DL-TBOA generates rhythmic depolarization

Generation of slow network oscillations in the developing rat hippocampus after blockade of glutamate uptake

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ABSTRACT

Cell surface glutamate transporters are essential for the proper function of early cortical networks as their dysfunction induces seizures in the newborn rat in vivo. We have now analyzed the consequences of their inhibition by DL-TBOA on the activity of the developing CA1 rat hippocampal network in vitro. DL-TBOA generated a pattern of recurrent depolarization with an onset and decay of several seconds duration in interneurons and pyramidal cells. These slow network oscillations (SNOs) were mostly mediated by GABA in pyramidal cells and by GABA and NMDA receptors in interneurons. However, in both cell types SNOs were blocked by NMDA receptor antagonists suggesting that their generation requires a glutamatergic drive. Moreover, in interneurons, SNOs were still generated after the blockade of NMDA-mediated synaptic currents with MK-801 suggesting that SNOs are expressed by the activation of extrasynaptic NMDA receptors. Long-lasting bath application of glutamate or NMDA failed to induce SNOs indicating that they are generated by periodic but not sustained activation of NMDA receptors. In addition, SNOs were observed in interneurons recorded in slices with or without the strata pyramidale and oriens suggesting that the glutamatergic drive may originate from the radiatum and pyramidale strata. We propose that in the absence of an efficient transport of glutamate, the transmitter diffuses in the extracellular space to activate extrasynaptic NMDA receptors preferentially present on interneurons that in turn activate other interneurons and pyramidal cells. This periodic neuronal co-activation may contribute to the generation of seizures when glutamate transport dysfunction is present.

Key Words: glutamate transporters, paroxysmal activity, extrasynaptic NMDA receptors.
INTRODUCTION

Dysregulation of glutamate homeostasis has been suggested to underlie several neurological disorders including amyotrophic lateral sclerosis, Alzheimer disease, and epilepsies (Kelly and Staley; 2001; Maragakis and Rothstein, 2004). This dysregulation may originate from an alteration in glutamate metabolism and also from a dysfunction of cell surface glutamate transporters. Factors that affect transporters function (e.g. mutation, pH, and phosphorylation) have been extensively reported (Gegelashvili and Schousboe, 1997; Takahashi et al., 1997; Danbolt, 2001; Kalandadze et al., 2004; Maragakis and Rothstein, 2004). Glutamate transporters are present at the surface of glial cells (i.e. GLT1 and GLAST), and neuronal cells (i.e. EAAC1, EAAT4, EAAT5), where they bind and transport glutamate into cells from the extracellular space (Danbolt, 2001). Thus, glutamate transporters play a key role in the clearance of extracellular glutamate and the prevention of glutamate excitotoxicity, receptor desensitisation and neuronal co-activation (Danbolt, 2001; Arnth-Jensen et al., 2002).

There is now compelling evidence that glutamate transporters play an important role during cortical development before and during the formation of synapses. For example, glutamate transporters are expressed at fetal stages in the human and rodent brains (Bar-Peled et al., 1997; Furuta et al., 1997; Ullensvang et al., 1997), functioning during cell proliferation, migration and differentiation since the deletion of genes encoding for GLT1 and GLAST strongly compromises the development of the cortex (Matsugami et al., 2006). Glutamate transporters are also essential for the proper function of early cortical networks since their inhibition generates partial seizures and recurrent paroxysmal activity in the EEG of newborn rats (Milh et al., 2007), a cortical pattern reminiscent of a “suppression burst” observed in some severe forms of neonatal epilepsy (Aicardi 1985; Schlumberger et al., 1992; Ohtahara and Yamatogi, 2003). Because of the clinical relevance of all these studies, it is important to clarify the role of these proteins during maturing cortical network activity. We have previously shown that inhibition of glutamate transporters by DL-TBOA generated slow recurrent depolarisation and burst of action potentials in developing neocortical pyramidal cells in vitro (Demarque et al., 2004). To evaluate whether this type of activity can also be generated in other brain regions and get more insights on the mechanism of this pattern, we focused our attention now on the CA1 region of the rat hippocampus, an area in which the development of synaptic and network activities are well documented. Studies have shown that...
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glutamate play a major role in the generation of network driven events i.e. the giant depolarizing potential (GDPs; Cherubini et al., 1991; Ben-Ari, 2001). However, its action is powerfully controlled by pre and postsynaptic mechanisms (Durand et al., 1996; Gasparini et al., 2000; Groc et al., 2002; Lauri et al., 2006) and glutamate transporters that prevent its diffusion in the extracellular space (Demarque et al., 2002, see also Marchionni et al., 2007). It is likely that this control is important for the maturation of hippocampal networks and that a deficiency of glutamate uptake would significantly alter networks activities. In the present study, we show that in developing hippocampus, as in neocortex, the inhibition of glutamate uptake dramatically modifies the pattern of activity and generates network oscillations of several seconds duration, in both interneurons and pyramidal cells a process that involves the activation of extrasynaptic NMDA receptors.
MATERIALS and METHODS

All the experiments performed in this study conformed to the French animal use legislation.

Electrophysiology

Experiments were performed on CA1 pyramidal neurons in hippocampal slices from Wistar rats obtained from the day of birth (P0) to postnatal day 20 (P20). Wistar rats were decapitated under chloral hydrate anaesthesia (20-40 mg/ Kg). Brains were rapidly removed and placed in oxygenated ice-cooled artificial cerebrospinal fluid (ACSF) with the following composition (in mM): 126 NaCl, 3.5 KCl, 2 CaCl$_2$, 1.3 MgCl$_2$, 25 NaHCO$_3$ 1.2 NaHPO$_4$, 10 glucose (95% O$_2$ and 5 % CO$_2$, pH 7.4). Coronal hippocampal slices (350-400 µm) were obtained with vibratome, and kept in oxygenated ACSF at room temperature at least one hour before use. Individual slices were then transferred to the recording chamber where they were fully submerged and superfused with ACSF at 32-34°C at a rate of 2-3 ml/min. Neurons were recorded under visual control with an axioskop Zeiss using patch-clamp technique in the whole cell-configuration. Microelectrodes had a resistance of 5-10 MOhm and were filled with a solution containing (in mM): 100 K$_2$SO$_4$ or KGlu; 40 KCl, 0.1 CaCl$_2$, 1.1 EGTA, 10 HEPES, 4 Mg$^{2+}$ATP, 0.3 Na$^{+}$GTP, pH 7.25; 270-280 mOsm with sometimes QX-314 to block voltage dependent Na$^+$ channels in the recorded cell. Biocytine was also added to the pipette solution for post-hoc reconstruction of the recorded cells (see Tyzio et al., 1999).

Some experiments have been performed with a CsGlu, filled pipette solution (in mM): 100 CsGlu, 40 CsCl, 0.1 CaCl$_2$, 1.1 EGTA, 10 HEPES, 4 Mg$^{2+}$ATP, 0.3 Na$^{+}$GTP. Whole cell measurements in voltage-clamp or current clamp mode were filtered at 3 kHz using an EPC-9 amplifier (HEKA). All electrophysiological data were digitized (1-2 kHz) with a digidata (Axon Instruments) interface card to a personal computer and analyzed with MiniAnalysis program (Synaptosoft). To obtain current/voltage curves (I/V) of slow network oscillations (SNOs), voltage ramps were applied for 1 sec from the resting membrane potential to 20 mV and performed with patch pipettes containing QX-314. In some experiments, SNOs were recorded at +30 mV with CsGlu-filled pipettes and voltage ramps were applied from this membrane potential to -100 mV. I/V curves were constructed by subtracting the ramp response in control from the maximal current response. The I/V relation of NMDA receptor-mediated response was performed with CsGlu-filled pipettes and we similar ramps protocol. The response was evoked by the pressure ejection of NMDA (30-100 µM) during 2 sec with a patch pipette connected to a picospritzer (general valve corporation, Fairfied, USA). NMDA
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was applied 4-5 times per cell and the I/V relations obtained in individual cells were averaged and expressed as a % of the mean value obtained at 30 mV and then pooled for each cell types (interneurons and pyramidal cells). Extracellular recordings were performed with a glass pipette (<1 MOhm) filled with ACSF in order to record multiple unit activity (MUA) and the signal recorded with a DAM80 amplifier (WPI).

**Morphological analysis**

Sections used for electrophysiological analysis were fixed for 24 Hrs (4% paraformaldehyde plus 0.5% glutaraldehyde) and subsequently stained with cresyl violet or with a Nissl fluorescent dye (NeuroTrace; Invitrogen, Molecular Probes) accordingly to manufacturers instructions. Slices were analyzed using a confocal microscope (Zeiss LSM 510) to evaluate the efficacy of the dissection of hippocampal sub-fields and layers.

**Statistics**

Data are expressed as mean ± SEM. Statistical significance of difference between means was assessed with Anova test and the level of significance was set at p < 0.05.

**Drugs**

DL-TBOA, NBQX, Bicuculline, D-APV, MK-801, NMDA, GABA were purchased from Tocris (Bristol, UK). TTX was purchased from Sigma Aldrich (Strasbourg, France).
RESULTS

We used DL-TBOA, a drug known to inhibit glial GLT1 and GLAST and neuronal EAAC1 glutamate transporters (Shimamoto et al., 1998), the main glutamate transporters expressed in the rat hippocampus (Furuta et al., 1997, Ullensvang et al., 1997). DL-TBOA is a non-transportable inhibitor that does not induce an artificial release of glutamate through hetero-exchange and it does not act as partial agonist of glutamate receptors (Shimamoto et al. 1998; Jabaudon et al., 1999, Demarque et al., 2004, also see Anderson et al., 2001).

Blocking glutamate transporters generates slow network oscillations in the hippocampus

In a first series of experiments we determined the consequence of glutamate transporter inhibition on the whole developing hippocampal network activity from the day of birth (P0) to the postnatal day 6 (P6). To this end, CA1 pyramidal cells were recorded using whole cell patch clamp technique in current clamp mode together with field recording of multiple unit activity (MUA) in CA1 (n = 7 slices) or CA3 (n = 17 slices) using an extracellular electrode placed in the stratum pyramidale. In addition, we performed dual field and whole cell recordings in CA3 (n = 4 slices). As already described in several studies the pattern of electrophysiological activity of the developing rat hippocampus during the firsts postnatal days of life is composed by fast synaptic events and also by the endogenous network driven events, the so called giant (Gabaergic) depolarizing potentials (GDPs) (for reviews see Cherubini et al., 1991; Ben-Ari, 2001). These rhythmic activities were reflected at the cellular level by a recurrent depolarization of 300 msec - 1sec duration associated with MUA and they occurred with a frequency of 1 GDP every 2 to 20 sec. In CA1, GDPs were observed in 15/24 slices while in CA3 they were observed in 20/21 slices. When CA1 and CA3 were recorded together, GDPs were observed concomitantly in both layers in 11/17 slices and were observed only in CA3 but not in CA1 in 6 slices. Bath application of DL-TBOA (50-100 µM) modified the pattern of activity and generated after 3-5 min of application, recurrent slow and long-lasting depolarization in CA1 and CA3 lasting 32 ± 4 sec (n = 28 slices) associated with long lasting recurrent MUA (mean 38 ± 13 sec) (fig.1A, B). Dual patch-clamp of CA1 pyramidal cells and field recordings in CA3 showed that MUA occurred in the same time window as the cellular depolarization (fig1C). These recurrent activities that we shall refer to as slow network oscillations (SNOs) occurred with a mean frequency of 1 oscillation every 136 ± 10 sec (n = 17 slices). SNOs in both layers were no longer observed after the washout of TBOA and they were always blocked by NBQX (10 µM) the antagonist of AMPA/kainate receptor.
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receptors and APV (80 µM) or MK-801 (10 µM), the antagonists of NMDA receptor (n = 5/5 slices; 3 with APV and 2 with MK-801) indicating that SNOs involved the activation of ionotropic glutamate receptors (data not shown).

Because the CA3 region is thought to drive most of the physiological and pathological network driven activity in the hippocampus (Ben-Ari et al., 1989; Menendez de la Prida and coll. 1998; Ben-Ari, 2001; De la Prida et al., 2006), we next wondered if the generation of SNOs in CA1 originated from CA3. We therefore disconnected CA1 from CA3 by a knife cut and tested the action of TBOA. GDPs were observed in both layers (n = 5/8 slices and n = 7/8 slices in CA1 and CA3 respectively) as already described in other studies (Garaschuk et al., 1998; Menendez de la Prida et al., 1998). DL-TBOA still generated SNOs in both regions (n= 8/8 slices, fig. 1D). In CA1 the disconnection from CA3 did not change significantly the frequency of SNOs (1 oscillation every 138 ± 6 sec). In CA3 the oscillations occurred at a frequency rate of 1 burst every 118 ± 6 sec. In the disconnected slices SNOs did not always occur concomitantly i.e. SNOs may be observed in CA3 but not in CA1 and vice versa (see fig.1D). Together, these data indicated that i) the CA1 region can generate rhythmic activities independently of the CA3 region; ii) the connection between the 2 areas allowed a better coherence of SNOs.

We then decided to focus our attention on the CA1 region to understand how SNOs may be generated specifically in this network. To this aim we conducted our experiments in CA1 mini slices in which CA3 and the dentate gyrus were completely removed.

In CA1 pyramidal cells, SNOs were mediated mostly by the activation of GABA receptors

SNOs were generated in 49/53 cells recorded in the mini slices independently of the presence or the absence of GDPs before the application of DL-TBOA (see fig. 2). SNOs had the following characteristics: i) in voltage clamp mode, SNOs were characterized by periodic slow inward currents that reach a maximum between 3 to 15 sec (mean 7 ± 2 sec) and with a duration that ranged between 10 and 60 sec (mean 28 ± 1 sec). They were associated with a 3-20 fold increase in the number of spontaneous synaptic events (fig. 2Ab, mean frequency rate of 18 ± 1 Hz); ii) SNOs reversed polarity at the same membrane potential as that evoked by bath application of GABA (-34 ± 2 mV, n = 16 and -37 ± 2 mV, n = 6, for SNOs and GABA respectively fig.2C); iii) in the large majority of pyramidal cells, SNOs were fully abolished by bicuculline (20 µM, n = 13/18 cells, fig.2B) or by gabazine (0.5 µM, n = 3/5 cells).
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Though in 7 cells, GABA<sub>A</sub> receptor antagonists failed to block SNOs, the antagonist strongly reduced their amplitude by 70-90% and in 3 of these cells it also reduced their occurrence (2-3 oscillations in 15 min). In all the cases, subsequent addition of APV blocked them (not shown); iv) in the absence of bicuculline, D-APV (80 µM) or MK-801 fully abolished them (n = 5/5 cells, 4 cells with APV, 1 cell with MK-801; fig.2D); v) they were blocked by tetrodotoxin (TTX, 1 µM, n = 3/3 cells, not shown), a blocker of voltage-dependent Na<sup>+</sup> channels indicating that SNOs were mediated by an action potential-dependent release of transmitters.

Altogether, these data indicated that in pyramidal cells SNOs were mediated mostly by GABA<sub>A</sub> receptors and required the activation of NMDA receptors for their generation. This suggests that the initiation of SNOs might take place in GABAergic interneurons.

In interneurons, SNOs are mediated by the activation of both GABA<sub>A</sub> and NMDA receptors

We have recorded interneurons in the oriens or radiatum strata of the CA1 mini slices. SNOs were generated after bath application of DL-TBOA (n = 32/38 cells). In interneurons, SNOs were associated with MUA in the stratum pyramidale indicating that SNOs concerned at the same time pyramidal cells and interneurons of the CA1 region (fig 3A). SNOs in interneurons have similar duration (33 ± 3 sec in current clamp and 31 ± 2 sec in voltage clamp), frequency (1 oscillation every 121 ± 12 sec) and time to peak (8 ± 1 sec measured in voltage clamp) as in pyramidal cells.

We then determined which receptors mediated SNOs in interneurons. Bicuculline failed to block SNOs in a large majority of cells (n = 15 /18 cells, fig.3B). The action of the antagonist was complex because from one cell to another it either decreased or increased or did not change the duration, the frequency and the amplitude of SNOs. However on average, bicuculline significantly reduced the duration of SNOs (in current clamp: 33 ± 3 sec vs 19 ± 2 sec in the absence and in the presence of bicuculline respectively; in voltage clamp: 31 ± 2 sec vs 21 ± 1 sec in the absence and in the presence of bicuculline respectively) but did not affect significantly their amplitude (-70 ± 12 pA and -57 ± 7 pA before and after bicuculline respectively) or frequency (1 oscillation every 121 ± 12 sec vs. 119 ± 20 sec before and after bicuculline respectively). The bicuculline insensitive component had the following characteristics: i) it had also a slow onset with the peak of the current/depolarization being reached in 7 ± 2 sec and blocked by D-APV (fig.3B); ii) it may be also devoid of EPSCs (see
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fig. 3B for example); iii) The I/V relationship displayed a region of negative slope at membrane potential more negative than -20/-30 mV and the current reversed polarity at 5 ± 2 mV (n = 5; fig.3Cb); iv) it was blocked by TTX (n = 4/4 cells, fig. 3Ca). Therefore this component was mediated by NMDA receptors, which were activated by an action potential dependent release of glutamate. In addition, application of APV alone, in the absence of bicuculline, also fully abolished SNOs (n= 5/5 cells, fig.3A).

These data suggest: a) in interneurons the activation of NMDA receptors is sufficient to generate SNOs; GABA receptor activation amplifies the oscillations; b) in pyramidal cells both NMDA and GABA_A receptor activation are necessary for expression of SNOs. Taken altogether, these data suggested that SNOs were initiated by the activation of NMDA receptors in interneurons that led to the firing of interneurons, the release of GABA, and the activation of GABA_A receptors of both interneurons and pyramidal cells.

SNOs are not generated by applications of glutamate agonists

How are SNOs initiated? Since blockade of glutamate transporters elevates the extracellular concentration of glutamate [glutamate]_o (Danbolt, 2001; O'Shea et al., 2002), a sustained increase of [glutamate]_o may directly trigger membrane oscillations in interneurons due to the voltage dependency of NMDA receptors and activation of K^+ channels as shown in several structure including the supraoptic nucleus, the spinal chord, the tractus solitarii (Hu and Bourque, 1992; Tell and Jean, 1993; Hochmann et al., 1994). A sustained elevation of [glutamate]_o could also provide a sufficient depolarization of interneurons to generate recurrent bursts of action potentials if these neurons have intrinsic rhythmic bursting properties as recently shown for some CA3 pyramidal neurons (Sipilä et al., 2005). An alternative hypothesis is that the elevation of glutamate in the extracellular space during SNOs is transient and not sustained.

To discriminate between these possibilities, we have analyzed the behaviour of interneurons in response to depolarizing current injection to mimic the depolarization produced by glutamate, or to long lasting bath application of NMDA, non transportable agonist or glutamate. As shown in fig.4A current injections generated a continuous firing of action potentials but failed to generate rhythmic bursts. None of the interneurons recorded for this study (n = 18) displayed intrinsic rhythmic activity whatever their membrane potential. Additionally, in contrast to DL-TBOA, long-lasting bath application of NMDA during 15 to 20 min depolarized the cell and generated a sustained firing of the cell and unit activities in the stratum pyramidale but no SNOs (fig.4B,C). Increasing NMDA concentration up to 30
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µM (n = 3) or decreasing the concentration of NMDA to 1 µM (n = 4) failed to produce SNOs (data not shown). Glutamate (10-100 µM) also produced a continuous cellular depolarization and an increase in the number of units but not SNOs (n = 4, fig. 4D,E). A similar protocol was applied for pyramidal neurons and they did not display any rhythmic oscillations in response to current injection (n = 5, see supplemental fig.1A) and SNOs were not generated after bath application of NMDA (n = 5) or glutamate (n = 4) (supplemental fig. 1B-E). Therefore a sustained elevation of glutamate in the extracellular space and tonic activation of glutamate receptors did not generate SNOs. These results suggested that SNOs are initiated by a periodic increase of [glutamate]o and recurrent activation of NMDA receptors located in interneurons.

**Extrasynaptic NMDA receptors contributes to SNOs**

The very slow kinetics of the NMDA component of SNOs (in the presence of bicuculline), the progressive growth of the current occurring sometimes in the absence of EPSCs, together suggested that synaptic NMDA receptors may not fully account for the development of these oscillations. If this hypothesis is true then SNOs should still be produced in a neuron in which NMDA-mediated synaptic currents were abolished. In order to test this hypothesis, we used MK-801 (20 µM), the non competitive and irreversible NMDA receptor antagonist that blocked the NMDA channel only when it is open. EPSCs were recorded in interneurons in the presence of bicuculline and at 30 mV where Mg²⁺ did not block the NMDA channel. In addition an extracellular electrode was placed in the stratum radiatum in order to record MUA in this layer. The mean decay time constant of glutamate-mediated synaptic currents recorded in 6 cells in this condition is shown in fig. 5B. To eliminate as much as possible NMDA-mediated EPSCs, the synaptic activity was enhanced by increasing the external concentration of KCl from 3.5 to 7 mM. This protocol augmented the synaptic activity by 2-5 times and MUA (fig. 5A). Moreover, in keeping with an other study (Sipilä et al; 2005), a rise in [K⁺]e generated GDPs like events as reflected by large outward currents associated with burst MUA (fig. 5A, 5 out of 6 cells). After 10 min, MK-801 was bath applied for also 10 min. This diminished progressively both the amplitude and the duration of the GDPs to a stable level that was reached after 7-9 min of application (from 274 ± 100 pA to 70 ± 35 pA, for the amplitude before and after the application of MK-801 respectively and from 1.8 ± 0.3 sec to 0.7 ± 0.08 sec for the duration before and during MK-801 respectively fig. 5A,B). This also largely decreased the mean decay time of EPSCs to a value that corresponded to pure AMPA mediated synaptic events (fig.5B) (see for example
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Cossart et al., 2002; Groc et al., 2002). DL-TBOA was applied 10-15 min after the re-perfusion of slices with the control ACSF (in bicuculline). At this time GDP like events were no more present (fig. 5Ad) while the mean decay time of EPSCs was similar to that during the application of MK-801 (fig. 5B). This indicated that synaptic events were exclusively mediated by AMPA receptor while NMDA-mediated EPSCs were still blocked by MK-801. In spite of this blockade, TBOA generated SNOs and recurrent bursts of units in 5 out of 6 cells (fig.5C) occurring on a tonic outward current (10-200 pA range) as described in several studies (Jabaudon et al., 1999; Arnth-Jensen et al.2002; Demarque et al., 2002; Cavelier et al. 2002; Le Meur et al., 2007). SNOs had duration of 32 ± 2 sec and occurred at a frequency rate of 1 oscillation every 215 ± 29 sec. SNOs were blocked by APV and displayed an I/V relation characteristic of NMDA receptor-mediated component (fig. 5C,D). The mean frequency of SNOs was significantly lower to that measured with functional synaptic NMDA receptor (see upper). In contrast the duration was similar to the isolated NMDA component of SNOs measured at 30 mV in separate experiments (34 ± 1 sec, n = 4 cells, data not shown, also see below)

These data suggested that the oscillations were expressed by the activation of extrasynaptic NMDA receptors.

**NMDA activates interneurons more efficiently than pyramidal cells.**

The contribution of extrasynaptic NMDA receptors to SNOs suggested that the glutamate released periodically diffused in the extracellular space. Why did it preferentially activate interneurons compared to pyramidal cells? Are both cell types activated with the same efficacy by NMDA? To answer these questions we compared the effects of exogenous application of NMDA to generate current or depolarization in pyramidal cells and interneurons. First, we analysed the I/V relationship of the NMDA receptor-mediated response evoked by pressure ejection of NMDA (60-100 µM). These experiments were performed in the presence of NBQX (10 µM) and bicuculline (20 µM) to block respectively the AMPA/kainate receptors and GABA_A receptors as well as TTX (1µM) and Cd^{2+} (100 µM) to block respectively the voltage dependent Na^+ and Ca^{2+} channels. In addition, voltage dependent K^+ channels were blocked by tetraethylammonium (TEA, 20mM) and CsCl (2 mM) and neurons recorded with a CsGlu solution filled electrode. In both interneurons and pyramidal cells, NMDA was ejected close to the soma in order to reduce space clamp errors and with the same pressure and duration. As shown in fig.6A, there was a significant
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difference in I/V curves of both interneurons and pyramidal cells at membrane potential more negative than -55 mV. NMDA receptors of pyramidal cells were less activated at more hyperpolarized membrane potentials than that of interneurons. Second, in order to confirm this result in more physiological conditions, neurons were recorded in current clamp mode with KM2SO4 filled pipette. We measured the input resistance and membrane capacitance of interneurons and pyramidal cells. Both cell types had the same capacitance (fig. 6F) but showed a significant difference in their input resistance, which was significantly higher in interneurons than in pyramidal cells (fig.6E).

Next, we have analysed the depolarization produced by NMDA application in interneurons and pyramidal cells in the presence of bicuculline and NBQX at the same membrane potential. To ensure that the depolarization evoked by pressure ejection of NMDA was mediated by NMDA receptors, the response was also evoked at the peak of the negative slope (~ -25 mV) and in some cases D-APV or MK-801 was applied at the end of the experiments. As shown in the fig.6B,D, NMDA (60 µM) evoked a depolarization in interneurons and pyramidal cells that was stronger at -25 mV than at -80 or -90 mV in keeping with the voltage dependency of the NMDA receptors mediated response. However, the depolarization was significantly larger around -80 mV in interneurons than in pyramidal cells. Third, the concentration of NMDA in the pipette of ejection was decreased in order to determine if the threshold concentration to generate a depolarization was the same in both cell types. As shown in fig.6C decreasing the concentration of NMDA to 30 µM in the pipette consistently evoked a depolarization in interneurons while it failed to depolarize pyramidal cells in more than 50% of the cells. In the other cells, the depolarization was significantly lower than in the interneurons (fig.6D). At 10 µM, NMDA failed to activate interneurons (n = 5 cells). Taken together, these data indicate that exogenous NMDA is more efficient to activate interneurons than pyramidal cells.

**SNOs do not require the presence of pyramidal neurons**

What is the source of glutamate release inducing SNOs? The blockade of the glutamate component of SNOs by TTX suggested that the release of glutamate had a neuronal rather than a glial origin as it required the generation of sodium action potentials. In addition, the fact that SNOs in pyramidal cells were largely blocked by bicuculline but still present in interneurons led us to determine if pyramidal neurons were necessary for the generation of SNOs.
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In order to test this hypothesis, the stratum radiatum were surgically isolated from the rest of the hippocampus and interneurons recorded. To ensure the total ablation of pyramidal neurons, slices were analyzed post-hoc with fluorescent Nissl staining or with cresyl violet. In this situation, DL-TBOA still generated SNOs (see supplemental fig.2A). The SNOs had the same properties as those recorded in control non-isolated mini slices: i) They occurred at a frequency rate of 1 oscillation every $108 \pm 10$ sec and their duration was $31 \pm 2$ sec; ii) they were fully blocked by APV; iii) they persisted in the presence of bicuculline and were subsequently blocked by TTX. To ensure that the APV sensitivity of SNOs reflected the presence of an NMDA component in the oscillations, we recorded in three different slices interneurons at 30 mV with CsGlu filled pipette and analyzed the I/V relation of SNOs. In addition, in order to confirm that these oscillations are observed in this restricted network, we also recorded MUA in the stratum radiatum. As shown in fig. 7, GDPs in control and SNOs during TBOA were both observed at the cellular and at the field levels indicating that these 2 rhythmic activities occurred concomitantly in a population of cells of this isolated interneuronal network. In the absence of bicuculline, SNOs had duration of $41 \pm 3$ sec, and they occurred at a frequency rate of 1 oscillation every $97 \pm 12$ sec. The current reversed polarity around -10 mV, a value more depolarized than $E_{\text{GABA}}$ (fig.7C,D). In the presence of bicuculline, SNOs occurred at a frequency rate of 1 oscillation every $90 \pm 7$ sec and they had a duration of $33 \pm 3$ sec. SNOs reversed polarity around 0 mV, the I/V relation displayed a region of negative slope and this component was blocked by APV indicating that SNOs were mediated by NMDA receptors (fig. 7C,D).

SNOs were also observed in interneurons recorded in the stratum oriens from slices containing both the strata pyramidale and oriens but not the strata radiatum (n = 3 /3 slices, see supplemental fig.2B). In contrast, SNOs were not generated in interneurons recorded in isolated stratum oriens slices (n = 3/3 slices, not shown).

Taken together these data support the idea that SNOs may be initiated in the absence of the main glutamatergic pyramidal cells and suggested that an additional glutamatergic drive is present in the stratum radiatum that provide a source of glutamate necessary for the generation of SNOs.

**The generation of SNOs is developmentally regulated**

Finally, we wondered if the generation of SNOs was restricted to immature networks or could be generated at adult stages. To this aim, we first performed extracellular recordings...
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in the stratum pyramidale in order to analyse the pattern of unit activities in these population of cells at different ages (P0-P20). At the beginning of the second postnatal week of life (P8-P9) TBOA consistently generated recurrent long-lasting burst of units characteristic of the SNOs (4/4 slices). GDPs were also observed in these slices in control (fig. 8A). After P12, GDPs were no longer observed and TBOA failed to generate SNOs (14/14 slices) (fig. 8B). Instead, the inhibition of glutamate transporters led to a continuous firing of units in the stratum pyramidale and a sustained depolarization and action potential discharge in the interneurons (3/3 cells, fig. 8C). Together, these data indicated that SNOs were initiated during a restricted period of development.
DISCUSSION

The present study shows that during development, cell-surface glutamate transporters exert a powerful control of the hippocampal network activity preventing the occurrence of long-lasting bursts of activity due to the co-activation of a large neuronal population in both CA3 and CA1 areas. A deficiency in the transport of glutamate leads to the replacement of GDPs by Slow Network Oscillations (SNOs) that includes most of the ongoing synaptic activity. We also show that the CA1 area can generate SNOs even when disconnected from CA3 that plays an important role in the generation of most of physiological and pathological network driven events in the hippocampus (Ben-Ari et al., 1989; Menendez de la Prida et al., 1998; Ben-Ari, 2001; De la Prida et al., 2006). The generation of SNOs is mediated by a periodic activation of extrasynaptic NMDA receptors primarily located on GABAergic interneurons. The interneurons then release GABA that is excitatory at that developmental stage (Ben-Ari et al., 1989; Cherubini et al, 1991; Ben-Ari, 2002; Tyzio et al., 2006) leading to the activation of large populations of interneurons and pyramidal cells. Interestingly, our isolation experiments strongly suggest that the glutamatergic drive generating SNOs originates from radiatum and pyramidale strata. We propose that the co-activation of neuronal ensembles occurring during SNOs may contribute to the generation of seizures that are observed when glutamate transport is reduced (Milh et al., 2007).

Comparison of SNOs and GDPs

The mechanisms that generate GDPs have been extensively investigated in the developing hippocampus (see for reviews, Cherubini et al., 1991; Ben-Ari 2001, 2002). The global picture that emerges from these studies is that a glutamatergic drive contributes to synchronize GABAergic interneurons that in turn generate the polysynaptic GDPs. Like GDPs, SNOs also require the activation of GABAergic interneurons by a glutamatergic neuronal source. Also, both patterns are observed in the disconnected CA1 and they do not require the presence of pyramidal cells. Finally, they are restricted to a limited developmental stage as both are not observed after the second postnatal week of life. However SNOs differ from the GDPs by their very slow kinetics, i.e the onset and the decay are of several seconds for SNOs whereas they are of a few hundred msec for GDPs. Several factors have been shown to modulate the frequency and/or the kinetics of GDPs (Strata et al., 1995, McLean et al. 1996; Sipilä et al., 2004; Bernard et al., 2005; Safiulina et al., 2005). At
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least in CA3, the blockade of GABA_B receptors (McLean et al. 1996), GABA transporters (Sipilä et al., 2004) or cannabinoid receptors (Bernard et al., 2005) increased the duration of the GDPs for only few hundred msec. However, none of these inhibitors produced modifications in the onset and duration of GDPs with the same magnitude as those generated after blockade of glutamate transporters. The time course of SNOs is reminiscent of that of the “paracrine” current that we have previously described in CA1 during the perinatal period and is evoked by the release and diffusion of glutamate and GABA into the extracellular space and subsequent activation of extrasynaptic receptors (Demarque et al., 2002). Our data provide evidences that SNOs are also built or expressed via the activation of extrasynaptic NMDA receptors. Particularly, we found that in the presence of bicuculline, SNOs were still present after the blockade of NMDA-mediated EPSCs by MK-801 and with duration similar to that observed with functional synaptic NMDA receptors. This procedure however decreases the occurrence of SNOs suggesting that synaptic NMDA receptors may also play an important role in the initiation of the slow network oscillations. Since MK-801 was applied in the presence of 7 mM KCl that will depolarize the network, it is likely that synaptic NMDA receptors were affected not exclusively in the recorded cell but also in other cells of the network. This probably will alter the occurrence of the network oscillations. We propose that GDPs and SNOs may be initiated by the same glutamatergic drive but that the deficiency in the transport of glutamate enlarges the range of action of this drive in the network due to the diffusion of the glutamate released and extrasynaptic activation of receptors. This “spill-over” may engage large populations of neurons in rhythmic activities including cells in which GDPs were absent.

**SNOs are generated by a periodic release of glutamate**

Blocking glutamate transporters enhances the extracellular concentration of glutamate (Danbolt, 2001; O’Shea et al., 2002) and a recent study strongly suggests that this rise may originate from glial cells (Le Meur et al., 2007). The consequence is a tonic activation of glutamate receptors (mostly NMDA) of neurons that is revealed at positive membrane potential by a sustained outward current Jabaudon et al., 1999; Arnth-Jensen et al.2002; Demarque et al., 2002; Cavelier et al. 2002; Le Meur et al., 2007). Our data indicate that this tonic activation is insufficient to generate SNOs. Indeed, we showed that the long-lasting application of NMDA or glutamate induces a sustained activation of both interneurons and pyramidal cells but does not lead to the generation of slow oscillations. Therefore our data are better explained if in addition to the persistent enhancement of [glutamate], there is a
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periodic release of glutamate that diffuse in the extracellular space. We cannot however exclude a contribution of tonic activation of glutamate receptors in the generation of SNOs. A recent study performed with dynamic two-photon microscopy and Ca\(^{2+}\) imaging described that the CA1 stratum pyramidale of neonatal mice hippocampus contains some neurons displaying intrinsic periodic burst of action potential (Crepel et al., 2007). Because these recurrent bursts were observed in a particular range of membrane potential, a tonic depolarization produced by the activation of NMDA receptors may help these cells in generating this pattern and generate SNOs when glutamate transport fails.

**Pyramidal cells are not required for the generation of SNOs**

An intriguing observation is that SNOs can be generated in the absence of pyramidal cells. These SNOs generated by GABAergic interneurons have identical properties with the SNOs recorded in the mini slices, i.e they are blocked by APV and TTX but not by bicuculline. Moreover their I/V relationship includes an NMDA-mediated component. This suggests that an action potential dependent release of glutamate occurs in spite of the absence of strata pyramidale/oriens. It is unlikely that this release comes from the Schaffer collaterals, perforant path or commissural fibers, that innervate interneurons and pyramidal cells in these layers otherwise SNOs in pyramidal cells should often contain a glutamate component. In addition, it is difficult to conceive that these axons generated spontaneous action potentials and released glutamate with the periodicity of SNOs while they are disconnected from their soma. Our observations are therefore best explained by the presence in stratum radiatum of intrinsically active neurons that release periodically glutamate. Glutamatergic neurons have been identified in the stratum radiatum of the CA1 region of the adult rat hippocampus and they were called “giant cells” (Gulyàs et al., 1998). Whether such cells are present in the developing hippocampus and constitute one source of the glutamatergic drive necessary for the initiation SNOs remain to be established.

**Interneurons and pyramidal cells are not activated by NMDA with the same efficacy**

Our data show preferential activation of extrasynaptic NMDA receptors of interneurons by glutamate during SNOs. The analysis of the sensitivity of interneurons and pyramidal cells to exogenous application of NMDA may provide some clues. First, in these cells, NMDA receptors display significant less voltage sensitivity to Mg\(^{2+}\) block compared with those of pyramidal cells. As a consequence, in interneurons, unlike pyramidal cells (Leinekugel et al., 1997), NMDA receptors can be activated at hyperpolarized membrane
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potentials without the requirement of concomitant depolarizing action of GABA. Consistent with this, we found that SNOs in interneurons present an NMDA component in the presence of bicuculline. However, a difference in the Mg$^{2+}$ block cannot solely explain the absence in pyramidal neurons of an NMDA receptor-mediated component in SNOs. Indeed, in a large majority of these neurons, SNOs were only mediated by GABA and fully blocked by the GABA$_A$ receptor antagonist (bicuculline) even when the pyramidal cells were depolarised at -30 mV, where the Mg$^{2+}$ block is alleviated. There are at least 3 possible explanations for this: i) The concentration of glutamate near pyramidal cells is lower than near interneurons and is insufficient to activate the NMDA receptors; glial cells could preferentially sheath pyramidal cells constituting a physical barrier for glutamate diffusion; ii) NMDA receptors located on pyramidal cells do not have the same sensitivity for glutamate than those located on interneurons. This could result from a different possible subunits composition of the NMDA receptor in the 2 populations of cells (see Monyer et al., 1994; Mori and Mishina, 1995). Another element that has to be taken into account is the higher input resistance of the interneurons compared to pyramidal cells. This may favour their depolarization and contribute to make these cells the main sensors of variation of glutamate in the extracellular space.

**The generation of SNOs is developmentally regulated**

We show that SNOs could not be induced after the second postnatal week of life. After this period, DL-TBOA generated a sustained activation of the neurons. A number of parameters could contribute to the change of the pattern. These include: i) disappearance or alteration of the properties of the pacemaker cellular elements that provide the glutamatergic drive, ii) a massive tonic release of glutamate that masks the periodic activation of NMDA receptors, iii) the formation of a high density of synapses that may be associated with a reduced density of extrasynaptic receptors. In addition, a switch in GABAergic signalling (from depolarizing to hyperpolarizing action) that occurred after the second post-natal week of life (see Khazipov et al., 2004) may also participate to the inability in generating SNOs at this period of time. Since GDPs disappear at the same time as SNOs, the change of pattern may have a common origin that remains to be clarified in further studies.

**Concluding remarks**

Recent observations suggest that glutamate transporters may exert a powerful control of the developing spinal chord and of neocortical networks activity. These 2 structures that display GDPs like events (O’Donovan et al., 1998; Garaschuk et al., 2000) respond to TBOA
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by glutamate dependent slow recurrent depolarizations/inward currents with duration and frequency similar to that described in the present study (Demarque et al., 2004; Scharifullina and Nistri, 2006). Therefore, the modulating actions of glutamate transporters could be a universal rule for developing networks and suggests that if glutamate transporters are not fully operational during development (see Maragakis and Rothstein, 2004) this will affect similarly the activity of several brain structures and transform the ongoing physiological patterns (GDPs) to slow and long lasting membrane oscillations. We propose that one important role of glutamate transporters during development is to reduce the impact of putative “generators” of rhythmic activity on the network. By preventing or reducing the spread of glutamate in the extracellular space, glutamate transporters act to limit the co-activation of the neurons and the number of cells that participate in network driven activity in immature brain. This regulation is fundamental because it prevents the generation of pathological activities and seizures. Indeed, large co-activation of neuronal activities is the hallmark of epilepsy. The recent observations that the inhibition of glutamate transporters generates in vivo NMDA receptor dependent recurrent paroxysmal bursts and partial seizures (Milh et al., 2007) support this hypothesis. We propose that the mechanism described here for the generation of SNOs may be similar to that leading to the “suppression burst” pattern observed in vivo in the rat pups after ICV injection of DL-TBOA (Milh et al., 2007).

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FIGURES LEGENDS

**Figure 1:** Inhibition of glutamate transporters generates SNOs in the hippocampal network.

**Aa)** The scheme shows the positions of both the extracellular field pipette (F.P.) in order to record multiple unit activity (MUA) and patch-clamp (P.C.) electrodes in the CA1 region of the hippocampus. The control activity was composed by GDPs (see at expanded time scale in b, black dot). DL-TBOA (100 µM) produces slow recurrent depolarisations associated with MUA. GDPs recovered after the washout of TBOA. One of the depolarisation (indicated by an asterisk) is shown in c at an expanded time scale. Note the slow onset of the oscillation shown at the same time scale than the GDPs. **B)** Example trace showing that SNOs are also generated in CA3 by DL-TBOA (100 µM). **C)** Traces showing that the recurrent depolarisations in CA1 occurred concomitantly with MUA in CA3. **D)** Slow depolarisations recorded in CA1 and MUA in CA3 in a slice after the cut of the Schaffer collaterals. SNOs are no more coordinated in the 2 regions.

**Figure 2:** Inhibition of glutamate transporters generates GABA<sub>A</sub> receptor-mediated SNOs in pyramidal cells.

**Aa)** Pyramidal cell recorded in CA1 mini slice. The trace shows SNOs recorded in voltage clamp in a pyramidal cell from a P1 old rat. Some events in control and one oscillation are shown at 2 expanded time scales below. No GDPs were observed in this cell prior to the application of DL-TBOA. Note also the progressive growing of the oscillation. **b)** Graph showing the effects of DL-TBOA on spontaneous events versus time (Bin size = 10 sec) and corresponding to the full experiment shown in a. Each SNOs is associated with a large increase in the number of spontaneous events. This effect is reversible after the washout of DL-TBOA. SNOs are reinstalled after a second application of TBOA. **Ba)** Pyramidal cell from P6 old rat recorded in current clamp mode. The SNOs are fully blocked by bicuculline (20 µM). **b)** Same cell recorded in voltage clamp after the washout of bicuculline and the recovery of SNOs. Bicuculline fully abolished SNOs at -70 mV and -30 mV. This cell has spontaneous and evoked EPSCs mediated by glutamate receptors. **c)** In the presence of bicuculline, the stimulation of the Schaffer collaterals evoked at -30 mV an EPSC that is not fully blocked by NBQX (10 µM) and requires APV (80 µM) to be fully abolished. **C)** I/V relationship of SNOs (a) and of GABA mediated current (b) in two different cells. GABA (10
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µM was bath applied for 20 sec). Cells were recorded with a pipette filled with KGlu solution containing QX-314 to block the voltage dependent Na⁺ channels. The I/V relationship was constructed by subtracting the ramp response in control (1) from that during the maximal current response (2) (see methods); D) Pyramidal cell from P1 old rat recorded in voltage clamp mode. SNOs are fully blocked by D-APV (80 µM).

**Figure 3:** SNOs in interneurons are mediated by GABA_A and NMDA receptors.

Aa) Interneuron from P0 recorded in the stratum radiatum (upper trace) with a KM_2SO_4 filled pipette solution and combined with the extracellular recording of MUA with a pipette located in the stratum pyramidale (lower trace). The activity of the CA1 region is composed by GDPs and associated with MUA in the stratum pyramidale (showed at an expanded time scale below the 2 traces). Bath application of DL-TBOA generates SNOs. All the SNOs generated in this experiment are not shown in this figure. This pattern is blocked by D-APV (80 µM). The trace is shown 5 min after the application of APV. GDPs recover 15 min after the washout of the drugs. b) Graph corresponding to the full experiment depicted in (a) and showing the effects of DL-TBOA on the number spikes in the interneuron and MUA in pyramidal cell layer (bin size 10 sec). B) Interneuron from P6 old rat recorded in the stratum oriens in voltage clamp mode. DL-TBOA generates SNOs that are not abolished by bicuculline (the trace depicted is shown 10 min after the application of the antagonist). The remaining component is blocked by D-APV. SNOs are shown at expanded time scales below. Note the absence of EPSCs in the bicuculline insensitive component of SNOs and the slow onset. Ca) TTX sensitivity of the isolated NMDA component of SNOs recorded in the presence of bicuculline in an interneuron of the stratum radiatum from P4 old rat. Few EPSCs are observed during this slow current. b) I/V relationship of SNOs constructed by subtracting the ramp response in control (1) from that during the maximal current response (2).

**Figure 4:** SNOs are not generated by glutamate receptor agonists in interneurons

A) Interneuron of P5 recorded in the stratum radiatum in current clamp mode with a KM_2SO_4 filled pipette solution. The cell was progressively depolarised by injection of current (lower trace). This leads to a sustained discharge of action potentials (upper trace). Ba) DL-TBOA induces recurrent depolarizations and bursts of action potentials in interneurons and units in the stratum pyramidale. Bb) Same recordings as in Ba. After the washout of DL-TBOA, NMDA was applied and generates a sustained cellular depolarization and action potential and unit discharges. C) Graphs of the full experiment partially shown in B representing the
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number of spikes in the interneuron and MUA in the stratum pyramidale versus time (bin size= 10 sec). The two lower graphs show at an expanded time scale the discharge during the application of the 2 drugs. Note the recurrent increase of the discharge during DL-TBOA and the continuous increase during NMDA. 

Da) Interneuron of P4 recorded in the stratum radiatum in current clamp mode with a KM$_2$SO$_4$ filled pipette solution combined with the extracellular recording of MUA in the stratum pyramidale. Both glutamate concentrations 10µM (a) and 30 µM (b) failed to generate oscillations. E) Graphs corresponding of the experiment shown in D.

**Figure 5:** NMDA extrasynaptic receptors contribute to the expression of SNOs

Concomitant recording of an interneuron of P6 located in the stratum oriens with a pipette filled with CsGlu solution at 30 mV in voltage-clamp mode and of MUA with an extracellular electrode placed in the stratum radiatum. The experiments were performed in the continuous presence of bicuculline (20 µM). 

A) a-d) One minute recording in control (a), 8 min after application of KCl 7 mM (b), 9 min after the addition of MK-801 (20 µM)(c), and 15 min after the re-perfusion of the slice with control solution (KCl 3.5 mM and without MK-801) (d). The synaptic activity recorded during five seconds in the different conditions is shown at higher magnification between the two continuous traces. GDPs like events recorded during KCl 7 mM and KCl + MK-801 are show at a higher time scale below the continuous traces. 

B) Summary of the effects of MK-801 on both the amplitude (first histogram) and the duration of GDPs like events (second histogram) generated by KCl 7 mM in 5 cells. The third histogram shows the mean decay of EPSCs recorded in the four conditions in 6 cells. 2 representatives EPSCs recorded before and after MK-801 are also shown. 

C) DL-TBOA generates a tonic current and SNOs after MK-801. Both are abolished by D-APV. The slow current and the associated field activity indicates with an asterisk is shown at an higher time scale in b. The beginning of the onset is also magnified. Rare EPSCs are present during the onset. They have kinetics of AMPA mediated synaptic current. The I/V relationship of the current constructed by subtracting the ramp response in control (2) from that during the maximal current response (1) is shown in (c). 

D) Mean I/V relationships ( 9 I/V) of SNOs recorded in 5 cells.
Figure 6: Characteristics of NMDA receptor-mediated responses in interneurons and pyramidal cells.
A) I/V relationships of NMDA receptor mediated current in interneurons (red) and pyramidal cells (black). The inserts show the protocol used to generate the I/V curves. Neurons have been recorded with pipette filled with CsGlu solution. The current was evoked by pressure ejection of NMDA. The NMDA current is significantly larger in interneurons than in pyramidal cells at membrane potential more negative than -55 mV. B,C) NMDA receptor-mediated response in interneurons and pyramidal cells recorded in current clamp mode in the presence of NBQX and bicuculline with patch pipette filled with K$_2$SO$_4$ solution. The depolarization was evoked by pressure ejection of NMDA during 2 sec close to the soma at 60 µM (B) and at 30 µM (C) with the same pressure in both cell types (6 psi). D) Histograms representing the mean depolarization produced by NMDA applied at 60 and 30 µM in interneurons and pyramidal cells at -80 and -25 mV. The depolarization is always significantly stronger in interneurons than in pyramidal cells. E) Histogram representing the mean input resistance (Rm) of interneurons and pyramidal cells. Left traces show representative responses of an interneuron (Int.) and a pyramidal cell (Pyr.) following incremental current steps of -5 pA. F) Histogram representing the mean capacitance (Cm) of interneurons and pyramidal cells.

Figure 7: SNOs occur in the absence of pyramidal cells.
A) Picture showing the isolated stratum radiatum of P5 stained with cresyl violet and the interneuron recorded with a CsGlu filled pipette solution containing biocytin and reconstructed post-hoc. An extracellular electrode was also placed in the stratum radiatum in order to record unit activity. Ba-d) The interneuron is recorded in voltage-clamp mode at 30 mV. GDPs are present in control (a) and TBOA generates SNOs (b). They persist in the presence of bicuculline (c) and are fully abolished by APV (d). GDPs and SNOs are shown at a higher time scale below the continuous traces. Tonic current is not shown. C) I/V relationships of one of the slow current recorded in this experiment in the absence and in the presence of bicuculline. Ramps are indicated by numbers. D) Mean I/Vs of SNOs recorded in the absence (6 I/Vs) and the presence of bicuculline(6 I/Vs) in 3 different isolated stratum radiatum slices.
**Figure 8:** The generation of SNOs is developmentally regulated

Extracellular recording of units at P8 with a pipette located in the stratum pyramidale. The control activity is composed of GDPs (an example is shown at an expanded time scale below the continuous trace). Bath application of DL-TBOA generates recurrent bursts of units. 

**B)**
Same type of recording performed at P12. There are no more GDPs in control and bath application DL-TBOA (100 µM) induces a sustained discharge of units. 

**Ca)** Interneuron of P20 recorded in the stratum radiatum combined with the extracellular recording (F.P.) of units in the stratum pyramidale. DL-TBOA induces a depolarization and a sustained discharge of action potentials in the interneuron and units in the stratum pyramidale. Note the presence of a burst at the end of the application of DL-TBOA. Traces indicated with symbols are shown at an expanded time scale in **(Cb)**. 

**Cc)** Graph corresponding to the experiment shown in **(Ca)**. The upper graph represents the number of spikes in the interneuron versus time. The lower graph represents the MUA recorded in the stratum pyramidale versus time (bin size :10sec).

**Supplemental figure 1:** SNOs are not generated by glutamate receptor agonists in pyramidal cells

**A)** Pyramidal neuron of P5 recorded in current clamp mode. The progressive injection of current generates a transient discharge in the cell but not oscillations. 

**B)** TBOA (a) but not NMDA (b) generates recurrent depolarizations and bursts of units. 

**C)** Graph representing the number of spikes in the pyramidal cell (upper graph) and MUA (lower graph) versus time corresponding of the full experiment partially shown in **B** (bin size :10sec). 

**D,E)** From another pyramidal cell of P6 showing that glutamate fails to generate SNOs.

**Supplemental figure 2:**

**Aa)** Scheme representing the experimental procedure. The dissection is made in order to isolate the stratum radiatum of CA1. 

**b)** Confocal image of fluorescent Nissl staining of the isolated neuropile layer at P5. Below: Reconstructed cell corresponding to the experiment displayed in **c** and **d** and recorded with KM₂SO₄ and rhodamine filled pipette solution. SNOs are still observed in the interneuron recorded in this layer. These activities are blocked by APV. Note the presence of GDPs in the control (they were observed in 7 out of 12 slices). 

**Traces indicated with symbols are shown at an expanded time scale on the right.** 

**d)** Same cell recorded in voltage clamp mode after the washout of DL-TBOA and APV. A subsequent application of DL-TBOA generates bicuculline insensitive SNOs that are fully abolished by TTX. 

**Ba,b)** Scheme (a) and confocal image (b) of a P6 hippocampal slice composed only by...
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the strata pyramidale and oriens. The cell recorded in the experiment displayed in e is shown right to the confocal image. SNOs are observed in the interneuron recorded in the stratum oriens. Note also the presence of GDPs in control prior to the application of TBOA (they were observed in 1 out of 3 slices). Traces with symbols are shown below at an expanded time scale (GDPs and SNOs are shown at two different time scale). Note that bicuculline reduces the duration of SNOs. Abbreviations: SR: stratum radiatum; SP: Stratum pyramidale; SO: stratum oriens.
Figure 1: Inhibition of glutamate transporters generates SNOs in the hippocampal network. Aa) The scheme shows the positions of both the extracellular field pipette (F.P.) in order to record multiple unit activity (MUA) and patch-clamp (P.C.) electrodes in the CA1 region of the hippocampus. The control activity was composed by GDPs (see at expanded time scale in b, black dot). DL-TBOA (100 μM) produces slow recurrent depolarisations associated with MUA. GDPs recovered after the washout of TBOA. One of the depolarisation (indicated by an asterisk) is shown in c at an expanded time scale. Note the slow onset of the oscillation shown at the same time scale than the GDPs. B) Example trace showing that SNOs are also generated in CA3 by DL-TBOA (100 μM). C) Traces showing that the recurrent depolarisations in CA1 occurred concomitantly with MUA in CA3. D) Slow depolarisations recorded in CA1 and MUA in CA3 in a slice after the cut of the Schaffer collaterals. SNOs are no more coordinated in the 2 regions.
Figure 2: Inhibition of glutamate transporters generates GABAA receptor-mediated SNOs in pyramidal cells. Aa) Pyramidal cell recorded in CA1 mini slice. The trace shows SNOs recorded in voltage clamp in a pyramidal cell from a P1 old rat. Some events in control and one oscillation are shown at 2 expanded time scales below. No GDPs were observed in this cell prior to the application of DL-TBOA. Note also the progressive growing of the oscillation. b) Graph showing the effects of DL-TBOA on spontaneous events versus time (Bin size = 10 sec) and corresponding to the full experiment shown in a. Each SNOs is associated with a large increase in the number of spontaneous events. This effect is reversible after the washout of DL-TBOA. SNOs are reinstalled after a second application of TBOA. Ba) Pyramidal cell from P6 old rat recorded in current clamp mode. The SNOs are fully blocked by bicuculline (20 μM). b) Same cell recorded in voltage clamp after the washout of bicuculline and the recovery of SNOs. Bicuculline fully abolished SNOs at -70
mV and -30 mV. This cell has spontaneous and evoked EPSCs mediated by glutamate receptors. c) In the presence of bicuculline, the stimulation of the Schaffer collaterals evoked at -30 mV an EPSC that is not fully blocked by NBQX (10 μM) and requires APV (80 μM) to be fully abolished. C) I/V relationship of SNOs (a) and of GABA mediated current (b) in two different cells. GABA (10 μM was bath applied for 20 sec). Cells were recorded with a pipette filled with KGlu solution containing QX-314 to block the voltage dependent Na+ channels. The I/V relationship was constructed by subtracting the ramp response in control (1) from that during the maximal current response (2) (see methods); D) Pyramidal cell from P1 old rat recorded in voltage clamp mode. SNOs are fully blocked by D-APV (80 μM).
Figure 3: SNOs in interneurons are mediated by GABAA and NMDA receptors. Aa) Interneuron from P0 recorded in the stratum radiatum (upper trace) with a KM2SO4 filled pipette solution and combined with the extracellular recording of MUA with a pipette located in the stratum pyramidale (lower trace). The activity of the CA1 region is composed by GDPs and associated with MUA in the stratum pyramidale (showed at an expanded time scale below the 2 traces). Bath application of DL-TBOA generates SNOs. All the SNOs generated in this experiment are not shown in this figure. This pattern is blocked by D-APV (80 μM). The trace is shown 5 min after the application of APV. GDPs recover 15 min after the washout of the drugs. b) Graph corresponding to the full experiment depicted in (a) and showing the effects of DL-TBOA on the number spikes in the interneuron and MUA in pyramidal cell layer (bin size 10 sec). B) Interneuron from P6 old rat recorded in the stratum oriens in voltage clamp mode. DL-TBOA generates SNOs that are not abolished by bicuculline (the trace depicted is shown 10 min after the application of the antagonist). The remaining component is blocked by D-APV. SNOs are shown at expanded time scales below. Note the absence of EPSCs in the bicuculline insensitive component of SNOs and the slow onset. Ca) TTX sensitivity of the isolated NMDA component of SNOs recorded in the presence of bicuculline in an interneuron of the stratum radiatum from P4 old rat. Few EPSCs are observed during this slow current. b) I/V relationship of SNOs constructed by subtracting the ramp response in control (1) from that during the maximal current response (2).
Figure 4: SNOs are not generated by glutamate receptor agonists in interneurons

A) Interneuron of P5 recorded in the stratum radiatum in current clamp mode with a KM2SO4 filled pipette solution. The cell was progressively depolarised by injection of current (lower trace). This leads to a sustained discharge of action potentials (upper trace). Ba) DL-TBOA induces recurrent depolarizations and bursts of action potentials in interneurons and units in the stratum pyramidale. Bb) Same recordings as in Ba. After the washout of DL-TBOA, NMDA was applied and generates a sustained cellular depolarization and action potential and unit discharges. C) Graphs of the full experiment partially shown in B representing the number of spikes in the interneuron and MUA in the stratum pyramidale versus time (bin size= 10 sec). The two lower graphs show at an expanded time scale the discharge during the application of the 2 drugs. Note the recurrent increase of the discharge during DL-TBOA and the continuous increase during
NMDA. Da) Interneuron of P4 recorded in the stratum radiatum in current clamp mode with a KM2SO4 filled pipette solution combined with the extracellular recording of MUA in the stratum pyramidale. Both glutamate concentrations 10µM (a) and 30 µM (b) failed to generate oscillations. E) Graphs corresponding of the experiment shown in D.
Figure 5: NMDA extrasynaptic receptors contribute to the expression of SNOs

Concomitant recording of an interneuron of P6 located in the stratum oriens with a pipette filled with CsGlu solution at 30 mV in voltage-clamp mode and of MUA with an extracellular electrode placed in the stratum radiatum. The experiments were performed in the continuous presence of bicuculline (20 μM). A) a-d) One minute recording in control (a), 8 min after application of KCl 7 mM (b), 9 min after the addition of MK-801 (20 μM) (c), and 15 min after the re-perfusion of the slice with control solution (KCl 3.5 mM and without MK-801) (d). The synaptic activity recorded during five seconds in the different conditions is shown at higher magnification between the two continuous traces. GDPs like events recorded during KCl 7 mM and KCl + MK-801 are shown at a higher time scale below the continuous traces. B) Summary of the effects of MK-801 on both the amplitude (first histogram) and the duration of GDPs like events (second histogram) generated by KCl 7 mM in 5 cells. The third histogram shows the mean decay of EPSCs recorded in the four conditions in 6 cells. 2 representatives EPSCs recorded before and after MK-801 are also shown. C) DL-TBOA generates a tonic current and SNOs after MK-801. Both are abolished by D-APV. The slow current and the associated field activity indicates with an asterisk is shown at an higher time scale in b. The beginning of the onset is also magnified. Rare EPSCs are present during the onset. They have kinetics of AMPA mediated synaptic current. The I/V relationship of the current constructed by subtracting the ramp response in control (2) from that during the maximal current response (1) is shown in (c). D) Mean I/V relationships (9 I/V) of SNOs recorded in 5 cells.
Figure 6: Characteristics of NMDA receptor-mediated responses in interneurons and pyramidal cells. A) I/V relationships of NMDA receptor mediated current in interneurons (red) and pyramidal cells (black). The inserts show the protocol used to generate the I/V curves. Neurons have been recorded with pipette filled with CsGlu solution. The current was evoked by pressure ejection of NMDA. The NMDA current is significantly larger in interneurons than in pyramidal cells at membrane potential more negative than -55 mV. B,C) NMDA receptor-mediated response in interneurons and pyramidal cells recorded in current clamp mode in the presence of NBQX and bicuculline with patch pipette filled with KM2SO4 solution. The depolarization was evoked by pressure ejection of NMDA during 2 sec close to the soma at 60 µM (B) and at 30 µM (C) with the same pressure in both cell types (6 psi). D) Histograms representing the mean depolarization produced by NMDA applied at 60 and 30 µM in interneurons and pyramidal cells at -80 and -25 mV. The depolarization is always significantly stronger in interneurons than in pyramidal cells. E) Histogram representing the mean input resistance (Rm) of interneurons and pyramidal cells. Left traces show representative responses of an interneuron (Int.) and a pyramidal cell (Pyr.) following incremental current steps of -5 pA. F) Histogram representing the mean capacitance (Cm) of interneurons and pyramidal cells.
Figure 7: SNOs occur in the absence of pyramidal cells. A) Picture showing the isolated stratum radiatum of P5 stained with cresyl violet and the interneuron recorded with a CsGlu filled pipette solution containing biocytin and reconstructed post-hoc. An extracellular electrode was also placed in the stratum radiatum in order to record unit activity. Ba-d) The interneuron is recorded in voltage-clamp mode at 30 mV. GDPs are present in control (a) and TBOA generates SNOs (b). They persist in the presence of bicuculline (c) and are fully abolished by APV (d). GDPs and SNOs are shown at a higher time scale below the continuous traces. Tonic current is not shown. C) I/V relationships of one of the slow current recorded in this experiment in the absence and in the presence of bicuculline. Ramps are indicated by numbers. D) Mean I/Vs of SNOs recorded in the absence (6 I/Vs) and the presence of bicuculline (6 I/Vs) in 3 different isolated stratum radiatum slices.
Figure 8: The generation of SNOs is developmentally regulated. Extracellular recording of units at P8 with a pipette located in the stratum pyramidale. The control activity is composed of GDPs (an example is shown at an expanded time scale below the continuous trace). Bath application of DL-TBOA generates recurrent bursts of units. B) Same type of recording performed at P12. There are no more GDPs in control and bath application DL-TBOA (100 μM) induces a sustained discharge of units. Ca) Interneuron of P20 recorded in the stratum radiatum combined with the extracellular recording (F.P.) of units in the stratum pyramidale. DL-TBOA induces a depolarization and a sustained discharge of action potentials in the interneuron and units in the stratum pyramidale. Note the presence of a burst at the end of the application of DL-TBOA. Traces indicated with symbols are shown at an expanded time scale in (Cb). Cc) Graph corresponding to the experiment shown in (Ca). The upper graph represents the number of spikes in the interneuron versus time. The lower graph represents the MUA recorded in the stratum pyramidale versus time (bin size: 10 sec).
Figure 1bis: Effect of Gabazine on SNOs and of isoguvacine-mediated current. A, B: Pyramidal cells recorded at 30 mV with a CsGlu filled pipette solution. In A SNOs were fully abolished by gabazine (0.5 μM). The current reversed polarity around -30 mV. In B gabazine failed to block SNOs but strongly decreased the amplitude. Note the absence of synaptic events during the onset of SNOs during gabazine. Note also that the I/V relationship has the characteristic of NMDA-mediated current. C) Gabazine fully abolished the current evoked by pressure ejection of isoguvacine.
Figure 2 bis: Two pyramidal cells were patched in CA1 at embryonic day 20, One cell was recorded at 30 mV with a CsGlu filled pipette in voltage clamp mode, the other one is recorded in current clamp mode at -40 mV with a KCl filled pipette electrode. In cell 1 synaptic activity is rare. In cell 2 the synaptic activity is more frequent but there is no GDPs. Addition of TBOA generated SNOs in both cells and as shown at an expanded time scale the growing is progressive with no apparent GDPs. In cell 1 synaptic events are even quasi absent.

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