Spontaneous synaptic activity is required for the formation of functional GABAergic synapses in the developing rat hippocampus

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Here we examine the role of the spontaneous synaptic activity generated by the developing rat hippocampus in the formation of functional γ-aminobutyric acid (GABA) synapses. Intact hippocampal formations (IHFs) were dissected at birth and incubated for 1 day in control or tetrodotoxin (TTX)-supplemented medium at 25°C. After the incubation, miniature $GABA_A$-mediated postsynaptic currents (m$GABA_A$-PSCs) were recorded in whole-cell voltage-clamped CA3 pyramidal neurones from IHF-derived slices. After 1 day *in vitro* in control medium, the frequency of m$GABA_A$-PSCs was similar to that recorded in acute slices obtained 1 day after birth, but significantly higher than the frequency recorded from acute slices just after birth. These results suggest that the factors required *in vivo* for the formation of functional GABAergic synapses are preserved in the IHFs *in vitro*. The frequency increase was prevented when IHFs were incubated for 1 day with TTX. TTX treatment affected neither the morphology of CA3 pyramidal neurones nor cell viability. The TTX effects were reproduced when IHFs were incubated in the presence of glutamatergic or GABAergic ionotropic receptor antagonists or in high divalent cationic medium. The present results indicate that the spontaneous synaptic activity generated by the developing hippocampus is a key player in the formation of functional GABAergic synapses, possibly via network events requiring both glutamatergic and GABAergic receptors.

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The proper development of highly organized structures in the central nervous system is a complex process that determines the functional integrity at adult stage. Neuronal circuits are formed over an extended period, both before and after birth. This process is under the control of both activity-independent and activity-dependent mechanisms. Most of our understanding of activity-dependent maturation of neuronal networks derives from studies on excitatory synapses. The classical examples include the neuromuscular junction (Lichtman & Purves, 1983), the climbing fibre–Purkinje cell synapse (Crepel *et al.* 1976) and the mammalian visual system (Stryker & Harris, 1986), where synaptic activity contributes to the refinement of initially coarse patterns of synaptic connections. Synaptic activity can also influence the functioning of neural circuits by scaling up or down the strength of excitatory synapses (Turrigiano *et al.* 1998). Nevertheless, synaptic inhibition can also be regulated by synaptic activity (Gaïarsa *et al.* 2002). In the auditory system for instance, the topographic organization of glycinergic projections is achieved through synapse elimination (Sanes & Siverls, 1991), a process involving activity-dependent mechanisms (Sanes & Tackacs, 1993). Manipulations inducing epileptic activity lead to a general increase in the level of GABAergic synaptic activity in the developing brain both *in vivo* and *in vitro* (Seil *et al.* 1994; Marty *et al.* 2000; Galante *et al.* 2000). Conversely, chronic blockade of synaptic activity during restricted periods of development reduces the amount of functional inhibition received by the target cells (Seil & Drake-Baumann, 1994; Rutherford *et al.* 1997; Galante *et al.* 2000; Kilman *et al.* 2002).

To date evidence for a role of synaptic activity in the functional maturation of inhibitory connections has

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been derived from experiments in which activity was altered for several days. However, whether acute blockade leads to the same outcome has been recently questioned (Craig, 1998; Zhu & Malinow, 2002). Moreover, most studies have been performed on neurones or explants of nervous tissue in culture. Although these preparations offer several advantages and have led to important information on the roles of chemical cues and neuronal activity in shaping neuronal connectivity, they also have some limitations. Following the dissociation procedure, cells in culture re-establish a neuronal network that is completely different to the *in vivo* situation, and cutting axons during explant preparation inevitably leads to a remodelling of connectivity in organotypic slice culture (Gahwiler *et al.* 1997).

A more complex preparation, in which the whole neuronal network is preserved, is therefore required to investigate the development of neuronal circuits under well-controlled conditions. In the present study, we used the neonatal intact hippocampal formation (IHF) (Khalilov *et al.* 1997), a preparation that offers the advantages of both the *in vitro* (control of the external medium) and *in vivo* (a complete preservation of the intra-hippocampal neuronal network) approaches. We found that the spontaneous synaptic activity generated by the developing hippocampus plays a key role in the formation of functional GABAergic synapses.

Methods

All experiments were carried out according to the guidelines laid down by the INSERM animal welfare committee.

Preparation of intact hippocampal formation

The procedure for the preparation of the intact IHFs was similar to that previously described (Khalilov *et al.* 1997). Brains were removed from anaesthetized (350 mg kg^{-1} chloral hydrate, administered intraperitoneally) Wistar rats at birth and submerged in artificial cerebrospinal fluid (ACSF) with the following composition (mM): NaCl, 126; KCl, 3.5; $CaCl_2$, 2; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; $NaHCO_3$, 25; and glucose, 11; pH 7.4, equilibrated with 95% O_2 and 5% CO_2. The hippocampi were then incubated at 25°C in 800 ml ACSF (oxygenated with 95% O_2 and 5% CO_2) alone or supplemented with different drugs. For the 48 h incubation periods, the ACSF was changed after 24 h. After the incubation, hippocampal slices (600 μm thick) were cut with a McIlwain tissue chopper and kept in ACSF at 25°C for 60 min before use.

Whole-cell recordings

Whole-cell patch-clamp recordings of CA3 pyramidal neurones were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Borosilicate microelectrodes (4–8 MΩ) were filled with the following solution (mM): CsCl, 110; potassium gluconate, 30; *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulphonic acid (Hepes), 10; EGTA, 1.1; $CaCl_2$, 0.1; MgATP, 4; NaGTP, 0.3; 5-(and-6)-tetramethylrhodamine biocytin (rhodamine, 0.5–1%); pH = 7.25, osmolarity = 275 mosmol l^{-1}. Series resistance (R_s), membrane capacitance (C_m) and input resistance (R_i) were determined by an online fitting analysis of the transient currents in response to a 5 mV pulse with Acquis 4.0 software (Bio-logic, Orsay, France). Criteria for accepting a recording included a resting potential < -55 mV, $R_i > 400$ MΩ, $R_s < 25$ MΩ.

Data acquisition and analysis

Miniature and spontaneous $GABA_A$ receptor-mediated postsynaptic currents ($GABA_A$-PSCs) were recorded at a holding potential of −70 mV. The currents were stored on an Axoscope 8.1 (Axon Instruments, Foster city, CA, USA) and analysed off-line with Mini Analysis program (Synaptosoft 5.1, Decatur, GA, USA). The fact that no false events would be identified was confirmed by visual inspection for each experiment. To generate the average m$GABA_A$-PSCs, multiple overlapping events were discarded, and the remaining events were aligned on their rising phase. The histogram and cumulative distributions were constructed using $GABA_A$-PSCs recorded for 10–30 min. For data presented as mean ± S.E.M., statistical analysis was performed using Student's unpaired *t* test. The level of significance was set as $P < 0.05$.

Morphological characterization of recorded cells

After the recording session, the slices were immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, 0.9% NaCl) overnight at 4°C. The slices were then rinsed in PBS, mounted on gelatin-coated slides and coverslipped with an aqueous mounting medium (Gel Mount, Biomeda, Foster City, CA, USA). The rhodamine-filled cells were analysed with an Olympus confocal microscope (Fluoview BX50WI, Germany) using a helium/neon laser ($\lambda_{excitation} = 543$ nm; $\lambda_{emission} = 560$ nm). Series of digitized optical sections (1024 pixels × 1024 pixels, step: 1.5 μm, lens: × 20 or × 40) were collected and maximum-intensity projections were derived using Olympus Fluoview software. The soma and apical dendrites of each neurone were reconstructed for morphometric analysis using Neurolucida 2000 software (Microbrightfield Inc., Colchester, VT, USA) from confocal optical sections. To establish possible differences in the dendritic branching pattern, Sholl analysis (Sholl, 1953) was carried out for the apical dendritic tree. The number of dendritic intersections within concentric rings (50 μm radius rings progressively more distal from the soma) was counted.

Cell death detection

Cell death was evaluated using propidium iodide (PI, Molecular Probes, Leiden, The Netherlands) which, on loss of membrane integrity, binds to nucleic acids and emits a bright red fluorescence with the rhodamine filter (Bevensee *et al.* 1995). PI (3 μM) was added to the ACSF for 2 h at room temperature after the 24 h incubation period. The IHFs were then immersed in a fixative solution containing 4% paraformaldehyde in 0.1 M PBS overnight at 4°C and stored in cryo-preservative solution (PBS with 20% sucrose and 0.01% sodium azide) at 4°C. Transverse slices (50 μm thick) were cut with a cryotome (Leica CM 1325, Switzerland). The sections were collected in PBS and mounted with an aqueous mounting medium (Gel Mount, Biomeda). Immunoreactivity was analysed with an Olympus confocal microscope (Fluoview BX50WI, Germany) (argon laser source: $\lambda_{excitation} = 543$ nm, $\lambda_{emission} = 560$ nm; lens: × 60). The density of PI-positive nuclei was determined in the CA3 stratum radiatum (view field area = 4300 μm^2) and pyramidal layer (view field area between 8127 and 22 882 μm^2). Counts were performed using ImageJ software from NIH on 8–15 view fields chosen randomly (4–6 independent experiments each).

Drugs

6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-2-amino-5-phosphovaleric acid (D-APV), and bicuculline were purchased from Tocris Cookson (Bristol, UK). Tetrodotoxin was purchased from Sigma (St Louis, MO, USA). 5-(and-6)-Tetramethylrhodamine biotin (rhodamine) was purchased from Molecular Probes.

Results

In the present study, the miniature $GABA_A$ receptor-mediated postsynaptic currents ($mGABA_A$-PSCs) were taken as an index of the formation of functional GABAergic synapses. The $mGABA_A$-PSCs were recorded under whole-cell voltage clamp ($V_h = -70$ mV) from identified (rhodamine) hippocampal CA3 pyramidal cells in the presence of the ionotropic glutamate receptor antagonists (CNQX, 10 μM, and D-APV, 40 μM) and TTX (1 μM) (Fig. 1*A* and *B*). The remaining activity was entirely blocked by bicuculline (10 μM) indicating that they were mediated by $GABA_A$ receptors (Fig. 1*A*). The amplitude distribution of the $mGABA_A$-PSCs recorded in each neurone was skewed toward large-amplitude events (Fig. 1*C*) and their inter-event intervals (IEI) had a random distribution (Fig. 1*D*).

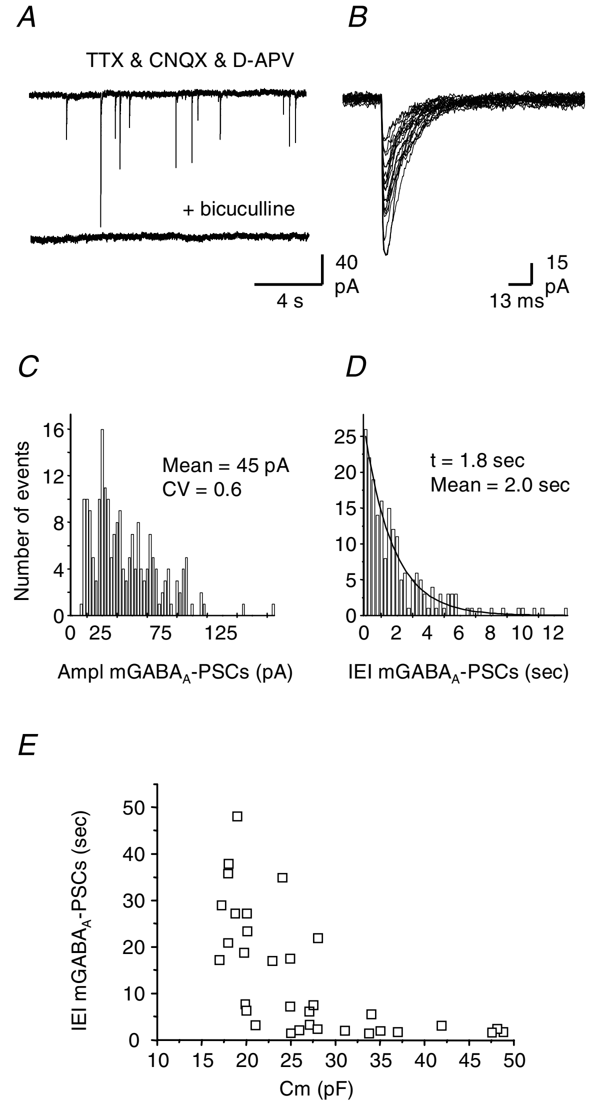

Figure 1. Properties of $mGABA_A$-PSCs in the developing CA3 pyramidal neurones
A, whole-cell voltage-clamp recording from a CA3 pyramidal cell (P1) held at −70 mV. The miniature inward currents recorded in the presence of 10 μM CNQX, 40 μM D-APV and 1 μM TTX were entirely blocked by a further application of bicuculline (10 μM).
B, 25 consecutive $mGABA_A$-PSCs recorded from the same cell as in *A*, superimposed and aligned to their rising phase. Note their large amplitude range. *C*, amplitude histogram of $mGABA_A$-PSCs recorded from the same cell as in *A*. The distribution is not Gaussian but skewed toward larger values. *D*, histogram of inter-event intervals (IEIs) for $mGABA_A$-PSCs recorded from the same cell as in *A*. The data are well described by an exponential function (smooth curve) with a time constant *(t)* close to the mean IEI, indicating the random occurrence of $mGABA_A$-PSCs. *E*, plot of the IEIs of $mGABA_A$-PSCs as a function of the membrane capacitance (C_m) of the CA3 pyramidal neurones recorded at P0.

Formation of functional GABAergic synapses *in vivo* and *in vitro*

To gain insight into the relevance of the *in vitro* intact hippocampal formation (IHF) to the *in vivo* situation, the $mGABA_A$-PSCs were recorded from acute slices at birth (postnatal day (P) 0) and 1 day after birth (P1) and these data were then compared to those obtained in slices from P0 IHFs incubated during 1 day *in vitro* in control conditions at 25°C.

The mean IEI of $mGABA_A$-PSCs changed dramatically, decreasing over 3-fold both after 1 day *in vivo* (P1) and after 1 day *in vitro* in ACSF (1 DIV ACSF) (Fig. 2*A*). The decrease in the IEI of $mGABA_A$-PSCs was reflected by the leftward shift of the cumulative IEI distribution (Fig. 2*B*). The mean coefficient of variation of $mGABA_A$-PSC amplitude (CV_a) increased significantly from P0 to P1 and 1 DIV ACSF (Table 1), while the mean amplitude and kinetics were not different (Table 1). These results suggest that the maintenance of the IHF for 1 day *in vitro* at a subphysiological temperature (25°C) did not significantly alter the formation of functional GABAergic synapses.

During our recording sessions we observed that the IEI of $mGABA_A$-PSCs was highly variable from cell to cell and, in fact, depended on the membrane capacitance (C_m) of the cells: the higher the C_m, the lower the IEI of $mGABA_A$-PSCs (Fig. 1*E*). Thus, to further strengthen the relevance of the IHF preparation, we decided to compare the IEIs of $mGABA_A$-PSCs among neurones having similar C_m at 1 DIV ACSF, P0 and P1. As illustrated in Fig. 2*C*, neurones with a $C_m \leq 25$ pF, accounting for the majority of the recorded cells, exhibited a higher IEI of $mGABA_A$-PSCs at P0 when compared to P1 ($P = 0.04$) and 1 DIV ACSF ($P = 0.05$). Moreover, the values obtained at P1 and after 1 DIV ACSF completely overlapped. In contrast, cells with a $C_m > 25$ pF showed the same level of $mGABA_A$-PSCs at P0, P1 and 1 DIV ACSF. Altogether these results indicate that the factors required for the formation of functional GABAergic synapses *in vivo* are preserved in the IHFs *in vitro*.

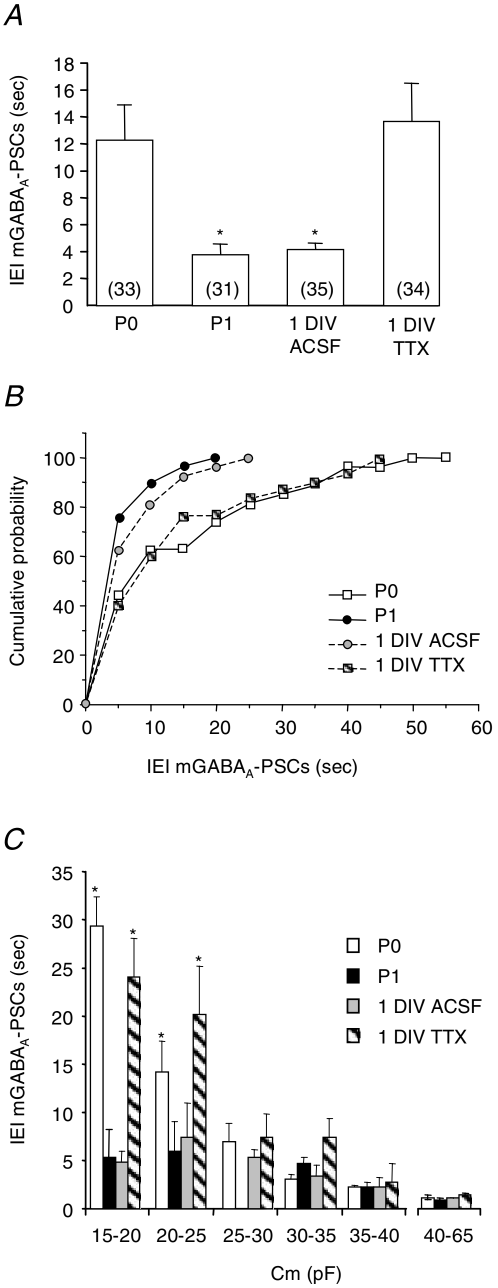

Figure 2. Spontaneous synaptic activity is required for the formation of functional GABAergic synapses
A, mean IEIs of $mGABA_A$-PSCs recorded at birth (P0), 1 day after birth (P1) and after 24 h *in vitro* in control conditions (1 DIV ACSF) or in the presence of TTX (1 DIV TTX). * $P < 0.05$ when compared to P0 and 1 DIV TTX. The numbers in parentheses are the total number of neurones recorded in each condition. *B*, cumulative distribution of the mean IEIs of the $mGABA_A$-PSCs recorded in the different conditions. *C*, plot of the IEIs of $mGABA_A$-PSCs as a function of the membrane capacitance (C_m) of the cells recorded in the different conditions. * $P < 0.05$ when compared to P1 and 1 DIV ACSF.

Activity deprivation alters the formation of functional GABAergic synapses *in vitro*

Having established the relevance of the IHF preparation to study the formation of functional GABAergic synapses, we next asked whether spontaneous synaptic activity does play a role in this process. To address this point, the P0 IHFs were incubated for 1 day *in vitro* in ACSF supplemented with TTX (1 DIV TTX). As shown in Fig. 2, the decrease in the mean IEI of $mGABA_A$-PSCs and the leftward shift of the cumulative IEI distribution of $mGABA_A$-PSCs observed after 1 DIV ACSF did not occur when spontaneous synaptic activity was blocked. The increase in the CV_a of $mGABA_A$-PSCs observed in control conditions was also prevented (Table 1). The amplitude and kinetics of $mGABA_A$-PSCs were, however, not affected by the treatment with TTX (Table 1). These data therefore suggest that activity deprivation affects the formation of

Table 1. Properties of mGABA$_A$-PSCs

	Amplitude (pA)	CV$_a$	10–90% rise time (ms)	Decay time (ms)	IEI (s)	C_m (pF)	*n*
P0	44 ± 3	0.53 ± 0.02	1.1 ± 0.08	10.1 ± 0.4	12.3 ± 2.6	27.8 ± 1.8	33
P1	42 ± 2	0.63 ± 0.02*	1.2 ± 0.08	9.5 ± 0.4	3.6 ± 0.7*	39.7 ± 2.4*	31
1 DIV ACSF	44 ± 3	0.65 ± 0.02*	1.2 ± 0.09	12.2 ± 0.6	4.7 ± 1*	28.9 ± 1.3	35
1 DIV TTX	47 ± 3	0.55 ± 0.03 †	1.1 ± 0.1	11.5 ± 0.4	12 ± 2.3 †	27.6 ± 1.7	34
2 DIV ACSF	39 ± 5	0.65 ± 0.04	1.3 ± 0.18	11.8 ± 0.7	5.4 ± 1.2	27.3 ± 1.1	12
2 DIV TTX	48 ± 6	0.52 ± 0.04 §	1.1 ± 0.08	10.6 ± 0.8	13.8 ± 2.9 §	28.4 ± 5	11
1 DIV TTX + 1 DIV ACSF	47 ± 5	0.65 ± 0.03	1.1 ± 0.04	11.2 ± 0.7	6.1 ± 1.6	31.1 ± 3	15

Values are means ± S.E.M. *n* represents the total number of neurones recorded in each condition. CV$_a$, coefficient of variation of mGABA$_A$-PSC amplitude; IEI, inter-event interval; C_m, membrane capacitance. * $P < 0.05$ when compared to P0. † $P < 0.05$ when compared to 1 DIV ACSF. § $P < 0.05$ when compared to 2 DIV ACSF.

functional GABAergic synapses. Comparing the IEI of mGABA$_A$-PSCs among neurones having similar C_m values further supports this hypothesis (Fig. 2*C*). This plot shows that the cells with a $C_m \leq 25$ pF exhibited a higher IEI of mGABA$_A$-PSCs after 1 DIV TTX than after 1 DIV ACSF. Moreover, the values obtained at P0 and after 1 DIV TTX were not significantly different.

Activity deprivation has no detectable effect on the morphological development of hippocampal CA3 pyramidal neurones

The decreased level of mGABA$_A$-PSCs after activity deprivation might be accounted for by an alteration in the morphological development of the target pyramidal neurones (Barbin *et al.* 1993; Luthi *et al.* 2001; Groc *et al.* 2002). Indeed, a smaller dendritic tree is likely to receive fewer GABAergic synapses and thus decrease GABAergic activity in the neurone.

To test this possibility, we performed a morphometric analysis of the rhodamine-loaded cells at 1 DIV ACSF ($n = 20$) and 1 DIV TTX ($n = 17$). Only cells with a $C_m \leq 25$ pF were included in this analysis – those were the cells showing the most significant IEI change after activity deprivation (see Fig. 2*C*). Furthermore, only the apical dendritic tree was analysed since the basal dendrite is absent or poorly developed at that stage (Tyzio *et al.* 1999). As illustrated in Fig. 3*A* and *B*, we found no significant difference in the total apical dendritic length ($P = 0.34$), the total number of apical segments ($P = 0.87$) and dendritic nodes ($P = 0.75$), and the mean apical dendritic segment length ($P = 0.08$). The complexity of the dendritic trees was further assessed using Sholl (1953) analysis (Fig. 3*C*). Again, no major differences were evident between control and TTX-treated cells.

Altogether these data show that the lower frequency of mGABA$_A$-PSCs after TTX treatment cannot be explained by an indirect effect of activity deprivation on the morphological development of the target CA3 pyramidal cells.

Activity deprivation does not lead to cell death

An important question that remained to be addressed was whether the maintenance of the IHFs in control or TTX-treated conditions affected neuronal survival. Indeed, selective GABAergic cell death in TTX could account for the lower level of mGABA$_A$-PSCs observed after activity deprivation. On the other hand, pyramidal cell death in ACSF could trigger a redistribution of GABAergic terminals on surviving neurones, leading to an increase in the level of mGABA$_A$-PSCs.

To explore these possibilities further, the occurrence of cell death was quantified by densitometric measurement of the cellular uptake of propidium iodide (PI, Fig. 4). The percentage of view fields (VFs, see Methods) with PI-fluorescent nuclei in the pyramidal layer and stratum radiatum was not different between control and TTX-treated IHFs (Fig. 4*B*). To test the sensitivity of our method, similar experiments were performed on IHFs after 2 days of *in vitro* incubation that should lead to a more significant cell death. Indeed, the percentage of VFs with PI-fluorescent nuclei significantly increased (Fig. 4*B*). It should be noted however, that, even in these conditions, the density of PI-fluorescent nuclei remained rather low (range 1–5 positive nuclei per VF, Fig. 4*C*).

To further exclude cell death, the 1 DIV TTX IHFs were incubated for a further 24 h in control ACSF (1 DIV TTX + 1 DIV ACSF) to restore spontaneous synaptic activity. If the additional incubation caused the GABAergic activity to recover to control values, a selective death of GABAergic interneurones could be definitely excluded. Values from IHFs incubated for 2 days in either control (2 DIV ACSF) or TTX medium (2 DIV TTX) were used as controls. As shown in Table 1, after 1 DIV TTX + 1 DIV ACSF the mean IEI of $mGABA_A$-PSCs recovered to a value similar to that obtained after 2 DIV ACSF and significantly different to that obtained after 2 DIV TTX. Moreover, the CV_a of $mGABA_A$-PSCs also recovered to the 2 DIV ACSF value.

Altogether, these data show that GABAergic cell death does not account for the decreased frequency of $mGABA_A$-PSCs observed after activity deprivation.

Characterization of the synaptic activity involved in the formation of functional GABAergic synapses

Having established that the formation of functional GABAergic synapses required spontaneous synaptic activity, we sought to specify the type of activity involved.

As a first approach to answering this question, we investigated the nature of the spontaneous synaptic activity generated in the IHF after 1 DIV ACSF at 25°C

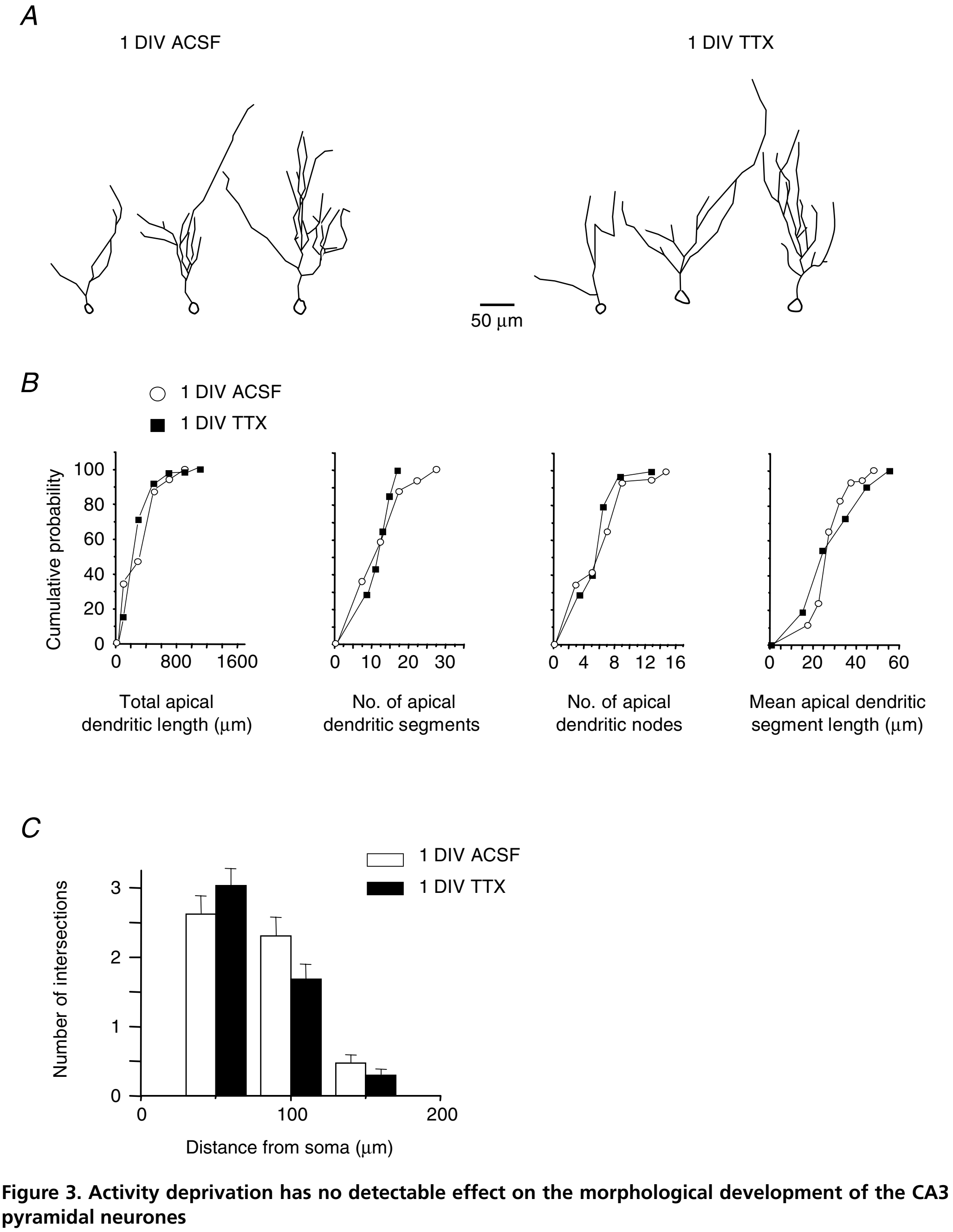

Figure 3. Activity deprivation has no detectable effect on the morphological development of the CA3 pyramidal neurones

A, representative examples of the three-dimensional reconstruction of rhodamine-filled CA3 pyramidal neurones obtained from control (1 DIV ACSF) and TTX (1 DIV TTX)-treated intact hippocampal formations (IHFs). *B*, cumulative distribution of the morphological parameters quantified in the different conditions. *C*, Sholl analysis of rhodamine-filled CA3 pyramidal neurones obtained from control and TTX-treated IHFs. The number of intersections within each concentric ring (50 μm beginning from the soma) is plotted *versus* the distance from the soma.

(the temperature at which the IHFs were incubated). Whole cell patch-clamp recordings showed that the giant depolarizing potentials (GDPs) that characterized the neonatal hippocampus (Ben-Ari *et al.* 1989) were present after incubation *in vitro* (Fig. 5*A*), and constituted the main synaptic activity of the incubated IHFs. They occurred at a mean IEI of 27 ± 4 s (range from 16 to 32 s, $n = 7$, 1 neurone per IHF). As already reported (Ben-Ari *et al.* 1989), these events were blocked by high divalent cation ACSF (6 mM Mg^{2+} and 4 mM Ca^{2+}, $n = 4$, not shown), by the ionotropic glutamatergic receptor antagonists (10 μM CNQX and 40 μM D-APV, $n = 6$, Fig. 5*A*), or by the $GABA_A$ receptor antagonist (10 μM bicuculline, $n = 5$, Fig. 5*A*). In the presence of CNQX and D-APV, spontaneous $GABA_A$-PSCs (s$GABA_A$-PSCs) occurred at a mean IEI of 2.15 ± 0.35 s (range from 0.8 to 3.2 s, $n = 6$, Fig. 5*A*). Bicuculline alone completely abolished all spontaneous synaptic activity in three out of five neurones (Fig. 5*A*) and led to the appearance of spontaneous epileptiform discharges in one neurone. In the remaining neurone, spontaneous glutamatergic PSCs were recorded at a mean IEI of 5.9 ± 1.2 s.

We next used these different pharmacological agents to identify the type of spontaneous activity involved in the formation of functional GABAergic synapses *in vitro*. The IHFs were incubated for 24 h in ACSF supplemented with CNQX (10 μM) and D-APV (50 μM), or with bicuculline (10 μM), or in high divalent cation

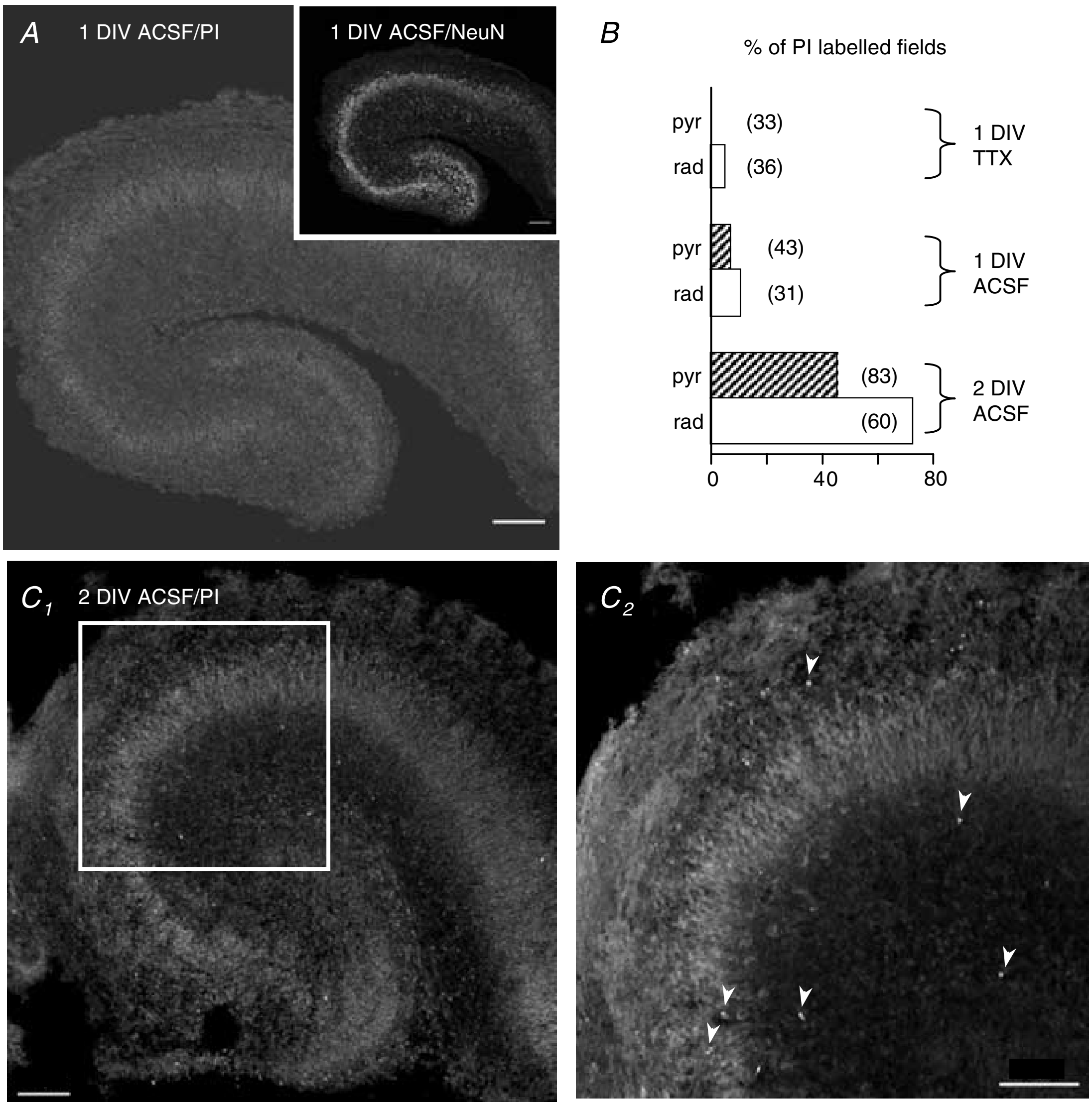

Figure 4. Activity deprivation does not lead to cell death
Immunofluorescent propidium iodide (PI)-positive nuclei on hippocampal slices obtained from intact IHFs incubated for one (*A*) or two (*C*) days in ACSF. C_2, enlargement of the field shown in C_1. Arrowheads point to PI-positive nuclei. Inset in *A* depicts neuronal nuclei (NeuN) staining of the same section. *B*, percentage of view fields exhibiting PI-positive nuclei in the different conditions. pyr, pyramidal layer; rad, stratum radiatum. The numbers in parentheses are the total number of view fields analysed. Calibration bars, 100 μm.

ACSF (6 mM Mg^{2+} and 4 mM Ca^{2+}), a procedure known to preferentially block network-driven polysynaptic activity (Berry & Penthreath, 1976). Only cells with a $C_m \leq 25$ pF were included in this analysis (see Fig. 2*C*). As illustrated in Fig. 5*B*, in all conditions the mean IEI of $mGABA_A$-PSCs was similar to that obtained after 1 DIV TTX ($P = 0.89, 0.98$ and 0.7, respectively) and significantly different to that obtained after 1 DIV ACSF ($P = 0.01, 0.01$ and 0.006, respectively). Similarly, the increase in the CV_a observed after 1 DIV ACSF was prevented by the different pharmacological manipulations ($CV_a = 0.49 \pm 0.04$ after 1 DIV CNQX + D-APV ($P = 0.005$), 0.51 ± 0.03 ($P = 0.02$) after 1 DIV bicuculline, and 0.49 ± 0.03 ($P = 0.005$) after 1 DIV $Mg^{2+} + Ca^{2+}$. Moreover, the amplitude and kinetics of $mGABA_A$-PSCs were not affected by the different pharmacological manipulations. It is worth pointing out that similar results were obtained when cells with $C_m > 25$ pF were included for quantification (not shown), as with P0 IHFs incubated in TTX.

Altogether, these results suggest that a network-driven activity requiring glutamatergic and GABAergic receptors is involved in the formation of functional GABAergic synapses in the developing rat hippocampus.

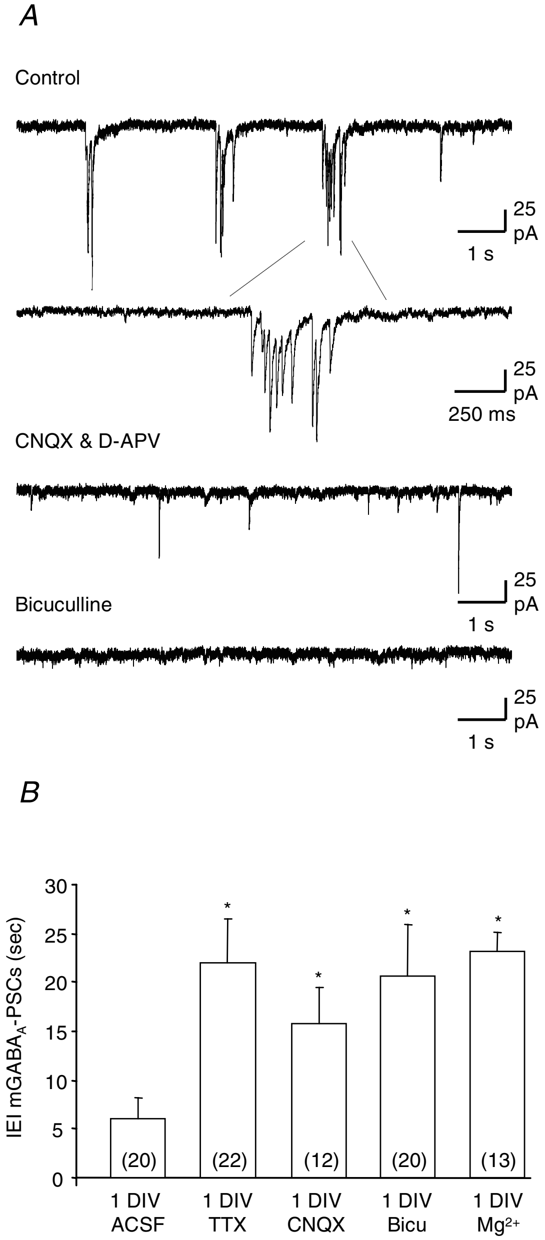

Figure 5. Effect of pharmacological agents on the formation of functional GABAergic synapses
A, whole-cell recording at 25°C of spontaneous synaptic activity generated by an IHF after 1 DIV ACSF. The network discharges that characterize the newborn rat hippocampus were present. These discharges were blocked by the ionotropic glutamatergic receptor antagonists CNQX (10 μM) and D-APV (40 μM), or by the $GABA_A$ receptor antagonist bicuculline (Bicu, 10 μM). *B*, mean IEIs of $mGABA_A$-PSCs recorded after 24 h *in vitro* in the different conditions. To construct this graph only the neurones with a $C_m \leq 25$ pF were selected. *$P < 0.05$ when compared to 1 DIV ACSF. The numbers in parentheses are the total number of neurones recorded in each condition.

Effect of activity deprivation on the spontaneous GABAergic synaptic activity

Several studies have reported that neuronal networks can compensate for activity deprivation to maintain the homeostasis of synaptic activity (Turrigiano, 1999; Burrone *et al.* 2002). To determine whether such a phenomenon occurs in our preparation, we have investigated the effect of activity deprivation on spontaneous $GABA_A$-PSCs ($sGABA_A$-PSCs) isolated in the presence of CNQX (10 μM) and D-APV (40 μM). During the first day of postnatal life *in vivo*, the IEI of $sGABA_A$-PSCs significantly decreased from 7.8 ± 1.2 s at P0 to 2.4 ± 0.8 s at P1 ($P = 0.03$) (Fig. 6). Similarly, after 1 DIV ACSF (Fig. 6) the IEI of $sGABA_A$-PSCs decreased to 2.6 ± 0.46 s, a value similar to that obtained at P1 ($P = 0.9$) and significantly different to that obtained at P0 ($P = 0.01$).

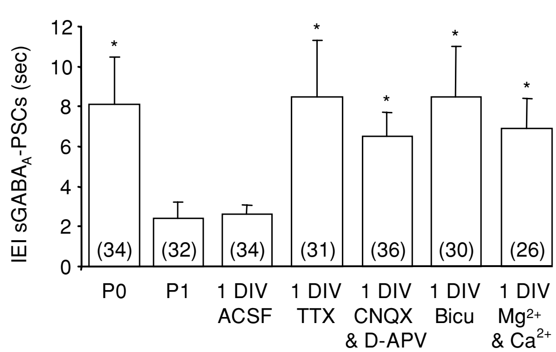

Figure 6. Effect of activity deprivation on spontaneous GABAergic synaptic activity
Mean IEIs of spontaneous $GABA_A$-PSCs recorded after 1 day *in vitro* in the different conditions. *$P < 0.05$ when compared to P1 and 1 DIV ACSF. The numbers in parentheses are the total number of neurones recorded in each condition.

As illustrated in Fig. 6, silencing the IHFs with different pharmacological agents prevented this decrease. In these conditions, the IEIs of $sGABA_A$-PSCs were similar to those obtained at P0.

These results suggest that the excitability of GABAergic cells does not compensate for the loss of functional GABAergic synapses following 24 h activity deprivation.

Discussion

In the present study, we used a combined morphological and physiological approach to investigate the role of synaptic activity in the formation of functional GABAergic synapses. Our results show that the *in vitro* intact hippocampal formation (IHF) is a useful preparation to address this question, and indicate that neuronal activity plays a crucial role in the formation of functional GABAergic synapses in the developing rat hippocampus.

The IHF to study activity-dependent maturation of GABAergic synapses

A common concern when studying activity-dependent maturation of neuronal networks *in vitro* is the relevance of the experimental model to the *in vivo* situation. In this study, we have used the intact hippocampal formation (IHF) (Khalilov *et al.* 1997) in which the whole intra-hippocampal network is preserved. Our first aim was to validate this preparation. We have shown that the IHFs survived for 24–48 h *in vitro*, with a good preservation of their morphological and physiological characteristics. The validity of this preparation would also depend on how closely the maturation *in vitro* mimics that *in vivo*. We have shown that, after 1 day *in vitro* in control medium, the overall mean frequency of $mGABA_A$-PSCs, and the frequency of $mGABA_A$-PSCs from cells of comparable C_m, are similar to those recorded in acute slices 1 day after birth. These observations indicate that, although the IHFs were incubated at a subphysiological temperature, the factors required for the formation of functional GABAergic synapses are preserved in this *in vitro* preparation. This preparation is therefore suitable to investigate the development of GABAergic connections in different well-controlled experimental conditions.

The formation of functional GABAergic synapses requires spontaneous synaptic activity

To determine whether the formation of functional GABAergic synapses only depends on the morphological state of the pyramidal neurones, or requires further environmental cues, we have compared the IEI of $mGABA_A$-PSCs at comparable C_m in the different conditions. Since C_m correlates with the dendritic extent (Tyzio *et al.* 1999; Hennou *et al.* 2002; I. Colin-Le Brun & J. L. Gaïarsa, unpublished results), we reasoned that the level of $mGABA_A$-PSCs would be the same at comparable C_m at birth and 1 day after birth if the formation of functional GABAergic synapses only depends on the dendritic extension of the pyramidal neurones. We found, however, a clear difference in the frequency of $mGABA_A$-PSCs between P0 and P1 or 1 DIV ACSF for cells with a $C_m \leq 25$ pF. In contrast, cells with a $C_m > 25$ pF did not show a significant difference. These data might suggest that the formation of only the first functional GABAergic synapses depends on the dendritic outgrowth. However, an alternative explanation is that the level of $mGABA_A$-PSCs is too high and the number of cells with a $C_m > 25$ pF too low to detect significant differences. To clarify this point, longer periods of incubation would be necessary.

When the IHFs were incubated for 24 h in TTX, the increase in $mGABA_A$-PSC frequency observed after 1 day *in vitro* in control medium was no longer present. The present study indicates that this effect does not result from an alteration in the morphological development of the CA3 target cells, nor from a modification of cell viability. Altogether, these results show that the formation of functional hippocampal GABAergic synapses is an active process requiring the spontaneous synaptic activity generated by the developing hippocampus.

Mechanisms of activity-dependent formation of functional GABAergic synapses

Activity deprivation has been shown to decrease the density of $GABA_A$ receptors at functional synapses, as proposed in dissociated cortical neurones in culture (Kilman *et al.* 2002; but see also Craig *et al.* 1994; Gally & Bessereau, 2003), or decrease the immunoreactivity for GABA or its synthesizing enzyme (Hendry & Jones, 1988; Benevento *et al.* 1995; Rutherford *et al.* 1997). These events appear unlikely, however, in the developing hippocampus as the two phenomena would have led to a decrease in both amplitude and frequency of $mGABA_A$-PSCs (Rutherford *et al.* 1997; Engel *et al.* 2001; Kilman *et al.* 2002). In the present study we observed that the formation of functional GABAergic synapses during the first day *in vivo* and *in vitro* is reflected by an increase in the frequency of $mGABA_A$-PSCs with no change in their mean amplitude, and silencing the IHFs prevents this increase. Although multivesicular release may also contribute (Ling & Benardo, 1999; Llano *et al.* 2000), it is worth pointing out that differences in $mGABA_A$-PSC frequency are usually attributed to differences in the total number of functional GABAergic synapses or probability of GABA release. In the present study, we observed an increase in the coefficient of variation of $mGABA_A$-PSC amplitude (CV_a) both *in vivo*, from P0 to P1, and *in vitro* after 24 h in control conditions. This increase in CV_a is prevented by activity deprivation, resulting in a smaller CV_a. As the variability in the amplitude of miniature currents has been attributed to different properties between releasing sites (Tang *et al.* 1994; Auger & Marty, 1997, 2000; Forti *et al.* 1997), a smaller CV_a in treated IHFs is consistent with fewer functional GABAergic synapses. This observation therefore suggests that activity-dependent maturation of GABAergic transmission is largely accounted for by an increase in the total number of functional synapses (Groc *et al.* 2003). Accordingly, silencing hippocampal or cerebral organotypic slice cultures has been reported to reduce GABAergic synaptogenesis onto hippocampal (Marty *et al.* 2000) and Purkinje (Seil *et al.* 1994) cells. However, this potential mechanism does not preclude an effect on the probability of transmitter release (Murthy *et al.* 2001).

In a recent study, activity deprivation has been reported to decrease the amplitude of $mGABA_A$-PSCs by decreasing the number of postsynaptic $GABA_A$ receptors (Kilman *et al.* 2002). The discrepancy with the present study may be due to differences in structure (visual cortex *versus* hippocampus), preparation (dissociated culture *versus* intact preparation), or medium (presence *versus* absence of serum). A more likely explanation relies on the different developmental stages at which spontaneous synaptic activity was manipulated. In the study of Kilman *et al.* (2002), activity was blocked after synapses had been re-established, while in our study activity was blocked during synapse formation. Thus the effects of activity deprivation on developing networks is likely to be different from those on established, more mature networks (Burrone *et al.* 2002). Sorting out these differences and the conditions under which $GABA_A$ clusters are or are not modified by activity is a fundamental issue that requires further investigation.

Characteristics of the synaptic activity involved in the formation of functional GABAergic synapses

Our patch-clamp recordings performed on IHFs at 25°C show that the spontaneous network-driven giant depolarizing potentials (GDPs) (Ben-Ari *et al.* 1989), are present after 1 day *in vitro* and constitute most of the synaptic activity recorded at that stage. Therefore GDPs and/or associated action potentials are the likely candidates. Reducing synaptic activity with ionotropic glutamatergic or GABAergic receptor antagonists, or with high divalent cation ACSF, leads to a reduced frequency of $mGABA_A$-PSCs compared to control. Although having different specific effects on synaptic activity, these pharmacological manipulations all block spontaneous GDPs (Ben-Ari *et al.* 1989; Khazipov *et al.* 1997) suggesting that they may play a role in the formation of functional GABAergic synapses. In a recent study, Lauri *et al.* (2003) have proposed that GDPs control the number of functional glutamatergic synapses in the developing rat hippocampus. The present study suggests that the same holds true for GABAergic synapses.

Conclusion

In summary, we conclude that spontaneous synaptic activity is a key player in the formation of functional GABAergic synapses in the developing rat hippocampus. Together with previous data showing that spontaneous activity also regulates the morphological development of the hippocampal neurones (Luthi *et al.* 2001; Groc *et al.* 2002) and the number of functional glutamatergic synapses (Lauri *et al.* 2003), our results point to a complex scenario in which synaptic activity modulates all aspects of hippocampal circuit formation.

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