Synaptic $GABA_A$ activation induces Ca^{2+} rise in pyramidal cells and interneurons from rat neonatal hippocampal slices

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1. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by activation of $GABA_A$ receptors (synaptic stimulation or application of the $GABA_A$ agonist isoguvacine) were studied on pyramidal cells and interneurons from hippocampal slices of rats from two age groups (postnatal days (P) 2–5 and P12–13) using the fluorescent dye fluo-3 and a confocal laser scanning microscope. Cells were loaded with the dye either intracellularly, using patch pipettes containing fluo-3 in the internal solution, or extracellularly, using pressure pulses applied to an extracellular pipette containing the permeant dye fluo-3 AM.

2. Interneurons and pyramidal cells from P2–5 slices loaded with fluo-3 AM responded by an increase in $[Ca^{2+}]_i$ to isoguvacine and to glutamate, in contrast to cells from P12–13 slices which responded to glutamate but not to isoguvacine.

3. The isoguvacine-induced rise in $[Ca^{2+}]_i$ was reversibly blocked by bath application of the $GABA_A$ receptor antagonist bicuculline (20 μM), suggesting the specific involvement of $GABA_A$ receptors. The sodium channel blocker tetrodotoxin (TTX, 1 μM in the bath) did not prevent the isoguvacine-induced rise in $[Ca^{2+}]_i$.

4. The isoguvacine-induced rise in $[Ca^{2+}]_i$ was reversibly blocked by bath application of the calcium channel blocker D600 (50 μM) suggesting the involvement of voltage-dependent Ca^{2+} channels.

5. Electrical stimulation of afferent fibres induced a transient increase in $[Ca^{2+}]_i$ in neonatal pyramidal cells and interneurons (P5) loaded non-invasively with fluo-3 AM. This elevation of $[Ca^{2+}]_i$ was reversibly blocked by bicuculline (20 μM) but not by APV (50 μM) and CNQX (10 μM).

6. During simultaneous electrophysiological recording in the current-clamp mode and $[Ca^{2+}]_i$ monitoring from P5 pyramidal cells, electrical stimulation of afferent fibres, in the presence of APV (50 μM) and CNQX (10 μM), caused synaptic depolarization accompanied by a few action potentials and a transient increase in $[Ca^{2+}]_i$. In voltage clamp (−70 mV) however, there was no increase in $[Ca^{2+}]_i$ following synaptic stimulation, showing that it is depolarization dependent.

7. Using a non-invasive method of $[Ca^{2+}]_i$ monitoring, we demonstrate here that in neonatal (P2–5) hippocampus, GABA is an excitatory neurotransmitter which can cause an elevation of $[Ca^{2+}]_i$ in interneurons and pyramidal cells via activation of voltage-dependent Ca^{2+} channels. This action may underlie the trophic role of GABA in hippocampal development.

In the mammalian adult central nervous system, GABA (γ-aminobutyric acid) is the main inhibitory neurotransmitter, acting on two main classes of receptors: $GABA_A$ and $GABA_B$. The bicuculline-sensitive $GABA_A$ receptors activate a channel permeable to chloride (Cl^-), underlying the fast IPSP in various brain structures, while postsynaptically localized $GABA_B$ receptors activate potassium conductances or decrease calcium currents through the activation of G proteins (for review, see Sivilotti & Nistri, 1991). Activation of presynaptic $GABA_B$ receptors depresses both glutamatergic and GABAergic synaptic transmission (Thompson, Capogna & Scanziani, 1993).

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Electrophysiological studies have shown that in the immature hippocampus, the excitatory synaptic drive is the result of GABAergic transmission: until postnatal day 5 (P5), the activation of $GABA_A$ receptors depolarizes pyramidal cells (Ben-Ari, Cherubini, Corradetti & Gaiarsa, 1989). This was attributed to an incomplete development of extrusion mechanisms for Cl^-, resulting in a reversal potential for $GABA_A$-activated currents (E_{Cl}) more positive than the resting potential (Misgeld, Deisz, Dodt & Lux, 1986; Zhang, Spigelman & Carlen, 1991).

One can therefore form the hypothesis that $GABA_A$ receptor stimulation could increase $[Ca^{2+}]_i$ in hippocampal neurons via depolarization and activation of voltage-dependent Ca^{2+} channels, as has been demonstrated for other depolarizing agents (Miyakawa *et al.* 1992).

Several studies have used non-invasive methods (loading of cells with esterified fluorescent calcium-sensitive dyes) to test whether GABA could increase $[Ca^{2+}]_i$. It has been demonstrated that bath application of $GABA_A$ receptor agonists could increase $[Ca^{2+}]_i$ in non-neuronal cells (Kirchhoff & Kettenmann, 1992; Nilsson, Eriksson, Rönnbäck & Hansson, 1993), in neurons in culture (Connor, Tseng & Hockberger, 1987; Segal, 1993; Hales, Sanderson & Charles, 1994; Reichling, Kyrozis, Wang & MacDermott, 1994; Wang, Reichling, Kyrozis & MacDermott, 1994) and in neurons from developing neocortex slices (Yuste & Katz, 1991). Evidence is still missing, to our present knowledge, that synaptically activated $GABA_A$ receptors can increase $[Ca^{2+}]_i$ in neurons.

We have therefore tested the hypothesis that synaptic or exogenous $GABA_A$ receptor activation leads to an increase of $[Ca^{2+}]_i$ in immature hippocampal cells. For this purpose, the $[Ca^{2+}]_i$ was monitored with a confocal laser scanning microscope in a non-invasive way: the cells were loaded by extracellular application of fluo-3 AM, which allowed for preservation of the physiological intracellular Cl^- concentration.

Here, we report that synaptic or exogenous activation of $GABA_A$ receptors induces an increase of $[Ca^{2+}]_i$ in pyramidal cells and interneurons from P3–5, but not P12–13, hippocampal slices through the activation of voltage-dependent Ca^{2+} channels.

A preliminary description of these results has been published previously in abstract form (Tseeb, Leinekugel, Ben-Ari, Conde & Bregestovski, 1994).

METHODS

Slice preparation

Brains were removed from male Wistar rats of 2–13 postnatal days of age (P2–13), that had been killed by decapitation, and thin transverse hippocampal slices (200–300 μm thick) were cut in ice-cold artificial cerebrospinal fluid (ACSF) using a vibrating slicer (FTB Vibracut). Slices were then incubated for 1–2 h in ACSF at room temperature for stabilization. Standard ACSF had the following composition (mM): NaCl, 126; KCl, 3·5; $CaCl_2$, 2; $MgCl_2$, 1·3; NaH_2PO_4, 1·2; $NaHCO_3$, 25; glucose, 11; pH = 7·3; bubbled with 95% O_2 and 5% CO_2.

$[Ca^{2+}]_i$ measurements

Thin hippocampal slices were transferred to a recording chamber located on the stage of an Axioscope Karl Zeiss microscope (×40 water-immersion objective lens) and perfused with standard ACSF (4 ml min^{-1}, ~32 °C). Fluorescence measurements were performed on cells loaded with the Ca^{2+}-sensitive dye fluo-3 AM or free acid using a confocal laser scanning microscope (MRC BIORAD 600) equipped with an argon–krypton laser and photomultiplier. Excitation was delivered at 488 nm and emission intensity was measured at > 500 nm.

The program 'SOM', provided by BIORAD, drove the laser acquisition of images. Subsequent modification of the program allowed for acquisition either in continuous mode or by 'frame'. A frame is a group of successive images, usually thirty-two, which could be recorded with a specified time interval between each other, usually 0·5 or 1 s. The continuous mode was used to control for the loading while the frame acquisition mode was used to record responses to stimulations.

The changes in fluorescence were quantified off-line on a computer with the program Fluo (IMSTAR, France). A specified portion of the image, corresponding to the limits of the cell of interest (mainly the border of the soma), was drawn on one image and quantification was done within these limits on all the images from the same frame of successive images. The first image of the frame (image 1) was used as the reference ('0 level' of fluorescence) for the subsequent thirty-one images (Fig. 2*C*). The value obtained for any image corresponds to the difference between the mean fluorescence intensities measured from the selected portion of this image and from the corresponding portion of image 1.

Because fluo-3 is a single-wavelength chromophore and fluorescence is a function of the concentration of Ca^{2+} and dye (Kao, Harootunian & Tsien, 1989) we have used this dye only for approximate estimation of $[Ca^{2+}]_i$ and included for analysis only experiments in which the fluorescence level recovered to the control value after cell excitation.

Fluo-3 AM loading

Stock solution of fluo-3 AM (about 500 μM) was obtained by adding 110 μl H_2O and 4·6 μl of either DMSO (< 0·1% final) or pluronic acid (20% in DMSO, < 0·02% final) to 50 μg of fluo-3 AM. It was diluted to the final concentration (0·5–10 μM) just before use by adding the corresponding amount of ACSF to a 7 μl aliquot of stock solution.

Three approaches were tested to load the cells. (1) Pre-incubation of the slices in fluo-3 AM (0·5–10 μM) containing oxygenated ACSF before transfer to the recording chamber. This did not prove to be a satisfactory method since there was no possibility to control the staining process. Thus, cells were seldom properly stained and staining was always located on the surface of the slice. Moreover, the signal-to-noise ratio was very low. (2) Circuit superfusion in the recording chamber with periodically re-oxygenated ACSF containing 1 μM fluo-3 AM (Fig. 1*A*). After 15–20 min of such treatment, a number of cells appeared to be loaded but the signal-to-noise ratio was quite low. Moreover, the penetration of the dye inside the slice looked quite poor as only cells on the surface of the slice appeared to be efficiently stained. (3) Pressure pulse application (using a Picospritzer; General Valve Corporation, USA; pressure pulses 0·3–1 s in duration and 0·2 Hz in frequency) of

fluo-3 AM (3·3 μM, 5–10 min) through a glass micropipette (Fig. 1*B*). An individual neuron was selected visually and the tip of the pipette containing fluo-3 AM solution was placed about 20–30 μm from the selected cell. Pressure was adjusted to see small movements of the cell after each pressure pulse application and the best staining was achieved with the tip of the pipette a few micrometres under the cell. This method had several advantages over the previous ones. (1) Following 5–15 min of such loading, the selected cell could easily be distinguished, with a much higher signal-to-noise ratio (Fig. 2*A*). In spite of the fact that fluo-3 AM was applied precisely nearby the desired cell, several surrounding cells, even far from this region (100–200 μm), were also labelled, most probably through diffusion of the staining solution (see cells 1 and 2 in Fig. 2*A*). (2) It was possible to load cells located inside the slice. (3) The intensity of staining could be tested by brief pulses of glutamate (20–100 ms, 100 μM) applied to the surface of the neuron: when the cell was properly loaded, glutamate induced a marked elevation in fluorescence that lasted for several seconds before returning to control levels (Fig. 2*B* and *C*). Since loading with fluo-3 AM resulted in heterogeneous field labelling (some spots around the target cell were also fluorescent), it was necessary to correlate the fluorescence image with the conventional optical image. This was done by mapping the fluorescence field with a calibration grid which corresponded to one on the bifocal of the microscope. Fluorescent spots were taken into account (i.e. considered as cells) only when it was possible to identify a neuron in the conventional optical field in the position corresponding to the spot of fluorescence.

Electrical stimulation

Electrical stimuli were delivered through a bipolar tungsten microelectrode. It was positioned between 150–300 μm from the soma, in the stratum radiatum. Four to five single voltage pulses (30–40 V, 33 μs) were delivered at a frequency of 4–5 Hz.

Application of drugs

Agonists (100 μM isoguvacine or 100 μM L-glutamate) were focally applied to the soma by brief pulses of pressure through a glass micropipette (1–5 MΩ) using a Picrospritzer. Antagonists (APV, 50 μM; CNQX, 10 μM; TTX, 1 μM; bicuculline, 20 μM; D600, 20–50 μM) were bath applied.

Reagents were purchased from Sigma (tetrodotoxin), Tocris Neuramin (bicuculline, APV, CNQX, L-glutamate and isoguvacine), and Molecular Probes Inc. (fluo-3 AM). D600 was a gift from Knoll AG (Ludwigshafen, Germany).

Electrophysiological recording and morphological monitoring

Pyramidal cells and interneurons from the CA3 region were studied using the conventional whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Electrical signals were recorded using an EPC-9 amplifier (HEKA-Elektronics, Germany) and stored on a digital tape-recorder DTR 1201 (Bio-Logic, France) for later analysis. The patch pipettes (5–10 MΩ) contained one of the following internal solutions. Solution 1 (mM): NaCl, 10; KCl, 100; $CaCl_2$, 0·25; EGTA, 5; Hepes, 10; glucose, 10; GTP, 0·2; MgATP, 2; pH 7·2; osmolality, 240–250 mosmol kg^{-1}. Lucifer Yellow (1%) was added to the internal solution and the morphology of the cells was monitored on-line by a confocal laser scanning microscope. Solution 2 (mM): KCl, 110; potassium gluconate, 50; $CaCl_2$, 0·4; EGTA, 1·1; Hepes, 10; MgATP, 2; pH 7·2; osmolality, 240–250 mosmol kg^{-1}. Fluo-3 (10 μM) was added to the internal solution and the fluorescence was monitored on-line by a confocal laser scanning microscope.

The osmolality of the solutions was measured routinely by using a KNAUER osmometer (Germany) and adjusted to the levels noted above by changing the concentration of KCl.

RESULTS

Distinction between interneurons and pyramidal cells in the CA3 region of hippocampus

Lamination of cell bodies in hippocampal slices is visible at P4–5 but less pronounced in slices from neonates (P2–3). Several studies have developed criteria (cell shape, geography and electrophysiology) to distinguish between pyramidal

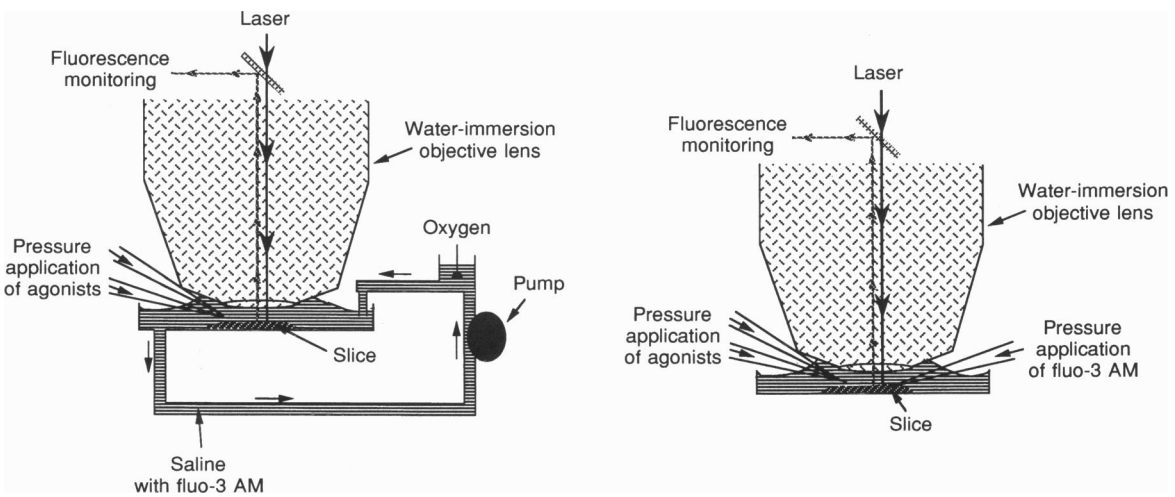

Figure 1. Two methods of loading neurons with fluo-3 AM under visual control in rat hippocampal slices

A, circuit superfusion with ACSF containing fluo-3 AM (1 μM). *B*, pressure pulses applied to the micropipette containing ACSF with fluo-3 AM (3·3 μM).

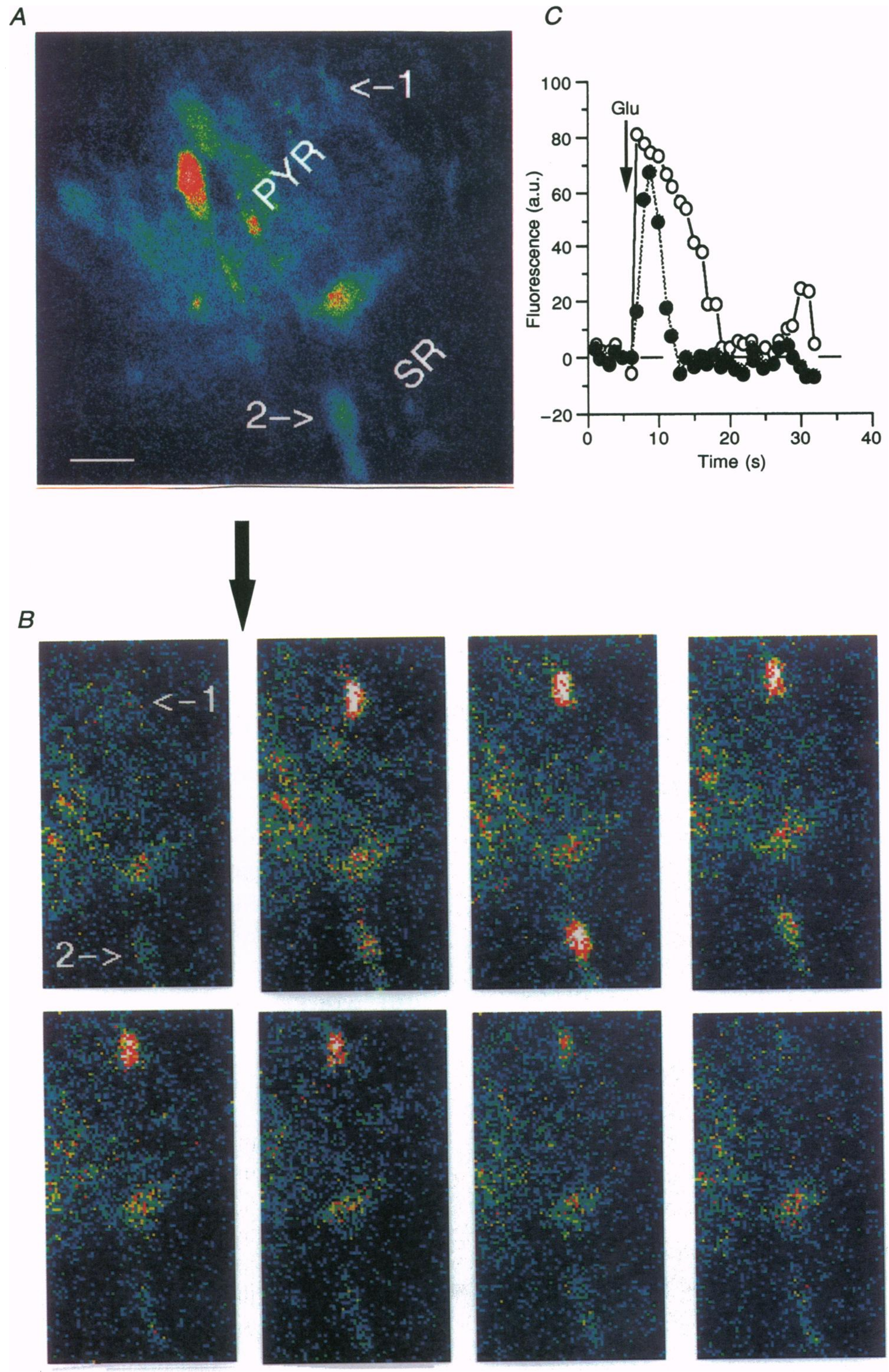

Figure 2. Calcium-dependent fluorescence responses to glutamate of cells extracellularly loaded with fluo-3 AM

A, the CA3 pyramidal layer (PYR) and part of the stratum radiatum (SR) are presented. Two distinct cells loaded by pressure pulse application during 15 min are shown with arrows. Scale bar, 15 μm. *B*, Ca^{2+}-dependent fluorescence was recorded every 1 s. Eight images (2 s between each image, from left to right) show the responses of cells 1 and 2 to glutamate. Glutamate (100 μM) was applied by brief (50 ms) pressure application between the first and second images (arrow). Cells 1 and 2 shown in *B* are the same as in *A*. The width of each panel corresponds to 55 μm. *C*, fluorescence measured from cells 1 and 2 during the whole 32 image frame. The moment of glutamate application is indicated by an arrow. Baseline level is represented as a dashed horizontal line. Fluoresence is given in arbitrary units (a.u.).

cells and interneurons in the hippocampus (Woodson, Nitecka & Ben-Ari, 1989; Lacaille, 1991). For the non-invasive method of $[Ca^{2+}]_i$ analysis, it was necessary to estimate the type of cell using visual but not electrophysiological criteria. Some preliminary studies were thus conducted to ensure the efficacy of visual discrimination between pyramidal cells and interneurons in neonatal hippocampal slices. Fifteen pyramidal cells and fifteen interneurons from two age groups (P2–4 and P11–13) were selected visually and electrophysiological activity was recorded in the whole-cell configuration. The presence of Lucifer Yellow in the pipette allowed 'on-line' visualization of cell morphology with a confocal laser scanning microscope (Fig. 3). Only cells with large somata were selected (Fig. 3*A* and *C*) and none of them exhibited the electrophysiological properties of glial cells. Interneurons were always selected in the stratum radiatum of CA3. In the current clamp mode, the spontaneous activity of interneurons exhibited a regular and high-frequency firing (Fig. 3*D*) while pyramidal cells exhibited an irregular pattern of activity (Fig. 3*B*). During a depolarizing step of current, pyramidal cells produced a burst of action potentials followed by a 'silent period' in contrast to interneurons which generated repetitive non-adapting activity (data not shown). These behaviours are similar to those described earlier in rat hippocampus (Lacaille, 1991; Perouansky & Yaari, 1993). The visual differentiation thus proved to be reliable and was used as the basis for distinguishing between interneurons and pyramidal cells in further experiments.

Isoguvacine increases $[Ca^{2+}]_i$ in CA3 interneurons and pyramidal cells from young but not mature hippocampal slices

Figure 4*A* and *B* shows the response of a pyramidal cell and an interneuron from the CA3 region of young hippocampal slices (P3–5) to brief pressure pulse applications of glutamate and isoguvacine: both stimulations increased the fluorescence signal for $[Ca^{2+}]_i$. In such cells which were tested for both agonists, the response to glutamate was

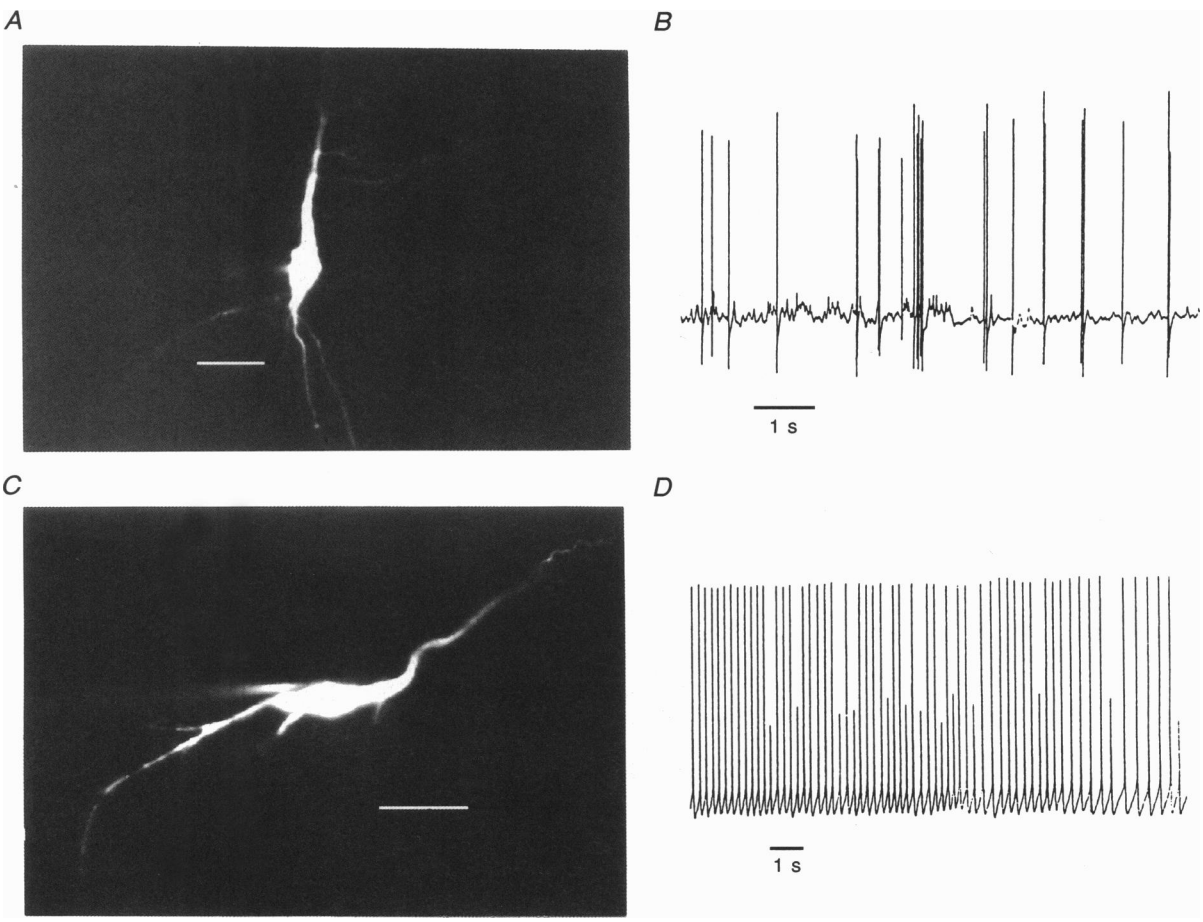

Figure 3. Electrophysiological recordings confirm the relevance of visual selection of P2 interneurons and pyramidal cells

A and *C*, visually selected cells loaded with Lucifer Yellow in the whole-cell patch-clamp mode (*A*, P2 CA3 pyramidal cell and *C*, P2 interneuron from CA3 stratum radiatum). Scale bars, 25 μm. *B* and *D*, spontaneous activities of typical P2 CA3 pyramidal cell (*B*) and P2 interneuron from CA3 stratum radiatum (*D*). Note that the pyramidal cell presented a typical irregular spiking activity in contrast to the regular non-adapting mode of spike firing of the interneuron.

larger (about 1·5–2 times) and of longer duration (about 2–3 times) than the response to isoguvacine.

Fifty-six cells were tested with test pulses of isoguvacine at P2–5: twenty-eight cells responded to isoguvacine by a 68 ± 6% increase in fluorescence (F; mean $\Delta F/F \pm$ S.E.M., $n = 25$). At P2, 100% (4/4) of the tested cells responded to isoguvacine, 60% (18/30) at P3–4, and 27% (6/22) at P5. The cells which did not respond to isoguvacine were systematically tested for glutamate, which produced a 78 ± 7% increase in fluorescence (mean $\Delta F/F \pm$ S.E.M., $n = 28$). Cells which did not respond to either agonist were discarded from the results. The responses had a fast time-to-peak and the fluorescence signal returned to the control level within several seconds of application of the agonists. When the cells were tested with several pulses of isoguvacine of different durations ($n = 4$), the amplitude of the $[Ca^{2+}]_i$ elevation in response to application of isoguvacine increased with the duration of the agonist application (Fig. 5*A*).

In contrast to young cells, neurons from juvenile hippocampus (P12–13) did not respond to application of isoguvacine ($n = 5$). However, in the same cells, glutamate induced a rise in $[Ca^{2+}]_i$, showing that they were healthy and properly loaded with fluo-3 AM (Fig. 4*C* and *D*).

Isoguvacine increases $[Ca^{2+}]_i$ through the activation of $GABA_A$ receptors and subsequent activation of voltage-dependent Ca^{2+} channels

To exclude non-specific effects in the generation of $[Ca^{2+}]_i$ transients by isoguvacine, we used the $GABA_A$ receptor antagonist bicuculline (20 μM). This compound selectively and reversibly inhibited the responses to isoguvacine in P3–5 neurons to 3 ± 3% of their amplitude in control conditions ($n = 7$; Fig. 5*B*) while it had no pronounced effect on the responses of the same cells to glutamate (Fig. 5*C*).

To exclude the possibility that increases in $[Ca^{2+}]_i$ induced in interneurons by isoguvacine were synaptically driven, we used the sodium channel blocker TTX (1 μM bath applied). Isoguvacine-evoked rises of $[Ca^{2+}]_i$ in P3–5 hippocampal interneurons could still be observed in the presence of TTX ($n = 5$, data not shown).

To examine the involvement of voltage-gated Ca^{2+} channels in the isoguvacine-evoked transient increases in $[Ca^{2+}]_i$, we used the calcium channel blocker D600 (Fleckenstein, 1977). This compound has no effect on isoguvacine-evoked currents in hippocampal neurons (D. Ragozzino & P. Bregestovski, unpublished observations). Figure 6 illustrates that bath application of D600 (50 μM) reversibly inhibited

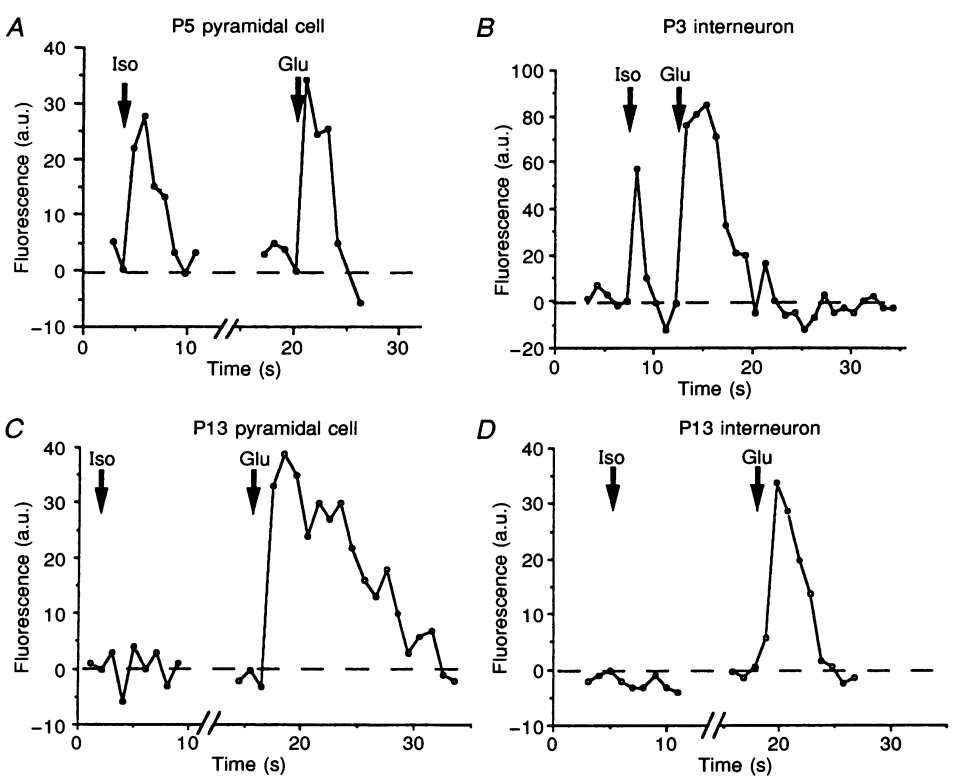

Figure 4. Isoguvacine increases calcium-dependent fluorescence in interneurons and pyramidal cells from neonate (P3–5) but not from more mature (P13) rat hippocampal slices

Glutamate (100 μM) or isoguvacine (100 μM) were applied by brief pressure applications (35 ms) on cells loaded with fluo-3 AM and Ca^{2+}-dependent fluorescence was monitored. *A*, P5 pyramidal cell; *B*, P3 interneuron; *C*, P13 pyramidal cell; and *D*, P13 interneuron. All cells were selected in the CA3 region (in stratum radiatum for interneurons). Baseline level of fluorescence is represented as a dashed horizontal line.

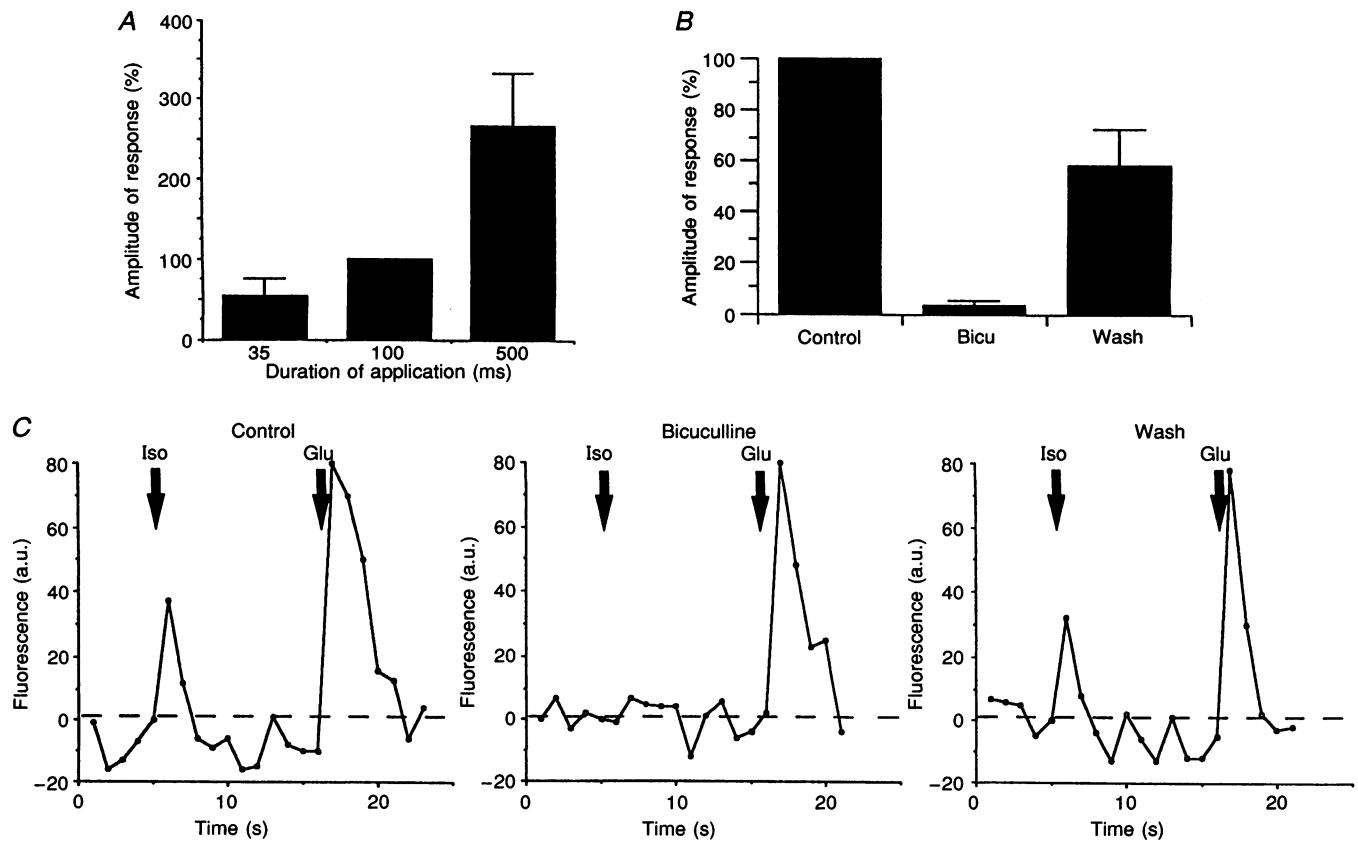

Figure 5. Isoguvacine increases calcium-dependent fluorescence by specific activation of $GABA_A$ receptors

Glutamate (100 μM) or isoguvacine (100 μM) were applied by brief pressure applications on P3–5 pyramidal cells and interneurons. *A*, pulses of isoguvacine were applied with durations ranging from 35 to 500 ms. This graph represents the responses of 2 cells tested for 35 and 100 ms applications and another 2 cells tested for 100 and 500 ms applications. The amplitudes of responses (Ca^{2+}-dependent fluorescence variations) are presented as percentages of the responses of the same cell to 100 ms applications (mean ± S.E.M.). *B*, the amplitudes of responses to pressure pulse applications of isoguvacine (100 μM, 50 ms) were estimated in control conditions, in the presence of bicuculline (Bicu, 20 μM bath applied) and after wash-out of bicuculline. Values are presented as percentages of the amplitude of responses of the same cell in control conditions (mean ± S.E.M., $n = 7$ cells). *C*, the responses to pressure pulse applications of isoguvacine (Iso, 100 μM, 50 ms) and glutamate (Glu, 100 μM, 50 ms) on a P3 pyramidal cell are shown under control conditions (left), during bath application of 20 μM bicuculline (centre), and after wash-out of bicuculline (right). Baseline level of fluorescence is represented as a dashed horizontal line. Note that bicuculline blocks the response to isoguvacine without affecting the response to glutamate.

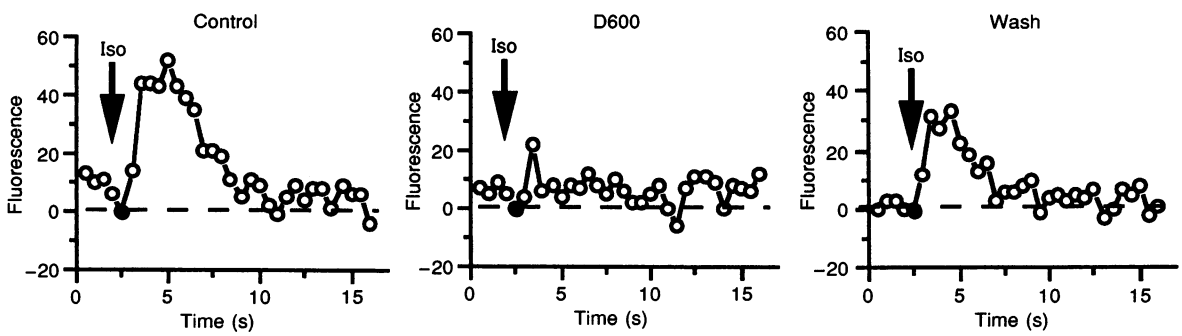

Figure 6. The voltage-dependent calcium channel blocker D600 reversibly inhibits the isoguvacine-induced increase in $[Ca^{2+}]_i$-dependent fluorescence

The responses to pressure pulse applications of isoguvacine (100 μM, 50 ms) on a P3 pyramidal cell are shown in control condition (*A*), during bath application of 50 μM D600 (*B*), and after wash-out of D600 (*C*). Baseline level is represented as a dashed horizontal line.

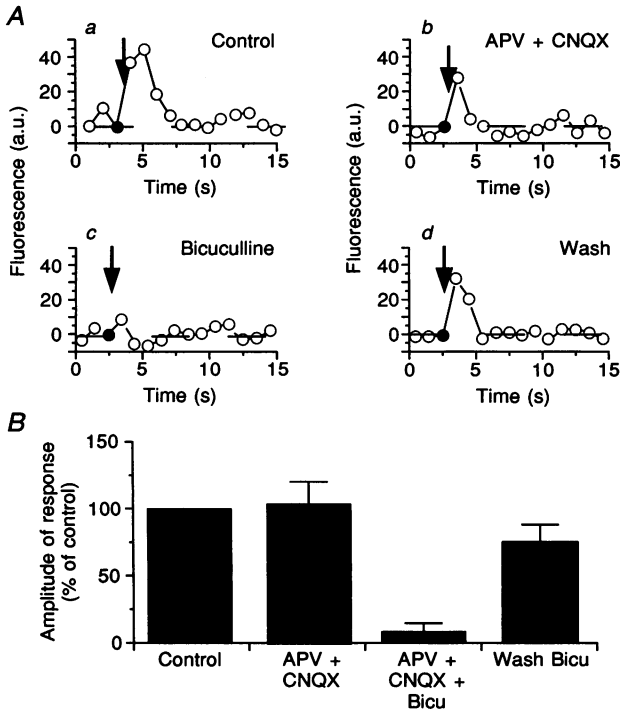

Figure 7. Electrical synaptic stimulation increases $[Ca^{2+}]_i$-dependent fluorescence through the activation of $GABA_A$ receptors in non-invasive conditions

Cells were loaded using extracellular pressure applications of fluo-3 AM. *A*, increase in $[Ca^{2+}]_i$ fluorescence from a CA3 pyramidal cell (P5) following electrical stimulation in control conditions (*a*), in presence of 50 μM APV and 10 μM CNQX (*b*), and during (*c*) and after (*d*) exposure to 20 μM bicuculline in the bath. Baseline level is represented as a dashed horizontal line. *B*, average of Ca^{2+}-dependent fluorescence responses to synaptic stimulations of 6 cells at P5. Amplitudes of responses at the maximum are presented in control conditions, in the presence of APV + CNQX (as percentage of responses of the same cell in control conditions), during further addition of bicuculline (as percentage of responses of the same cell in APV + CNQX), and after wash-out of bicuculline (as percentage of responses of the same cell in APV + CNQX). Values are presented as means ± S.E.M.

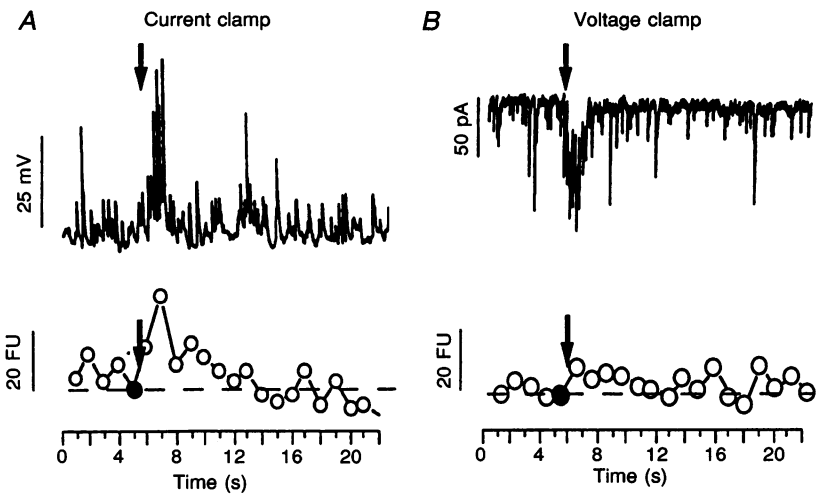

Figure 8. The synaptic $GABA_A$-induced $[Ca^{2+}]_i$ increase is mediated by postsynaptic depolarization

Whole-cell recordings (upper traces) and $[Ca^{2+}]_i$-dependent fluorescence measurements (lower traces) were performed simultaneously on a CA3 pyramidal cell (P5). The cell was loaded with fluo-3 (10 μM) via the recording pipette and electrical stimulations were delivered in the stratum radiatum (30–50 V; distance 150–200 μm; 5 Hz during 1 s, arrows). *A*, membrane potential responses in the current-clamp mode and corresponding simultaneous changes in $[Ca^{2+}]_i$-dependent fluorescence; resting potential, −80 mV. APV (50 μM) and CNQX (10 μM) were present in the bath. *B*, membrane currents in the voltage-clamp mode (membrane potential, −70 mV) and corresponding simultaneous changes in $[Ca^{2+}]_i$-dependent fluorescence. Baseline levels of $[Ca^{2+}]_i$ fluorescence are represented as dashed horizontal lines.

isoguvacine-induced elevation of $[Ca^{2+}]_i$ in a pyramidal neuron from young (P5) hippocampus. Similar observations were obtained on five pyramidal cells and two interneurons from slices at the age P3–5. This suggests that the isoguvacine-induced increase in $[Ca^{2+}]_i$ is mediated by the activation of voltage-gated Ca^{2+} channels.

Electrical stimulation of afferents increases $[Ca^{2+}]_i$ via $GABA_A$ receptor activation in interneurons and pyramidal cells from the young hippocampal CA3 region

Finally, to determine whether synaptically released GABA can induce elevation of $[Ca^{2+}]_i$, we analysed fluorescence transients in hippocampal neurons following electrical stimulation of the stratum radiatum.

P5 cells were loaded non-invasively with fluo-3 AM. Figure 7*A* shows that in these conditions, electrical stimulation of the stratum radiatum markedly increased the signal for $[Ca^{2+}]_i$. This effect was resistant to bath application of APV and CNQX (Fig. 7*A b*) but was reversibly blocked by 20 μM bicuculline (Fig. 7*A c* and *d*), suggesting the specific involvement of $GABA_A$ receptors. On average, amplitude of response was $104 \pm 16{\cdot}5\%$ of control amplitude values in presence of APV + CNQX, and further addition of bicuculline reduced the amplitude of response to $9{\cdot}2 \pm 5{\cdot}8\%$ ($n = 6$) of its value in presence of APV + CNQX (Fig. 7*B*).

In order to show directly that the increase in $[Ca^{2+}]_i$ depends on the $GABA_A$-induced depolarization, P5 pyramidal cells ($n = 2$) were recorded in the whole-cell configuration with fluo-3 in an internal solution containing about 110 mM Cl^- (E_{Cl} about -10 mV). In the current-clamp mode and in presence of glutamate receptor antagonists (50 μM APV with 10 μM CNQX), electrical stimulation of the stratum radiatum produced a depolarization and firing of a few action potentials (Fig. 8*A*, upper trace). The simultaneous monitoring of fluorescence showed that the electrophysiological response to electrical stimulation was associated with a transient $[Ca^{2+}]_i$ increase (Fig. 8*A*, lower trace). Keeping the cell at -70 mV in the voltage-clamp mode abolished the $[Ca^{2+}]_i$ response to electrical stimulation (Fig. 8*B*).

DISCUSSION

Our results show that activation of $GABA_A$ receptors can cause elevation of $[Ca^{2+}]_i$ through the activation of voltage-gated Ca^{2+} channels in interneurons and pyramidal cells from immature (P3–5) but not mature (P12–13) rat hippocampal slices. Furthermore, we present here the direct evidence that transient elevations of $[Ca^{2+}]_i$ can be evoked by synaptically released GABA in immature hippocampal slices.

It is known that $GABA_A$ receptor-activated currents are strongly dependent on intracellular Cl^- concentration and can be modulated by protein kinase-dependent phosphorylation (Leidenheimer, Browning & Harris, 1991). The results we present here were mainly obtained using a non-invasive method of $[Ca^{2+}]_i$ estimation. This approach allowed us to avoid possible modulations of GABA responses due to intracellular dialysis during whole-cell patch-clamp recording.

Source of $[Ca^{2+}]_i$ elevation following activation of $GABA_A$ receptors

The increases in $[Ca^{2+}]_i$ we report here have the following pharmacological properties: (i) the synaptic stimulation-induced increase in $[Ca^{2+}]_i$ is resistant to antagonists of glutamatergic ionotropic receptors (APV and CNQX); (ii) it is mimicked by the $GABA_A$ agonist isoguvacine; and (iii) reversibly blocked by the $GABA_A$ antagonist bicuculline. Our results therefore strongly suggest that the increases in $[Ca^{2+}]_i$ we observed on P3–5 pyramidal cells and interneurons in response to exogenous or synaptic stimulation is due to specific activation of $GABA_A$ receptors.

The isoguvacine-induced increase in $[Ca^{2+}]_i$ is reversibly blocked by D600, suggesting the involvement of voltage-dependent Ca^{2+} channels. During experiments in which E_{Cl} was set to -10 mV, simultaneous monitoring of electrical activity and Ca^{2+} -dependent fluorescence during whole-cell recording showed a clear association between the depolarization induced by synaptic $GABA_A$ stimulation and the increase in $[Ca^{2+}]_i$. This is in agreement with previous results reporting that: (i) $GABA_A$ receptors are linked to a Cl^- conductance which evokes depolarizing current in immature cells due to an inverted Cl^- gradient (Misgeld *et al.* 1986; Ben-Ari *et al.* 1989; Zhang *et al.* 1991), and (ii) stimulation of $GABA_A$ receptors can increase $[Ca^{2+}]_i$ through the activation of voltage-dependent Ca^{2+} channels via depolarization (Connor *et al.* 1987; Yuste & Katz, 1991; Kirchhoff & Kettenmann, 1992; Horvath, Acs, Mergl, Nagy & Makara, 1993; Yamashita & Fukuda, 1993; Reichling *et al.* 1994).

We report here that activation of $GABA_A$ receptors by isoguvacine produces a depolarization strong enough to activate voltage-dependent Ca^{2+} channels, without the involvement of TTX-sensitive sodium channels. This is in agreement with the previous observation that in the presence of TTX, activation of $GABA_A$ receptors could increase $[Ca^{2+}]_i$ in cultured rat dorsal horn neurons via high threshold voltage-dependent Ca^{2+} channels (Reichling *et al.* 1994).

Since interneurons from P3–5 rats responded to isoguvacine in the presence of TTX, which blocks synaptic transmission, the responses to isoguvacine in our experiments could not result from disinhibition or direct activation of afferent

pyramidal cells. The responses thus most probably reflect a depolarizing action of $GABA_A$ receptors activation on immature interneurons. The fact that bicuculline-sensitive synaptically induced transient elevations of $[Ca^{2+}]_i$ were observed in immature interneurons in the presence of glutamate antagonists supports the idea that immature interneurons can excite each other through $GABA_A$ synapses.

Physiological relevance for pyramidal cells and interneurons

Development of the hippocampal network. Several lines of evidence have shown that in the hippocampus, as well as in the neocortex, interneurons mature before pyramidal cells. GABAergic interneurons become postmitotic before pyramidal and granule cells do (Bayer, 1980; Soriano, Cobas & Fairen, 1986). Biochemical and morphological studies have shown that the GABAergic system is well developed at birth (Coyle & Enna, 1976; Rozenberg, Robain, Jardin & Ben-Ari, 1989; Seay-Lowe & Clirborne, 1992) in contrast to the glutamatergic system (Amaral & Dent, 1981; Pokorny & Yamamoto, 1981; Richter & Wolf, 1990; Hosokawa, Sciancalepore, Stratta, Martina & Cherubini, 1994). We report here that, in spite of an early morphological development of interneurons when compared with pyramidal cells, these two kinds of cells (in studied areas) display a common, immature type of response to $GABA_A$ receptor stimulation at P2–5. Both types of neurons demonstrated elevation of $[Ca^{2+}]_i$ following synaptic stimulation of $GABA_A$ receptors or application of the $GABA_A$ agonist, isoguvacine.

Electrophysiological studies have shown that, until P5, a powerful excitatory synchronized activity termed giant depolarizing potentials (GDP) is present in the immature hippocampus, at a time when the glutamatergic system is poorly developed (Ben-Ari *et al.* 1989). It has been shown that this excitatory synaptic drive is in fact the result of GABAergic transmission. However, several points remain unclear about the mechanisms which support the activity of the immature hippocampal network. Our results show that the number of cells responding to isoguvacine by an increase in $[Ca^{2+}]_i$ progressively decreased from 100% at P2 to less than 30% at P5 and finally 0% at P12–13. This progressive transition from depolarizing to hyperpolarizing $GABA_A$ within about 48 h around P4–5 is in agreement with previous results (J. L. Gaïarsa, personal communication). The conclusion of our work that immature interneurons can excite each other through $GABA_A$ synapses is of important relevance to the understanding of the immature hippocampal network activity.

Trophic effect of GABA. The $GABA_A$-induced increase in $[Ca^{2+}]_i$ may reflect an important role of GABA-releasing interneurons in the development of hippocampus. It has been shown that transmitters, such as glutamate, can regulate dendritic outgrowth (Lipton & Kater, 1989; Meier, Hertz & Schousboe, 1991), presumably through membrane depolarization leading to an increase in intracellular Ca^{2+} concentration (Kater, Mattson, Cohan & Connor, 1988; Mattson, 1988). It has also been shown that GABA can evoke ultrastructural changes during early development (Belhage, Hansen, Schousboe & Meier, 1988; Hansen, Belhage, Schousboe & Meier, 1988) and that the $GABA_A$ antagonist, bicuculline, can inhibit the neuritic growth of hippocampal cells in culture (Barbin, Pollard, Gaïarsa & Ben-Ari, 1993). It seems possible that the neurotrophic effect of $GABA_A$ receptor stimulation may be mediated by an increase in $[Ca^{2+}]_i$ due to depolarization and activation of voltage-dependent Ca^{2+} channels.

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Acknowledgements

We wish to thank Drs J. L. Gaïarsa, R. Miles, A. Represa, H. McLean and S. L. Shorte for valuable comments and critical reading of the manuscript. We are also grateful to B. Martin and E. Telle for modification of the programs for acquisition and analysis of images from the confocal microscope. The authors thank Knoll Laboratories for the gift of D600. X.L. and V.T. were supported by grants from Ministéré de la Recherche et l'Espade (DRE) and Direction des Recherches, Etudes et Techniques (DRET).

Received 31 October 1994; accepted 28 February 1995.