ELSEVIER

Brain Research 913 (2001) 195–200

BRAIN RESEARCH

www.elsevier.com/locate/bres

Short communication

Recurrent CA1 collateral axons in developing rat hippocampus

Laurent Aniksztejn, Michaël Demarque, Youri Morozov, Yehezkel Ben-Ari, Alfonso Represa*

INMED-INSERM U29, Parc scientifique et technologique de Luminy, 163 Route de Luminy BP 13, 13008 Marseille Cedex, France

Accepted 16 July 2001

Abstract

Rat perinatal (E20–P0) CA1 pyramidal neurons were either synaptically active or silent. We show here that, during this developmental period, active but not silent cells form recurrent axon-collaterals that invade the radiatum and the lacunosum moleculare strata. These recurrents were never observed in adult rats. We propose that these transient recurrent axons may participate in the activity-dependent modulation of the synaptogenesis © 2001 Published by Elsevier Science B.V.

Theme: Development and regeneration

Topic: Cell differentiation and migration

Keywords: Synaptogenesis; Sprouting; Axonal retraction

In an effort to better understand the organisation of developing networks we have investigated the maturation of synaptic responses in CA1 pyramidal cells. We previously demonstrated [26] that at birth up to 80% of pyramidal cells did not have dendrites in the radiatum or oriens strata and were silent in that they exhibited no spontaneous or evoked synaptic currents. Synaptic responses mediated by $GABA_A$ receptors only could be detected in around 10% of pyramidal cells, having as a requisite an apical dendrite in the stratum radiatum. Finally, 10% of recorded cells displayed both GABAergic and glutamatergic synaptic responses; their dendritic arbor was more developed and dendrites penetrated in the stratum lacunosum moleculare. These and other observations [26] suggest that the first synapses are established on the apical dendrites of the pyramidal neurons and are GABAergic followed by the establishments of glutamatergic synapses, only once the postsynaptic target has reached a certain degree of maturation.

The majority of pyramidal cells recorded at P0 displayed a relatively well developed axon that originated from the cell body and reached the stratum oriens. This pattern was independent of the degree of maturation of the dendritic tree and synaptic activity [26]; these observations support the notion that axons develop before dendrites. In adults the axons of CA1 pyramidal cells originate a few collaterals that may distribute within the stratum oriens and seldom the stratum pyramidale [10,16,19,25]. The recurrent excitatory network is therefore relatively poor in this field and synchronisation of CA1 pyramidal cells (e.g., genesis of ictal discharges) is consequently a rare event [9] as compared with the CA3 area, where recurrent synapses between pyramidal cells drive polysynaptic responses after removal of GABAergic inhibition [4,17].

In the course of our study, we observed that in contrast to adult neurons, CA1 pyramidal axons in the embryonic and early postnatal hippocampus might have an axonal arbor that extended to the distal parts of stratum radiatum and even to lacunosum moleculare.

Experiments were performed on coronal CA1 hippocampal Vibratome slices (400-μm-thick) obtained from embryos (E19 to E21), newborn (P0) young (P27) or adult (P60–P120) Wistar rats. Slices were incubated in oxygenated artificial cerebrospinal fluid [ACSF composed of (in mM): 126 NaCl, 3.5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$ 1.2 $NaHPO_4$, 10 glucose] for at least 1 h. Individual slices were fully submerged and superfused in

*Corresponding author. Tel.: +33-4-9182-8114; fax: +33-4-9182-8114.

E-mail address: represa@inmed.univ-mrs.fr (A. Represa).

0006-8993/01/$ – see front matter
PII: S0006-8993(01)02817-7

the recording chamber with oxygenated ACSF at 30–32°C at a rate of 2–3 ml/min.

Pyramidal cells were recorded under visual control (with an Axioscope, Zeiss) using patch-clamp technique in the whole-cell configuration [14] with an Axopatch 200A (Axon Instruments, USA) or EPC9 amplifiers (HEKA). Microelectrodes had a resistance of 5–10 MOhm and filled with a solution of the following composition (in mM): 130 CsGlu, 10 CsCl, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 4 Mg^{2+}ATP, 0.3 Na^{+}GTP, and biocytine (0.5–0.8%) or biocytin Alexa fluor (0,1%; Molecular Probes, Eugene, OR, USA), pH 7.25, 270–280 mOsm. Slices were stimulated by a bipolar twisted nichrome electrode placed in the stratum radiatum of CA1 with an intensity that ranged between 10 and 100 V, 30 μs duration and at a frequency of 0.05– 0.033 Hz. Synaptic currents and agonist-evoked responses were analysed using Acquis software (Gérard Sadoc, France).

Drugs used were: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), 6-cyano-7-nitroquinoxaline-2,3 dione (CNQX) and D-2-amino-5-phosphonovalerate (D-APV) all purchased from Tocris Coolson Ltd., Bristol, UK. Bicuculline and biocytine were purchased from Sigma–Aldrich (St Quintin Fullavier, France).

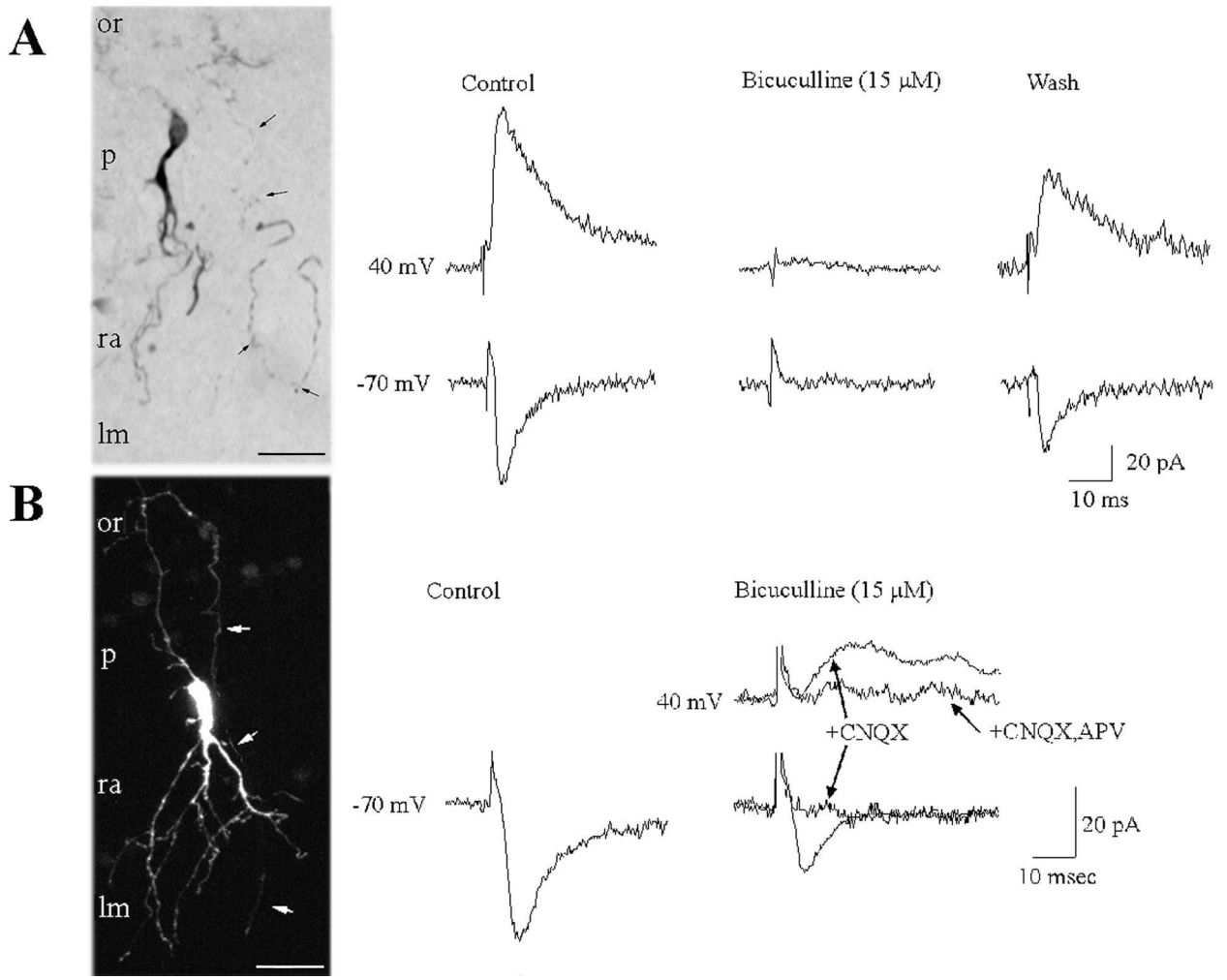

Fig. 1. Embryonic cells with recurrent axons were synaptically active. (A) Pyramidal cell recorded at E20 and injected with biocytin (0.05%). The apical dendrite reaches the stratum radiatum but not the lacunosum moleculare. The recurrent axon (indicated by arrows) crosses the stratum pyramidale and the stratum radiatum. A drawing of this cell is represented in Fig. 2 (no. 3). Synaptic responses were evoked by the stimulation of the stratum radiatum (20 V, 30 μs duration, 0.033 Hz) and were mediated by $GABA_A$ receptors only since bicuculline (15 μM) abolished reversibly this response at both potentials recorded, even when the stimulus intensity was increased to 100 V (traces represent the mean of five consecutive responses evoked at 100 V, 5 min after the application of bicuculline). (B) Pyramidal cell recorded at P0 and injected with Alexa (0.1%). The apical dendrite branched within the stratum radiatum and lacunosum. The recurrent axon (arrows) that crossed the stratum pyramidale also reached the stratum lacunosum. Note that Alexa gave a bright and precise fluorescent staining that is visible in living cells, prior to fixation. A drawing is reproduced in Fig. 2 (no. 7). Synaptic activity was mediated by $GABA_A$, AMPA and NMDA receptors. Each trace corresponds to the mean of five consecutive responses evoked by stimulation of stratum radiatum at 30 V. At −70 mV bicuculline reduced but did not block completely the synaptic response. The remaining postsynaptic current (PCS) was suppressed by CNQX (15 μM). In the presence of the two antagonists, the stimulation evoked at 40 mV PSCs sensitive to D-APV (50 μM). lm, stratum lacunosum moleculare; or, stratum oriens; p, stratum pyramidale; ra, stratum radiatum. Scale bars=50 μm.

To reveal biocytine, slices were immersed in a fixative solution of paraformaldehyde (4%) and glutaraldehyde (0.2%) overnight, rinsed in 0.05 M Tris-buffered saline, pH 7.4 (TBS) containing 0.3% Triton X-100 for 30 min and incubated overnight at 4°C in an avidin–biotin–peroxidase solution prepared in TBS (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA). After a 30 min wash in TBS, slices were processed in 0.06% 3,3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. They were then rinsed and mounted with Permount. Stained cells were reconstructed using a camera lucida.

CA1 pyramidal cells were recorded from embryonic day (E) 20 to P0 (day of birth) under visual control that allowed us to distinguish cells with an anlage of apical dendrite from those that had a more developed apical dendrite that reach the stratum radiatum or lacunosum-moleculare [26]. In keeping with our previous study we found that the first group of cells ($n=31$) were synaptically silent (i.e., they had neither spontaneous nor evoked responses by the stimulation of the stratum radiatum synaptic currents). The second group of cells had synaptic activity mediated only by $GABA_A$ receptors ($n=48$) since both spontaneous and evoked synaptic responses were fully blocked by bicuculline (15 μM), the $GABA_A$ receptor antagonist. Finally, the third group of cells had synaptic activities mediated by both $GABA_A$ and glutamate receptors ($n=16$), since in addition to bicuculline, CNQX (10 μM) was required to completely block the synaptic responses at −70 mV, indicating the participation of AMPA receptors. The involvement of NMDA receptors to the synaptic activity in these cells was revealed at 40 mV, by the presence of a D-APV (50 μM)-sensitive component.

Axons of these pyramidal cells, revealed by biocytin or Alexa staining, reached the stratum oriens and also the alveus. In addition we found that some of these axons generated recurrent collaterals that originated within the stratum pyramidale or the stratum oriens, crossed the pyramidal cell layer and distributed within the stratum radiatum and eventually the lacunosum moleculare (with one or two axon terminals; Figs. 1 and 2). While silent cells did not show any recurrent collateral, 12% of GABA only responsive cells (six out of 48 stained cells) and 37% of GABA+glutamate responsive cells (six out of 16) developed this axonal arborisation.

Pyramidal neurons from young (P27; $n=4$) and adult (P60–120; $n=69$) rats were analysed to investigate whether recurrent collaterals persisted or not in adults. Cells recorded displayed a more complex dendritic tree (Fig. 3), with basilar dendrites branching within the stratum oriens and a long radial apical dendrite reaching the hippocampal fissure. The axons of these cells, originating from cell bodies or basilar dendrites, reached the

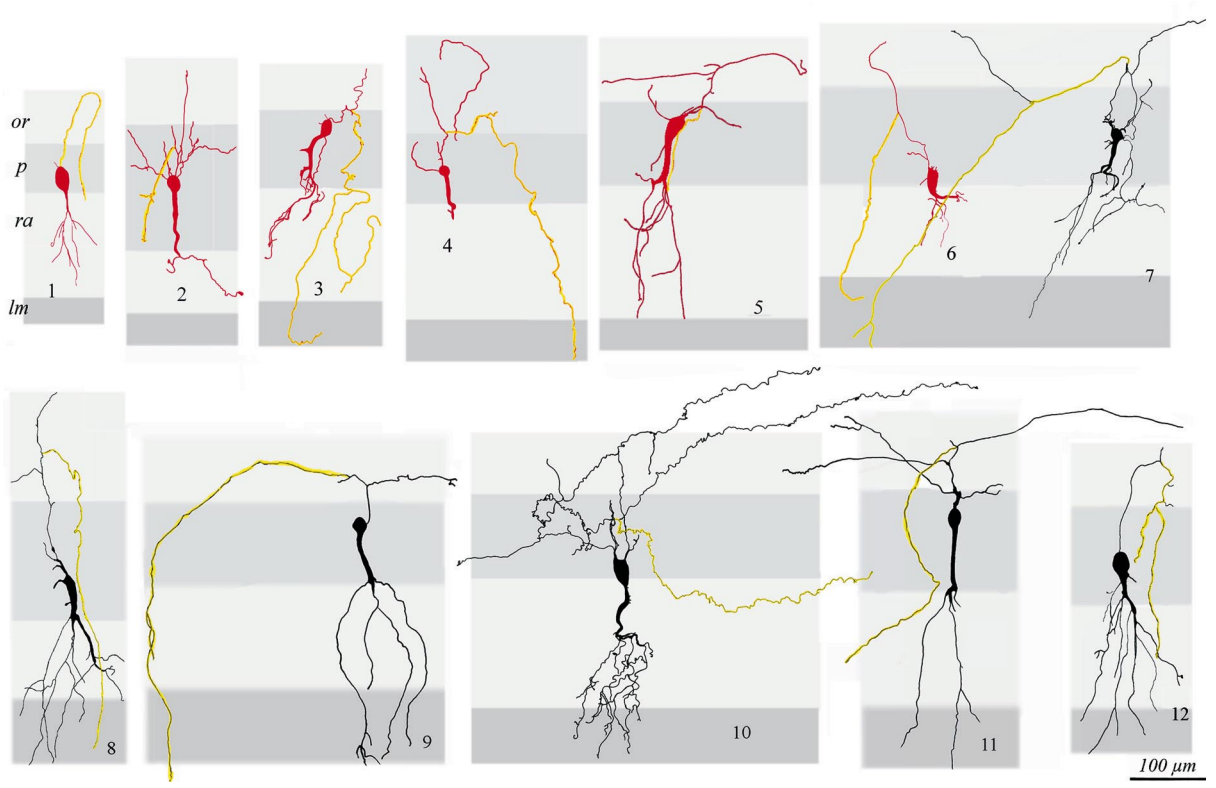

Fig. 2. Camera lucida reconstruction of perinatal CA1 pyramidal cells with GABA-mediated PSCs (in red) or GABA plus glutamate -mediated PSCs (in black) displaying recurrent axons (in yellow). Cells were recorded and injected from E20 (1, 3, 5), E21 (6, 7) or P0 (2, 4, 8–12) hippocampus. The limits of cell layer are shown in grey. or, oriens; p, pyramidale; ra, radiatum, lm, lacunosum moleculare. Scale bar=100 μm.

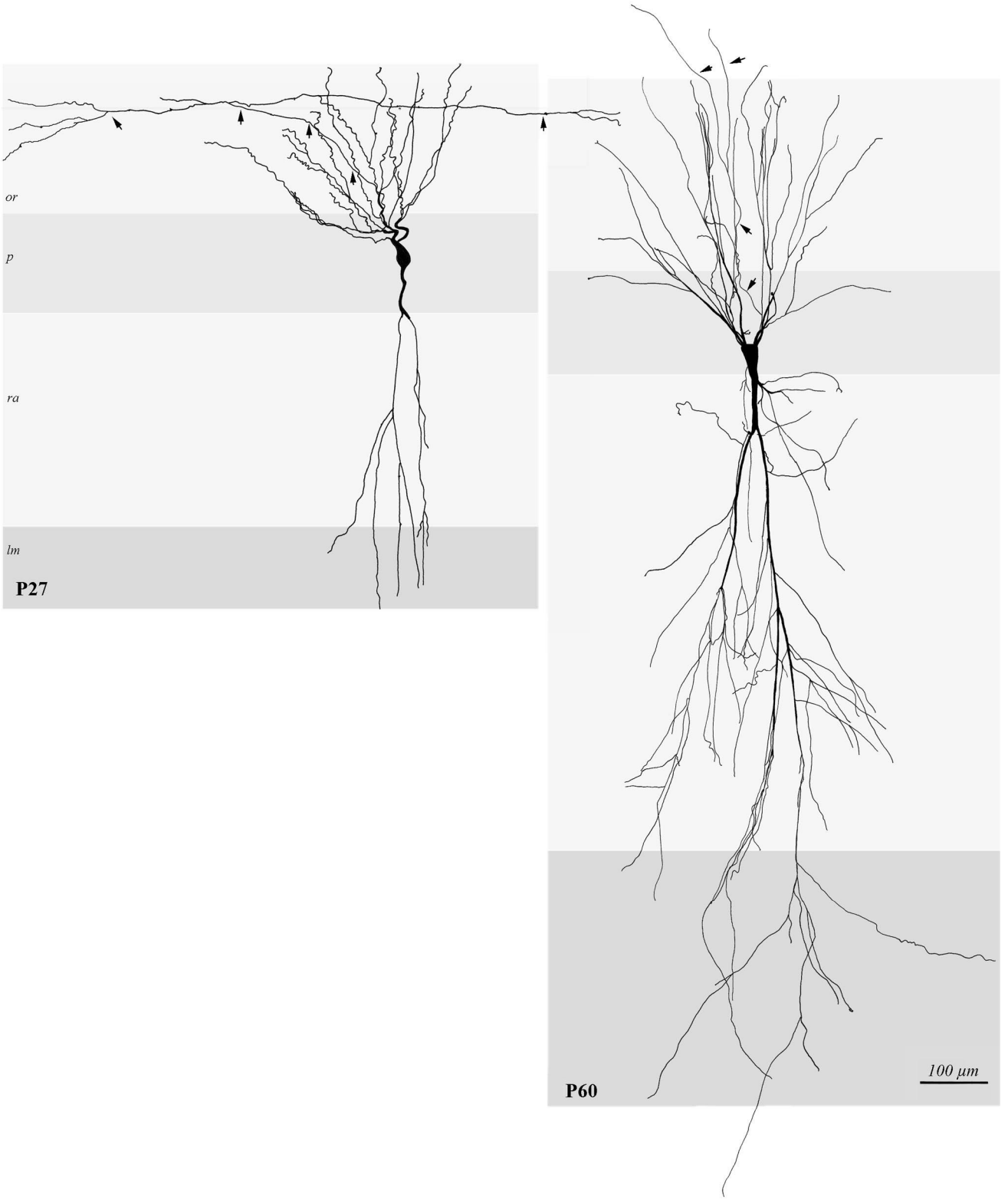

Fig. 3. Camera lucida reconstruction of CA1 pyramidal cells recorded from P27 and P60 days old. Axons (arrows) were observed in the stratum oriens and alveus but no recurrent collaterals were observed in stratum radiatum. Scale bar=100 μm.

alveus and could be followed for relatively long distances. They may originate a few collaterals within the stratum oriens (Fig. 3A), but we never observed recurrent collaterals invading the pyramidale or the radiatum strata. The mean density of axon collaterals did not significantly change with age [3.1±1.8 in E20–P0 (n=135 rats) and

3.7±2.8 in adult, (n=69 rats)], so that the changes between perinatal (E20–P0) and adult (P60–120) rats are rather more qualitative (i.e., area of distribution) than quantitative (i.e., elimination of supernumerary branches). However, this conclusion should be tempered since cells were recorded from the surface of 400 μm sections and we cannot exclude that some collaterals branched out of the slice. In addition, though biocytine is relatively well transported along neuronal extensions, it may be that some collaterals are not stained.

The present data strongly suggest that recurrent collaterals are transiently developed during the maturation of the dendritic tree and that a minimum degree of maturation is required (because it is absent from silent cells). If these collaterals establish functional synaptic contacts it is presently unknown, and only electrophysiological analysis coupled to electron microscopy (EM) would help to clarify this point. If CA1 transient axonal branches disappear without contributing to the formation of developing networks, they would represent navigation errors of growing axons that affect more than 10% of maturing pyramidal cells.

Previous reports on adult hippocampi demonstrated that axon collaterals, that are mainly restricted to the oriens layer, innervate both interneurons [1,2] and basilar dendrites of CA1 pyramidal cells [10]. It is therefore plausible that recurrent collaterals present in the stratum radiatum and lacunosum of perinatal rats innervate both interneurons and apical dendrites of pyramidal cells. However, while GABA interneurons, that proliferate in the hippocampus before E17 [22], are relatively well developed by the end of gestation, pyramidal cells, that proliferate after E17 [3], almost never displayed dendrites in the stratum lacunosum moleculare before birth. Therefore, if recurrent axons made synapses in this layer, interneurons and Cajal–Retzius cells would constitute the more plausible target. In the stratum radiatum in contrast, recurrent axons would innervate both interneurons and apical dendrites of pyramidal cells. Interestingly, it has previously been shown [24] that in P0–P5 mice the majority of CA1 afferents synapse, in these layers, calretinin- (a marker for Cajal–Retzius cells) or GABA-positive cells and it was consequently suggested that these cells constitute transiently the main targets for glutamatergic fibres.

Transient profusion of axonal branches, followed by regression, constitutes a common event in developing CNS structures, including the optic nerve [11], the callosal connections [6,8,12,18] the reticulogeniculate pathway [21,23], the association projections in the visual cortex [20] or the striatothalamic projections [15]. The axonal refinement may result from neuronal degeneration due to a genetic program inducing cell death and/or from the competition for target-derived neurotrophic support and it may also result from the elimination —retraction— of collateral branches [7]; this occurs in the retinogeniculate pathway during the establishment of the binocular segregation [21] or in the postcentral gyrus neurons that in the rhesus monkey foetuses retract callosal collaterals while maintaining ipsilateral projections [8]. Although there is presently no evidence for postnatal CA1 pyramidal cell death, it is plausible that programmed cell death is responsible for the elimination of these recurrent collaterals. Interestingly, developmental cell death has been reported for a class of GABAergic cells by Super et al. [24]; these cells distribute within the radiatum and lacunosum moleculare stratum and have been considered as pioneer neurons involved in the formation of layer-specific connections. If these cells are the target for the recurrent pyramidal axons, their postnatal death (which takes place during the first postnatal week) may underlie the elimination of the inappropriate collateral projection.

Recent data have shown a reactive sprouting of CA1 axon collaterals that profusely distributed within the stratum pyramidale [13,19] and eventually the stratum radiatum in epileptic animals [13]. These recurrent axons may be responsible, at least in part, for the epileptiform bursts evoked in CA1 by stimulation of the associational pathway [13]. Our present data, showing an innervation of radiatum moleculare strata by CA1 recurrent axons in developing rats, reinforce the suggestion that reactive sprouting recapitulates developmental programs and that it may provide some clues for a better understanding of the developmental changes. Thus, it is tempting to suggest that recurrent collaterals in immature hippocampi contribute to the synchronisation of CA1 neurons which generates giant depolarising potentials (GDPs), the main network activity during this period of life [5].

Acknowledgements

We express our gratitude to Dr J. Hirsch for her advice, help and critically reading of the manuscript and to Isabel Jorquera for technical assistance.

References

[1] A.B. Ali, J. Deuchars, H. Pawelzik, A.M. Thomson, CA1 pyramidal to basket and bistratified cell EPSPs: dual intracellular recordings in rat hippocampal slices, J. Physiol. 507 (1998) 201–217.
[2] A.B. Ali, A.M. Thomson, Facilitating pyramide to horizontal oriens-alveus interneuron inputs: dual intracellular recordings in slices of rat hippocampus, J. Physiol. 507 (1998) 185–199.
[3] J. Altman, S.A. Bayer, Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale, J. Comp. Neurol. 301 (1990) 343–364.
[4] Y. Ben-Ari, M. Gho, Long lasting modification of the synaptic properties of rat CA3 hippocampal neurones induced by kainic acid, J. Physiol. 404 (1988) 365–384.
[5] Y. Ben-Ari, R. Khazipov, X. Leinekugel, O. Caillard, J.L. Gaïarsa, $GABA_A$, NMDA and AMPA receptors: a developmentally regulated ménage à trois, Trends Neurosci. 20 (1997) 523–529.

[6] P. Berbel, G.M. Innocenti, The development of the corpus callosum in cats: a light- and electron-microscopic study, J. Comp. Neurol. 276 (1988) 132–156.
[7] M. Bernstein, J.W. Lichtman, Axonal atrophy: the retraction reaction, Curr. Opin. Neurobiol. 9 (1999) 364–370.
[8] L.M. Chalupa, H.P. Killackey, Process elimination underlies ontogenetic change in the distribution of callosal projection neurons in the postcentral gyrus of the fetal rhesus monkey, Proc. Natl. Acad. Sci. USA 86 (1989) 1076–1079.
[9] V. Crepel, R. Khazipov, Y. Ben-Ari, Blocking GABA(A) inhibition reveals AMPA- and NMDA-receptor-mediated polysynaptic responses in the CA1 region of the rat hippocampus, J. Neurophysiol. 77 (1997) 2071–2082.
[10] J. Deuchars, A.L. Thomson, CA1 pyramid–pyramid connections in rat hippocampus in vitro: dual intracellular recordings with biocytin filling, Neuroscience 74 (1996) 1009–1018.
[11] S.A. Dunlop, Transient axonal side branches in the developing mammalian optic nerve, Cell Tissue Res. 291 (1998) 43–56.
[12] T. Elliott, C.I. Howarth, N.R. Shadbolt, Axonal processes and neural plasticity. II: Adult somatosensory maps, Cereb. Cortex 6 (1996) 789–793.
[13] M. Esclapez, J.C. Hirsch, Y. Ben-Ari, C. Bernard, Newly formed excitatory pathways provide a substrate for hyperexcitability in experimental temporal lobe epilepsy, J. Comp. Neurol. 408 (1999) 449–460.
[14] O.P. Hamill, A. Marty, E. Neher, B. Sakmann, Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches, Pflügers Arch. 391 (1981) 85–100.
[15] T. Hattori, M. Takada, T. Moriizumi, K.J. Campbell, Direct striatothalamic projections in the neonatal rat, Brain. Res. Dev. Brain. Res. 54 (1990) 137–141.
[16] W.D. Knowles, P.A. Schwartzkroin, Axonal ramifications of hippocampal CA1 pyramidal cells, J. Neurosci. 1 (1981) 1236–1241.
[17] R. Miles, R.K.S. Wong, Excitatory synaptic interactions between CA3 neurones in the guinea-pig hippocampus, J. Physiol. 373 (1986) 397–418.
[18] C.R. Norris, K. Kalil, Development of callosal connections in the sensorimotor cortex of the hamster, J. Comp. Neurol. 326 (1992) 121–132.
[19] Y. Perez, F. Morin, C. Beaulieu, J.C. Lacaille, Axonal sprouting of CA1 pyramidal cells in hyperexcitable hippocampal slices of kainate-treated rats, Eur. J. Neurosci. 8 (1996) 736–748.
[20] D.J. Price, C. Blakemore, The postnatal development of the association projection from visual cortical area 17 to area 18 in the cat, J. Neurosci. 5 (1985) 2443–2452.
[21] K.F. So, G. Campbell, A.R. Lieberman, Development of the mammalian retinogeniculate pathway: target finding, transient synapses and binocular segregation, J. Exp. Biol. 153 (1990) 85–104.
[22] E. Soriano, A. Cobas, A. Fairen, Neurogenesis of glutamic acid decarboxylase immunoreactive cells in the hippocampus of the mouse. I: Regio superior and regio inferior, J. Comp. Neurol. 281 (1989) 586–602.
[23] D. Sretavan, C.J. Shatz, Prenatal development of individual retinogeniculate axons during the period of segregation, Nature 308 (1984) 845–848.
[24] H. Super, A. Martinez, J. Del Rio, E. Soriano, Involvement of distinct pioneer neurons in the formation of layer-specific connections in the hippocampus, J. Neurosci 18 (1998) 4616–4626.
[25] A.M. Thomson, S. Radpour, Excitatory connections between CA1 pyramidal cells revealed by spike triggered averaging in slices of rat hippocampus are partially NMDA receptor mediated, Eur. J. Neurosci. 3 (1991) 587–601.
[26] R. Tyzio, A. Represa, I. Jorquera, Y. Ben-Ari, H. Gozlan, L. Aniksztejn, The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite, J. Neurosci. 19 (1999) 10372–10382.