Interneurons are the Source and the Targets of the First Synapses Formed in the Rat Developing Hippocampal Circuit

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In hippocampal CA1 pyramidal neurons, GABAergic synapses are established before glutamatergic synapses. GABAergic interneurons should therefore develop and acquire synapses at an earlier stage to provide the source for GABAergic synapses. We now report that this is indeed the case. At birth and *in utero*, when nearly all pyramidal neurons are not yet functional, most interneurons have already either GABAergic only or GABAergic and glutamatergic postsynaptic currents. At birth, the morphological maturation of interneurons parallels their individual functional responses. In addition, the formation of functional interneurons types appears to be a sequential process. Interneurons that innervate other interneurons acquire $GABA_A$ synapses before peridendritic interneurons, but also before perisomatic interneurons that are not yet functional at birth. Therefore, interneurons are the source and the targets of the first synapses formed in the developing circuit. Since GABA was shown to be excitatory *in utero*, interneurons provide all the excitatory drive at a time when the principal cells are silent. They could therefore play a central role in the formation of the cortical circuit at early developmental stages.

Introduction

Once neurons have finished their migration and differentiation, they start shifting from a largely genetically oriented sequence to one in which the environment plays a progressively determinant role via synaptic currents. The shift from an ensemble of neurons that act independently to one in which neurons integrate external and internal information to generate a coherent behaviour via billions of synaptic connections is a formidable task that necessarily involves intermediate steps. One of the first problems that the developing neuron must solve is how to establish and preserve equilibrium between excitation and inhibition. In adult neurons, inhibition is principally mediated by GABAergic synapses that generate a hyperpolarization via chloride permeable channels, whereas excitation is mediated by glutamatergic receptors permeable to cations. Agents that augment GABAergic inhibitory postsynaptic currents (PSCs) are sedative and anticonvulsant. Conversely, agents that reduce or block GABAergic synapses generate seizures and hyperactivity. Similarly, excessive glutamatergic activity is neurotoxic and generates seizures, whereas blockade of glutamatergic receptors is life-damaging by virtue of a blockade of ongoing network driven activity. Therefore, the formation of synapses must have an endowed system that prevents at any time an imbalance between excitation and inhibition, thus excluding an earlier development of pure GABAergic or glutamatergic synapses. One theoretically possible solution to this problem could have been a parallel development of both types of synapses with an inborn device that titrates the strength of each family of PSCs. This solution has, however, not been retained because it has major limitations, including the need to completely pre-program all the mechanisms that control GABAergic and glutamatergic synaptic efficacy. In fact, in CA1 pyramidal neurons GABA synapses were observed at early developmental stages when a small anlage of apical dendrites emerges in stratum radiatum (SR). Excitatory synapses appeared later when dendrites reached stratum lacunosum moleculare (SLM) (Tyzio *et al.*, 1999). We therefore postulated that, if GABAergic synapses were formed before glutamatergic ones, the source of these synapses, interneurons, should develop following the same sequence, but at an earlier stage. We have now tested this hypothesis in the same region of the hippocampus.

Materials and Methods

Slice Preparation

Wistar rats were killed on day of birth (P0) or at embryonic stages between E18 and E20. Brains were removed and placed in oxygenated ice-cooled artificial CSF (ACSF) and hippocampal transversal slices (400 μm) were cut using a Vibratome (VT 1000E; Leica, Germany). Slices corresponding to the middle third were generally taken for patch-clamp experiments. Individual slices were then transferred to the recording chamber where they were fully submerged and superfused with oxygenated ACSF at 31 ± 1°C at a flow rate of 3.5 ± 0.1 ml/min.

Electrophysiological Recordings

Stimulation of the afferent pathway was achieved with a bipolar twisted nichrome electrode, placed in SR or stratum oriens (SO) of CA1. Patch pipettes were filled with a potassium gluconate solution to simultaneously visualized $GABA_A$ and AMPA receptor-mediated responses, containing (in mM): K-gluconate, 135; EGTA, 1.0; HEPES, 10; $CaCl_2$, 0.1; $MgCl_2$, 2; Mg-ATP, 2; pH = 7.3. After addition of 5 mg/ml biocytin, the osmolarity of the solution was adjusted between 270 and 280 mOsm. The resistance of the pipettes was 4–10 MΩ and the reverse potential of $GABA_A$ currents was around –60 mV. Capacitance values were determined by curve fitting of the capacitive current generated by a 5 mV depolarizing voltage step. Patch-clamp experiments were usually performed blindly, but in a small number of slices, interneurons were also patched under visual control (Zeiss Axioscope microscope and a Hamamatsu camera with an incident light filtered to pass visible and infrared).

Neuronal Reconstructions

After recording, the patching electrode was slowly removed from the cell and the slice was fixed overnight at 4°C in a solution of 4% fresh paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer and then left for at least 24 h in a 30% sucrose solution containing 0.1% NaN_3 in 0.1 M phosphate buffer. Neurons were then visualized using the avidin–biotinylated horseradish peroxidase complex reaction (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) using 3,3′-diaminobenzidine (DAB) as chromogen. An experimenter unaware of the physiological data performed reconstructions of stained cells.

Immunocytochemistry

After transcardial perfusion of P0 rats with paraformaldehyde (4%) and glutaraldehyde (0.2%), brains were kept overnight in 4% paraformaldehyde. Vibratome sections 40 μm thick were incubated overnight with primary antibodies (see below) in PBS containing 5% normal goat serum.

After washing, sections were sequentially incubated with biotinylated anti-rabbit antibody (1: 400; Vector Laboratories) and avidin–biotin–peroxIdase solution. Sections were then processed in DAB tetrahydrochloride (0.06%) and hydrogen peroxide (0.01%) and then rinsed, dehydrated and mounted with Permount. Adjacent sections were incubated in the absence of either primary or secondary antibodies to check the specificity of the peroxidase staining. Non-specific staining was prevented by preincubating the sections with gelatine (0.5 %) and normal goat serum (10%; Vector Laboratories). Primary antibodies for indirect immunohistochemistry included polyclonal rabbit anti: parvalbumin (1:5000), calbindin D-28k (1:10 000) and calretinin (1:2000), all from Swant (Switzerland); and somatostatin (1:200, Peninsula Laboratories, San Carlos, CA). Antibodies against CCK-8 (1:5000) and substance P (1:2000) were kindly provided by Dr J.J. Benoliel (INSERM U288, Paris, France).

Results

Three Developmental Types of Interneurons at Birth

A birth, 131 interneurons were blindly patched in all CA1 areas of the hippocampus. They were classified into three types depending on their spontaneous and evoked PSCs and their size after morphological reconstructions.

Silent Interneurons

These neurons corresponded to non-innervated cells because, when recorded over 30–60 min, they had no spontaneous PSCs. Furthermore, repetitive electrical stimulation of afferent fibres at high intensity (70–90 V) and frequency (100 Hz, 1s) also did not evoke PSCs (Fig. 1*Aa*). In addition, there were also no action currents in long-lasting cell-attached recordings (not shown). They were identified as neurons (versus glia) by the tetrodotoxin (TTX)-sensitive inward sodium spikes evoked by depolarizing steps. The six silent interneurons found in the total population of interneurons at birth had a small soma and their axons and dendrites were poorly developed, remaining in SR (Fig. 2*A*).

We have determined that the lack of synaptic responses was not due to synapses that rarely release transmitters spontaneously or that were difficult to activate with a stimulating

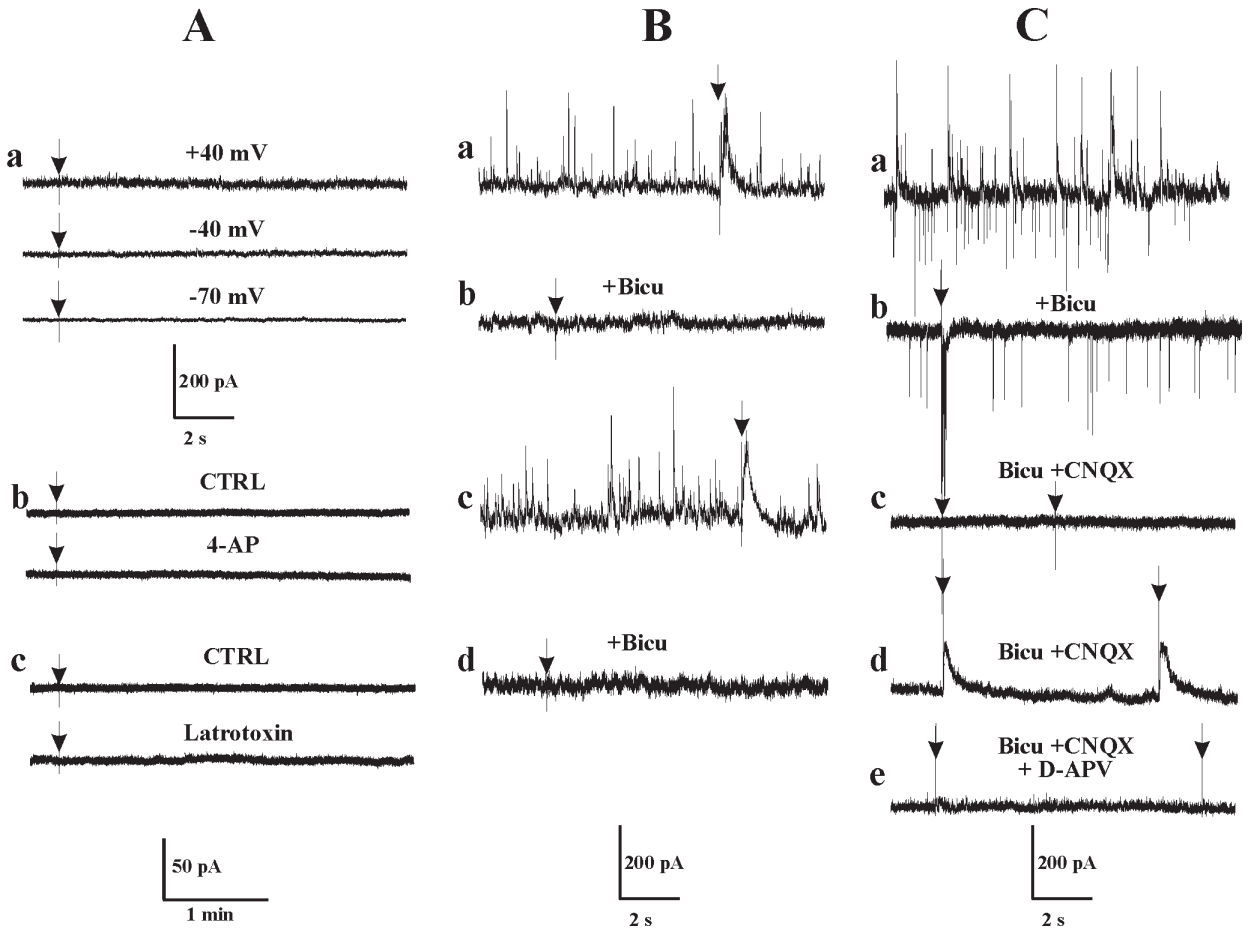

Figure 1. Functional responses of CA1 interneurons at birth. (*A*) Silent interneurons. (*a*) No spontaneous or evoked activity (arrow, 80 V, 60 μs duration) could be recorded at three different membrane potentials in some interneurons blindly patched from SR. (*b*) Small interneurons from SR selected under visual guidance were also found to have no spontaneous or evoked (arrow, 80 V, 60 μs) synaptic responses in control conditions (CTRL) and remain 'silent' even if the release of neurotransmitter is increased by 4-aminopyridine (4-AP, 100 μM, 30 min) or α-latrotoxin (0.6 nM, 1 min). (*B*) G interneurons. Spontaneous and evoked outward PSCs mediated by $GABA_A$ receptors were recorded in SR at –45 mV (*a*) and at +40 mV (*c*) with K-gluconate-filled electrodes. The reversal potential for $GABA_A$ currents was –61 mV. (*b*, *d*) Application of bicuculline (10 μM, 10 min) completely suppressed spontaneous and evoked (arrow, 80 V, 60 μs) $GABA_A$ responses at these two potentials. Note the absence of interictal seizures and that no NMDA response was evoked at +40 mV in these conditions. (*C*) GG interneurons. (*a*) Spontaneous and evoked $GABA_A$ (outward) and AMPA (inward) PSCs were recorded at –45 mV in K-gluconate-filled electrodes (reversal potential for $GABA_A$ currents was around –59 mV). Outward GDPs were present in this record. (*b*) Application of bicuculline (10 μM, 10 min) completely suppressed outward but not inward spontaneous or evoked currents and induced an interictal-like evoked response. (*c*) Addition of CNQX (10 μM, 10 min) to bicuculline suppressed AMPA receptor-mediated spontaneous and evoked currents at –45 mV. (*d*) In the presence of AMPA and $GABA_A$ receptor antagonists, large evoked and spontaneous outward currents were recorded at +40 mV. (*e*) These were abolished by concomitant addition of D-APV (20 μM, 10 min).

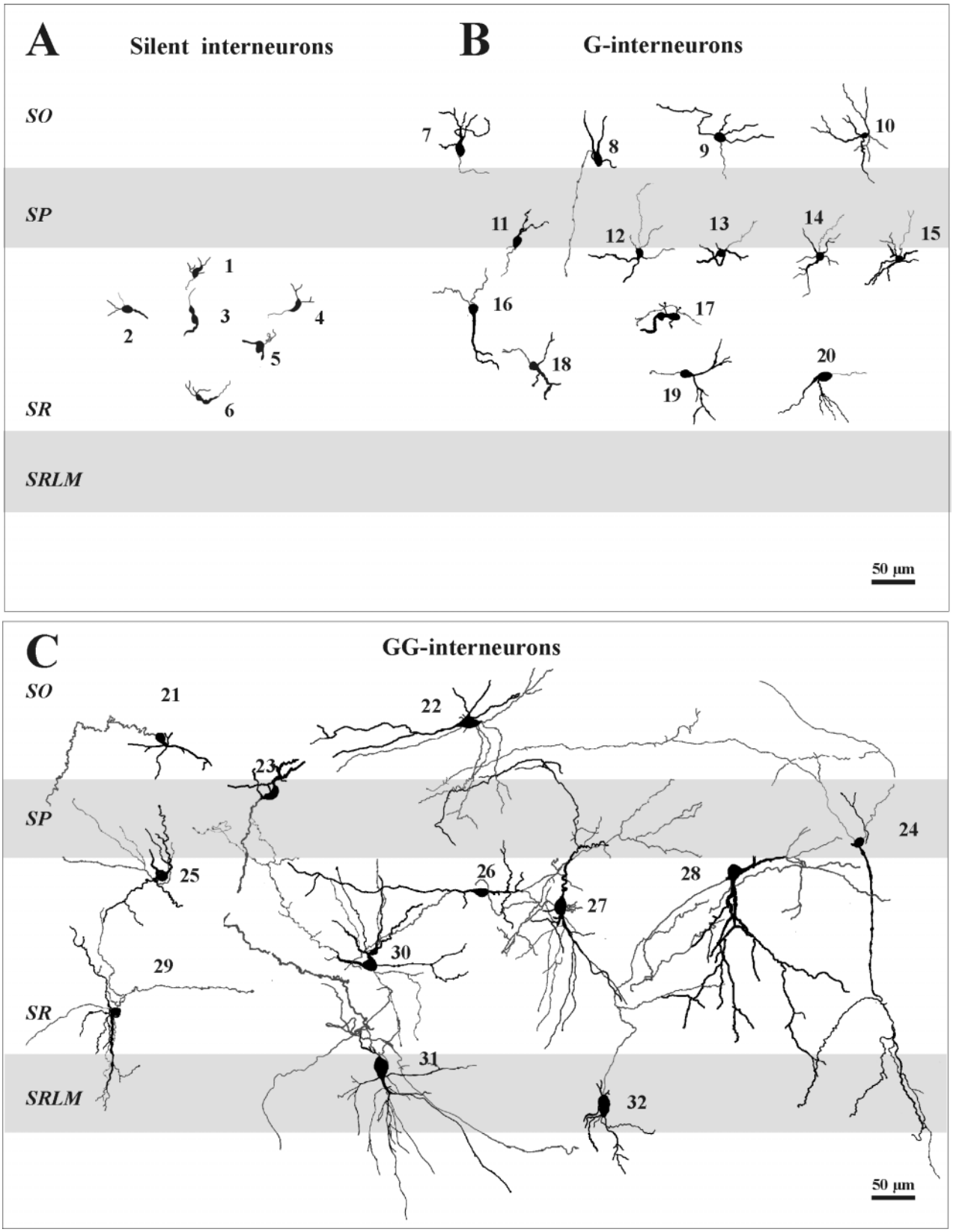

Figure 2. Morphology of interneurons at birth. (*A*) Silent interneurons. Six non-innervated interneurons were filled with biocytin (cells 1–6) and reconstructed. They were all located in SR. (*B*) G interneurons. Some of the 22 interneurons with only $GABA_A$ PSCs were reconstructed and represented in their layers. Cells 12–15 are examples of G interneurons that are located at the border with SP.They have a stellate morphology and their axons invade SP.They represented the majority of G interneurons in SR. Cell 9 may correspond to an IS-1 type of interneuron. (*C*) GG interneurons. Twelve of the 103 GG interneurons are represented to illustrate their morphological heterogeneity. Their size, axonal arbor and dendritic trees are different from cell to cell. Cells 22 and 29 may resemble an IS-1 interneuron. Cells 31 and 32 located in SLM may correspond to IS-2 interneurons, whereas cell 24 with a cell body in SP may correspond to the IS-3 subtype of interneuron.

electrode. As our sample of blindly patched silent interneurons was limited to six cells, we used the visual infrared technique to select under visual control silent interneurons from SR. 4-Aminopyridine (4-AP, 100 μM, 60 min), a K^+ channel blocker (Storm, 1988) that augments transmitter release (Perreault and Avoli, 1991; Pena and Tapia, 1999), did not generate any spontaneous or evoked PSCs in these cells (n = 6; Fig. 1*Ab*). In addition, no current was detected over 15–30 min in additional silent interneurons (n = 3) superfused with α-latrotoxin (0.6 nM, 1 min), which depletes vesicular pools (Sudhof, 2001) (Fig. 1*Ac*). In spite of the absence of synapses, silent cells did generate large currents upon bath application of AMPA, isoguvacine or NMDA in the presence of TTX. These currents were respectively blocked by CNQX, bicuculline and APV, indicating the presence of non-synaptic receptors (not shown).

G Interneurons

This second population of interneurons corresponded to a group of 22 cells (16.8%). They displayed only spontaneous and evoked GABAergic PSCs (Fig. 1*Ba*) that were fully blocked by bicuculline (10 μM, 10 min). Spontaneous and evoked AMPA receptor-mediated PSCs were not observed even with strong stimuli elicited at various membrane potentials (Fig. 1*Bb*). Furthermore, no spontaneous or evoked epileptic discharges were observed upon application of bicuculline (n = 8). G interneurons were morphologically heterogeneous and were found in all CA1 layers, but not in SLM (Fig. 2*B*). They correspond to cells that were clearly more developed than silent interneurons. However, their short dendrites and axons had generally no ramifications. At this stage of development it is hard to recognize an adult morphologic type described previously (Freund and Buzsáki, 1996; Parra *et al.*, 1998). However, some neurons in SO and SR could correspond to immature IS types (see Fig. 2*B*).

GG Interneurons

The majority of CA1 interneurons (103 cells, 78.6%) were functional at birth, with GABA and glutamate PSCs. Thus, spontaneous and evoked PSCs mediated by $GABA_A$, AMPA and NMDA receptors were usually recorded in the first 2–5 min, in addition to currents corresponding to the so-called 'giant depolarized potentials'. Thus, at –45 mV, bicuculline blocked GABAergic outward currents and induced spontaneous and evoked interictal responses. The remaining spontaneous and the large evoked inward responses (Fig. 1*Ca*) were abolished by CNQX (10 μM, 10 min; Fig. 1*Cb*). At +40 mV, the current remaining after CNQX application (Fig. 1*Cc*) was subsequently blocked by D-APV (20 μM; Fig. 1*Cd*). During this analysis we made two observations that have not been previously made in pyramidal cells (Tyzio *et al.*, 1999): (i) the voltage-dependence of AMPA PSCs displayed a rectification at positive voltages, suggesting that in interneurons AMPA receptors may be calcium permeable and (ii) in contrast to pyramidal cells (Tyzio *et al.*, 1999) 'silent glutamatergic synapses' that expressed NMDA only and no AMPA PSCs (Isaac *et al.*, 1999), have not been identified in interneurons. This is unlikely to be due to a sampling problem as glutamatergic synapses are 10 times more frequent in interneurons than in pyramidal cells. Minimal stimulations should be used to confirm this observation that suggest that the sequential formation of NMDA and AMPA synapses may be different in the two types of neurons: in essence, it is possible that synapses formed on interneurons are mixed NMDA/AMPA from the start. This does raise important questions concerning the trafficking and targeting of receptors in pyramidal neurons and interneurons and may be in line with the abundant information that suggests differences between plasticity in both types of neurons.

GG interneurons have very heterogeneous shapes with round, oval, fusiform and pyramidal somata. They were more frequently recorded in SR (n = 64) and were also found in the other CA1 layers (SO, n = 8; SP, n = 10; SLM, n = 21). GG interneurons from SR with radial dendrites were more frequently encountered (45%) than cells with horizontal dendrites (15%), suggesting that GG interneurons probably received their glutamatergic inputs from Schaffer and commissural fibres. The morphology of immature interneurons appeared to be highly variable, but some adult types could be already recognized at birth. Thus, although less ramified, some horizontal interneurons (five cells) including those extending in three CA1 areas were found in SR. A large number of interneurons resemble IS-1, IS-2 and IS-3 types (29 cells) that have previously been described (Freund and Buzsáki, 1996). They were found in SO, SP, SR and SLM. In addition, neurons projecting outside CA1 such as immature O-LM (two cells), back projections (four cells) and interneurons projecting to the subiculum (two cells) were also recognized at birth (see Figs 2*C* and 3). In spite of this morphological diversity, functional glutamatergic synapses were always found in interneurons with axonal and dendritic arbours that are clearly more developed than those of G interneurons (Fig. 2*B*,*C*).

An important unresolved issue is the source of afferent GABAergic and glutamatergic fibres and, in particular, the possible contribution of inter-hemispheric connections. It is at present not known at which stage these fibres become functional, although there are some suggestions from electron microscopy that commissural synapses may be formed first on interneurons (Super *et al.*, 1998), but see Pleasure *et al.* (Pleasure *et al.*, 2000). Using interconnected hippocampi *in vitro* (Khazipov *et al.*, 1999) and recordings from both hemispheres suggests that there are indeed early functional synaptic connections from one hippocampus to the other (Khalilov *et al.*, in preparation).

Sequential Formation of Synapses In Utero

Our results suggest that at birth there is a sequential developmental program of synapses formation in both interneurons and pyramidal cells (Tyzio *et al.*, 1999). However, the interneuronal network may mature at an earlier stage since 78% of interneurons but only 8% of pyramidal neurons already expressed both $GABA_A$ and glutamatergic synapses (Fig. 4*A*). We next determined whether this template is also valid at an earlier developmental stage and in fetal slices (E18–E20) 52 pyramidal cells and 69 interneurons were recorded in parallel. The majority of pyramidal neurons in utero were found to be silent. Thus, 88% of pyramidal neurons had no spontaneous or evoked PSCs with only 6% of neurons with both $GABA_A$ and glutamate PSCs. In contrast, 65% of interneurons recorded in the same slices had functional synapses (37.6% of G interneurons and 27.5% of GG interneurons; Fig. 4*B*). These observations suggest that interneurons are in a more advanced developmental stage than pyramidal cells *in utero*. They also extend the conclusion that GABA synapses are formed before glutamate ones as the ratio of G to GG interneurons was more than six times higher at E18–E20 (1.36) than at P0 (0.21).

GABA is Already Excitatory In Utero

The above observations indicate that, compared to pyramidal neurons, interneurons have more active neurons and acquired GABAergic but also glutamatergic functional synapses earlier, in

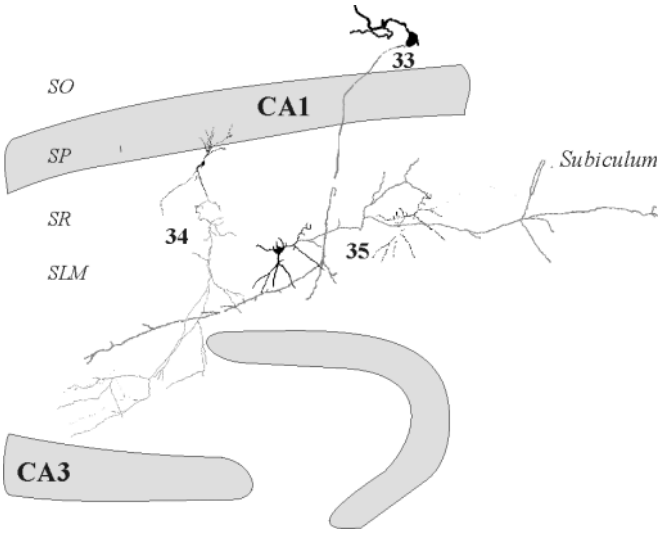

Figure 3. Interneurons projecting outside CA1. The three neurons were identified as GG interneurons at birth. Cell 33 represents an immature O-LM cell with its axon invading SLM of CA1 and then the CA3 area. Cell 34 may correspond to a type called back-projection, with its axon crossing the fissure and extending with ramification to the CA3 area. Cell 35 sends a long and ramified axon that emerges in the subiculum area.

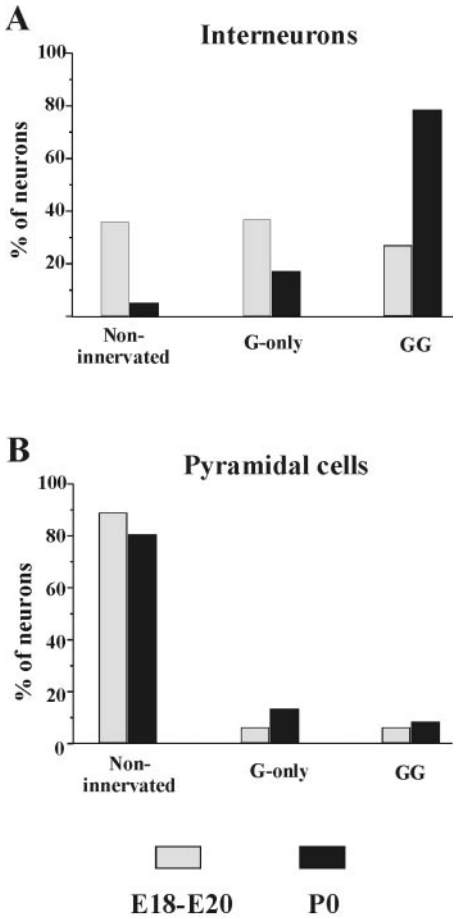

Figure 4. Relative proportions of the three types of interneurons and pyramidal cells at two developmental stages. (*A*) A large number (65%) of embryonic interneurons is already functional (45 cells) and this proportion greatly increases (95%) at birth (125 cells). Note that the proportion of G interneurons decreases at birth. (*B*) In embryonic slices, 88% of pyramidal cells (52 cells) are silent and this proportion is slightly reduced at birth [78%, 59 cells, data from Tyzio *et al.* (Tyzio *et al.*, 1999)].

agreement with our initial suggestion. Our developmental hypothesis also implied that GABA provided the initial excitatory drive in the absence of glutamatergic synapses. GABA is well known to be excitatory during early developmental stages in a large variety of tissues and species (Ben-Ari, 2002) and in postnatal rats, GABA was found to be excitatory in P2–P6 CA3 pyramidal neurons (Khazipov *et al.*, 1997). We have now extended this observation to CA1 embryonic interneurons. They were recorded from E19–E20 slices in cell-attached configuration in conditions that should not have perturbed $[Cl^-]_i$ and were then characterized in whole cell configuration. In GG interneurons (n = 4), electrical stimulation generated four to six action potentials that were blocked in part by D-APV (20 mM) and CNQX (10 mM) and in part by bicuculline (10 mM; Fig. 5). In G-only interneurons (n = 3), stimulation of the synaptic inputs evoked a pure $GABA_A$ receptor-mediated action potential discharge that was not affected by the addition of glutamate receptor antagonists. Therefore, activation of GABAergic synapses excites interneurons that are devoid of glutamatergic synapses, indicating that GABA is already excitatory in embryonic stages.

Immunocytochemistry of Peptides in P0 Slices

In addition to their morphology and functional responses, interneurons have been identified by their peptide content (Freund and Buzsáki, 1996). In this study, we have performed an immunocytochemical detection of some peptides to determine if it was possible to recognize at birth the type of more mature interneurons. Slices were incubated with polyclonal antibodies raised against calretinin, calbindin, parvalbumin, somatostatin, CCK-8 and substance P (Fig. 6). Calretinin immunoreactivity was intense in SLM of P0 rats, reflecting the presence in this layer of Cajal–Retzius cells. In addition, scattered small neurons were immunoreactive in the SO, SP and SR (Fig. 6*A*). Calbindin-positive cells were intensely stained in SO, but less so in SR, where cell bodies and small thin dendrites were immunoreactive. A few cells were also stained in the pyramidal layer, but

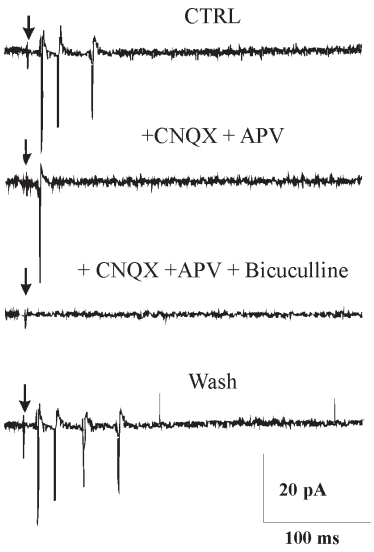

Figure 5. GABA contributes to the excitatory drive in CA1 interneurons. Traces of responses evoked in GG interneurons by electrical stimulation are recorded in cell-attached configuration in embryonic slices. Glutamatergic antagonists (CNQX, 20 μM; D-APV, 40 μM) reduce the number of spikes evoked in control conditions (CTRL). They are completely suppressed by further addition of bicuculline (10 μM) and are again evoked after washout of the drugs.

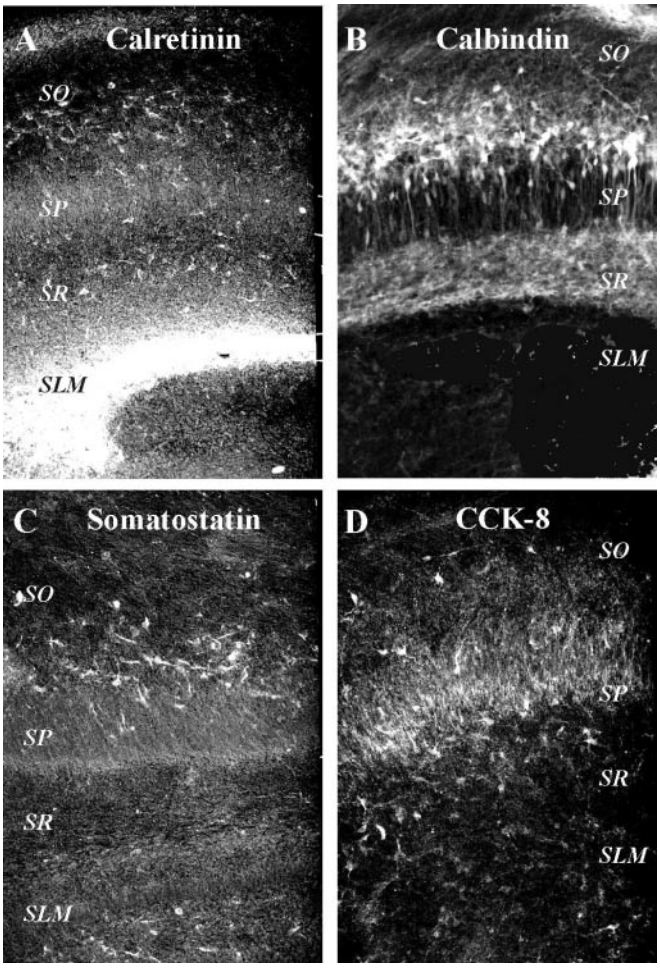

Figure 6. Immunocytochemistry in P0 slices. Adjacent hippocampal slices were incubated with polyclonal antibodies (see Materials and Methods). Only the CA1 region is shown (SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum moleculare). (*A*) Calretinin. The intense labelling corresponds to Cajal–Retzius cells. A less intense labelling is observed in SO and SR in cells characterized by a small dendritic tree and no specific orientation. (*B*) Calbindin. Immunoreactive cells are observed mainly in SO (the more intensely stained) and SR, where cell bodies and thin dendrites are labelled. A few pyramidal cells are also stained; within SP, cell bodies and the apical dendrites are lightly immunoreactive. (*C*) Somatostatin. Positive somata and dendritic extension are labelled in SO. In addition, few axons are observed in SLM. (*D*) CCK-8. Positive-cells are found in SO, SP and SR, but only cell bodies and some dendrites and not axons are labelled.

their cell bodies and apical dendrites were only lightly immunoreactive (Fig. 6*B*). Somatostatin-positive cells were observed in the SO, as reported for adults (Freund and Buzsáki, 1996) and in SLM only a few axons were immunoreactive. In SO, the dendrites of somatostatin-positive cells were frequently horizontal, suggesting that they may be the target of pyramidal recurrent axons (Fig. 6*C*). Several CCK8-inmunoreactive cells were located in SO and SR, less frequently in SP but not in SLM and their axons were generally not stained (Fig. 6*D*). Parvalbumin and substance P appeared not to be expressed at birth in CA1 hippocampal sections. In contrast, in slices from P7 rats taken as positive controls, different types of cells were labelled with the same antibodies (not shown). Therefore, already at birth, interneurons expressed some calcium binding proteins and peptides that characterized adult types interneurons.

Discussion

Analysis of morpho-functional characteristics of CA1 interneurons and pyramidal cells suggests that there is a developmental program of synapse formation with the sequential establishment of $GABA_A$ synapses prior to glutamatergic ones. This sequence occurs first in interneurons and then in pyramidal neurons (summarized in Fig. 7). In fetal slices, the first functional synapses are GABAergic synapses and are established between interneurons, then between interneurons and pyramidal cells dendrites. The inter-neuronal network is therefore already operative through excitatory GABAA synapses at a time when most principal cells are quiescent with no synaptic connections. Our results provide a general framework for the maturation of a cortical network and suggest that the consequence of these developmental gradients is that interneurons are in a position to exert a key role in the early maturation of the hippocampal network. Therefore, interneurons that have a different site of origin (Pleasure *et al.*, 2000) and divide before the principal cells (Soriano *et al.*, 1986; Altman and Bayer, 1990) also mature and become operative at an earlier stage. They are thus in a position to modulate, by the release of GABA, the maturation of the principal cells and the cortical circuit.

Sequential Formation of $GABA_A$ and Glutamatergic Synapses on Interneurons

At birth, functional $GABA_A$ and glutamatergic synapses were already formed. However, interneurons are functionally and morphologically heterogeneous and can be classified into three functional developmental stages: silent interneurons with no synapses and a small size; G interneurons with $GABA_A$ synapses only and soma and processes that are larger than those of silent neurons; and GG interneurons with $GABA_A$, AMPA and NMDA synapses and that are more morphologically developed than G interneurons. $GABA_A$ synapses appeared before glutamatergic synapses, as none of the recorded embryonic and P0 interneurons express glutamatergic synapses only. In addition, the majority of functional interneurons in embryonic slices were G-only interneurons, whereas the proportion of GG interneurons dramatically increases at birth with a simultaneous reduction of G-only interneurons.

The same sequence of synapse formation has been reported in pyramidal cells from rat (Tyzio *et al.*, 1999) and macaque (Khazipov *et al.*, 2001) hippocampus. However, interneurons acquired these synapses much earlier than pyramidal cells. Thus, in fetal neurons (E18–E20) 65% of interneurons are already functional, whereas 88% pyramidal neurons are still silent. This difference between the two types of cells is also observed at birth, since almost all interneurons (95%), but only 19% of pyramidal cells (Tyzio *et al.*, 1999) are functional. These observations indicate that interneurons that are born prior to pyramidal neurons (Soriano *et al.*, 1986; Altman and Bayer, 1990) also acquire synapses before principal cells.

This sequential formation of synapses has been observed in rat neocortex (Owens *et al.*, 1999) and isolated rat spinal cord (Nishimaru *et al.*, 1996), suggesting that this is a general property of developing neurons that has been preserved across evolution from rodents to primates. In addition, $GABA_A$ receptors were also detected before glutamatergic receptors by application of specific agonists in rat embryonic spinal cord cells (Walton *et al.*, 1993) and in cell cultures from rat hypothalamus (Chen *et al.*, 1995), hippocampus, septum and neocortex (Koller *et al.*, 1990), suggesting that even non-synaptic receptors follows the same developmental sequence.

The mechanisms underlying this sequential formation of

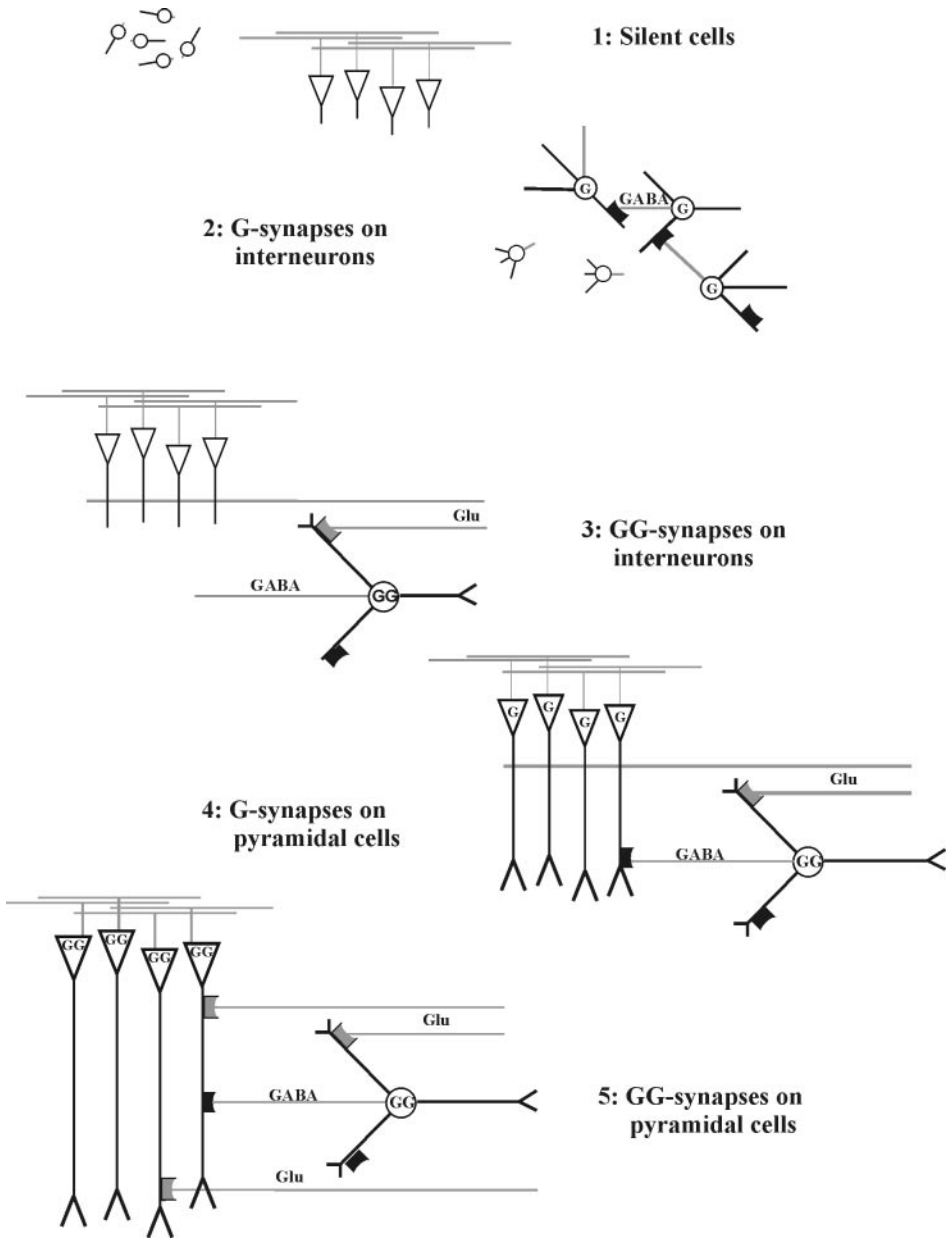

Figure 7. Sequential formation of synapses in interneurons and pyramidal cells during development. Interneurons are represented by a round cell body and pyramidal cells by a triangle. Step 1: both types are not yet innervated and have no functional synapses. Steps 2 and 3: interneurons, but not pyramidal cells, express sequentially $GABA_A$ (step 2) and glutamatergic (step 3) synapses; note that excitatory fibres selectively synapse with interneurons and not with the poorly developed apical dendrites of pyramidal cells. Steps 4 and 5: pyramidal neurons express sequentially $GABA_A$ (step 4) and glutamatergic (step 5) synapses on apical dendrites. Note that the apical dendrites of pyramidal cell progressively extended in SR (step 4) and SLM (step 5).

synapses are presently unknown. However, recent studies suggest that the formation of GABA synapses is an activity-dependent process. Thus, application to silent pyramidal cells that have no PSCs of repetitive intracellular pulses of current relevant to the physiological condition generate calcium currents leading to the expression of functional $GABA_A$ (Gubellini *et al.*, 2001). Thus, a rise of intracellular Ca^{2+} produced by intracellular postsynaptic stimulation is sufficient to activate the machinery required for the operation of GABA synapses.

Our results suggest that the degree of maturation of the target is crucial for the formation of glutamatergic synapses, as already suggested from cell cultures studies (Fletcher *et al.*, 1994). This degree of maturation may also involve the initial formation of $GABA_A$ synapses. In agreement with this hypothesis, glutamatergic synapses were not observed on interneurons or pyramidal cells, although afferent intrinsic and extrinsic afferent fibres are already present at birth in several CA1 layers of the rat (Diabira *et al.*, 1999) and mouse (Super and Soriano, 1994) hippocampus. Furthermore, the elongation of the apical dendrite of pyramidal cells is clearly required for the acquisition of glutamate synapses (Tyzio *et al.*, 1999).

Sequential Formation of Functional Interneuronal Types

The present morpho-functional study has suggested that there is also a sequential development of various populations of interneurons. At an early stage, synapses are likely to be formed between interneurons only. Indeed, few pyramidal cells have acquired $GABA_A$ synapses *in utero* (4%), whereas $GABA_A$

currents are already recorded in 38% of interneurons, suggesting that GABAergic synapses between interneurons and pyramidal cells are formed later. In agreement with this hypothesis, the morphology of a large number of G-only or GG interneurons resembles that of the different IS-types described previously (Freund and Buzsáki, 1996). These neurons are specialized to control other interneurons in the rat hippocampus (Gulyás *et al.*, 1996) and they contain calretinin (Gulyás *et al.*, 1996). Accordingly, we found that calretinin is clearly expressed in P0 interneurons from SR, confirming previous results showing that this calcium-binding protein was present at early postnatal stages (Jiang and Swann, 1997). Since pyramidal cells are almost silent at this developmental stage, these observations suggest that, for a short period of time, GABAergic synapses between interneurons are the only operative transmitter gated mode of communication between cells in CA1. They are therefore the source and the target of the first synapses formed in the hippocampus. In agreement with the group of Soriano (Super *et al.*, 1998), we suggest that interneurons constitute, to some extent, the pioneers in the establishment of a network that will primarily operate via excitatory GABAergic synapses.

Peridendritic interneurons contribute then to early $GABA_A$ synapses located on the proximal part of apical pyramidal dendrites. Such interneurons are known to contain calbindin and we found that this calcium binding protein was expressed in P0 slices, in agreement with previous studies (Soriano *et al.*, 1994; Freund and Buzsáki, 1996; Gulyás and Freund, 1996). Finally, at birth, interneurons that innervate the soma of pyramidal cells (perisomatic interneurons) are not functional. Indeed, pyramidal cells with a short dendrite remaining in the pyramidal layer have no $GABA_A$ synapses (Tyzio *et al.*, 1999). In addition, none of the 162 reconstructed interneurons extended a plexus of axonal ramification around the pyramidal cell layer, a hallmark of adult perisomatic cells (Freund and Buzsáki, 1996). Furthermore, blind patch directly in the pyramidal layer did not reveal interneurons with a basket- or chandelier-like morphology and, accordingly, we found no parvalbulmin-like immunoreactivity in P0 slices, as previously reported (Nitsch *et al.*, 1990; Solbach and Celio, 1991). As the expression of parvalbumin is dependent on the establishment of physiological activity (Solbach and Celio, 1991), we suggest that baskets cells containing parvalbumin may be present at birth but are not functional. However, in adult rats, there is a second population of basket cells that is devoid of parvalbumin but contains CCK (Freund and Buzsáki, 1996). At birth, CCK-positive cells have been observed mainly in SO, SR and, less frequently, in SP of CA1. Although some of them located in SP may correspond to non-functional basket cells because of their poorly developed axonal tree, those located in SO and SR may rather correspond to a subpopulation of dendrite-targeting cells (Cope *et al.*, 2002; Pawelzik *et al.*, 2002). Therefore, the late formation of functional perisomatic interneurons is in agreement with the observation that basket cells are generated after other interneurons types (Soriano *et al.*, 1986, 1994).We suggest that there is also another sequence, namely the formation of dendritic following by somatic inhibition. Since these two forms of inhibition convey different functional roles – the former controls the input of the hippocampus and the latter the output (Miles *et al.*, 1996) – we suggest that the dendrites are the site of initial interactions between glutamate and GABA synapses. Perhaps the early elaboration of a set of GABAergic synapses on the dendrites participates in the determination of glutamatergic synapses subsequently

An Early Operative GABAergic Network

Previous studies have shown that GABA is excitatory in P2 hippocampal (Khazipov *et al.*, 1997) and hypothalamic neurons (Wang *et al.*, 2001) and that synaptically released GABA increases $[Ca^{2+}]i$ (Leinekugel *et al.*, 1995). We have now extended this observation to the embryonic period. Indeed, we observed that GABA, through $GABA_A$ receptors, could trigger action potentials in E19– E20 interneurons from SR, even in the presence of glutamate antagonists. Therefore, at an early stage, when few glutamatergic synapses are functional, excitatory GABAergic synapses may provide the initial source of depolarization and increase intracellular calcium concentrations. Further studies are in progress to determine whether the network of interconnected interneurons could elicit oscillations at early developmental stages. An additional interesting point is the presence of interneurons that project across large parts of the developing hippocampal circuit (Fig. 3). These neurons most likely have developed at an early stage and may correspond to the neurons observed by Soriano and colleagues (Super *et al.*, 1998) and suggested to be pioneer cells that are the target of the first glutamatergic synapses and destined to die subsequently. These neurons are quite similar to the neurons that in cultures are required for the generation of GDPs (Voigt *et al.*, 2001) and are thus good candidates to provide distal synaptic connections required for the generation and propagation of this pattern.

In conclusion, our results suggest that the GABAergic interneuronal network is functional prior to that of the principal cells. Interneurons follow a different course of migration than pyramidal cells, but differentiate and become functional prior to the principal cells. However, they still abide by the same rules as to the expression of GABA and glutamate synapses. This observation adds an important element to accumulating evidence that reflects the central role of GABAergic synapses in brain maturation. In the adult brain, several behaviourally relevant patterns are generated by interneurons at a time when GABA is the principal inhibitory transmitter. We suggest that excitatory GABA exerts a similar role in the formation of the hippocampal network when pyramidal cells are silent.

Notes

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