. Therefore, in addition to $GABA_A$ receptors that provide the fast excitatory drive to pyramidal neurons at birth (Cherubini *et al.*, 1991; Ben-Ari *et al.*, 1997), there is also a significant contribution of NMDA and AMPA receptors to this activity. An early functional glutamatergic transmission will have important consequences for the activity-dependent development of the hippocampus, and this observation is in line with previous reports showing that ionotropic glutamate receptors influence the regulation of neuronal circuitry and cyto-architecture during neuronal differentiation (McDonald & Johnston, 1990), and that non-NMDA receptors are involved in neurite outgrowth (Mattson *et al.*, 1988) or DNA synthesis (LoTurco *et al.*, 1995). Finally, the use of intact preparations and field recordings at early developmental stages will allow determination of the physiological and pathophysiological relevance of immature networks. This approach will also allow the properties of neuronal plasticity in embryonic and early postnatal circuits to be determined.

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Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CA, *Cornu ammonis*; CNQX, 6-cyano-7-nitro-quinoxaline2,3-dione; CPA, N^6-cyclopentyl adenosine; D-APV, D(–)-2-amino-5-phosphonopentanoic acid; DiI, 1,1′-dioctadecyl-3,3,3′, 3′-tetra-methylindocarbocyanine perchlorate; DPCPX, 8-cyclopentyl-1,3-dipropyl-xanthine; E, embryonic day; fEPSP, field excitatory postsynaptic potential; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GYKI-53655, 1-(4-aminophenyl)-3-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine; IHF, intact hippocampal formation; NBQX, 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione; NMDA, *N*-methyl-D-aspartate; P, postnatal day; PSC, postsynaptic current; TTX, tetrodotoxin.

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