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Paracrine Intercellular Communication by a Ca^{2+}- and SNARE-Independent Release of GABA and Glutamate Prior to Synapse Formation

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Summary

GABA and glutamate receptors are expressed in immature "silent" CA1 pyramidal neurons prior to synapse formation, but their function is unknown. We now report the presence of tonic, spontaneous, and evoked currents in embryonic and neonatal CA1 neurons mediated primarily by the activation of $GABA_A$ receptors. These currents are mediated by a nonconventional release of transmitters, as they persist in the presence of calcium channel blockers or botulinium toxin and are observed in Munc18-1-deficient mice in which vesicular release is abolished. This paracrine communication is modulated by glutamate but not GABA transporters, which do not operate during this period of life. Thus, a Ca^{2+}- and SNARE-independent release of transmitters underlies a paracrine mode of communication before synapse formation.

Introduction

A central issue in developmental neurobiology is whether neurons communicate via transmitter release prior to the formation of conventional synapses. Studies performed in a wide range of preparations—notably the neuromuscular junction—suggest that receptors to neurotransmitters are operative before the formation of synapses (Xie and Poo, 1986; Hall and Sanes, 1993). Other observations suggest that the principal transmitters of the brain—i.e., GABA and glutamate that, respectively, mediate most of the inhibitory and excitatory synapses in adult circuits—provide important communication signals during development. Thus, in immature neurons, GABA, which is excitatory at an early stage of development, and glutamate may modulate proliferation, migration, and dendritic outgrowth (for reviews, see Nguyen et al., 2001; Ben-Ari, 2001, 2002; Owens and Kriegstein, 2002). To mediate these actions, transmitters must be released at an early developmental stage and diffuse to stimulate receptors. Several observations support this hypothesis: (1) functional GABA and glutamate receptors are expressed by neuronal precursors and neurons of several brain areas at a very early stage (Nguyen et al., 2001; Ben-Ari, 2001, 2002; Owens and Kriegstein, 2002); (2) GABA and glutamate are released by growth cones in developing networks (Taylor and Gordon-Weeks, 1991; Soeda et al., 1997; Gao and Van Den Pol, 2000); (3) ambient glutamate activates NMDA receptors on cortical plate neurons in embryonic rats (Lo Turco et al. 1991); (4) ambient GABA activates cultured embryonic hippocampal neurons (Valeyev et al., 1993); (5) blockade of NMDA receptors reduces spontaneous Ca^{2+} elevation and alters the rate of migration of cerebellum granular cells (Komuro and Rakic, 1993); and (6) blockade of $GABA_A$ receptors generates an outward current and produces an enhancement of DNA synthesis in proliferating neuronal precursors of the neocortical ventricular zone (Lo Turco et al., 1995). If transmitters mediate intercellular communication prior to synapse formation, a tonic, spontaneous, and evoked nonsynaptic current should be observed in maturing neurons.

We have now investigated this issue in CA1 hippocampal neurons of embryonic and neonatal slices. This region is particularly suitable since at birth most pyramidal neurons have just an anlage of apical dendrite and have no synapses and no excitatory or inhibitory postsynaptic currents. These cells (synaptically silent cells) have, however, functional GABA and glutamate receptors, as they respond to applications of GABA or glutamate (Tyzio et al., 1999). In addition, GABA and glutamate fibers are present already in utero and may provide a source of transmitter (Rozenberg et al.,1989; Super and Soriano, 1994; Dupuy and Houser, 1996; Diabira et al., 1999). We report that these neurons exhibit a tonic form of GABAergic activation and that the evoked release of GABA and glutamate generates an early slow current (ESC) primarily mediated by the activation of $GABA_A$ receptors and to a lesser extent NMDA, but not AMPA, receptors. The mechanisms of release include an important Ca^{2+}- and SNARE-independent component as the tonic current, and ESC persisted after a treatment with Ca^{2+} channel blockers or botulinium toxin and are observed in Munc18-1 deficient mice in which vesicular release is abolished (Verhage et al., 2000). We suggest that this nonconventional form of release may provide an early form of intercellular communication that enables a distal flooding type of action prior to the topic synaptic mode of interaction.

Results

In keeping with our previous study (Tyzio et al., 1999), large numbers of CA1 pyramidal neurons recorded during the perinatal period (E18–P1) are synaptically silent. They responded neither to electrical stimulation nor to α-latrotoxin, which stimulates vesicular exocytosis (Südhof, 2001), by postsynaptic currents (PSCs) (Figures 1C, 2A, and 2B). Bath or focal applications of GABA and glutamate did, however, generate large currents (Figure 1D) (Tyzio et al., 1999) that were, respectively, blocked by bicuculline or picrotoxin (two antagonists of $GABA_A$ receptors) and MK-801 or APV (two antagonists of NMDA receptors) plus CNQX (an antagonist of AMPA/kainate receptors) (data not shown). These neurons had

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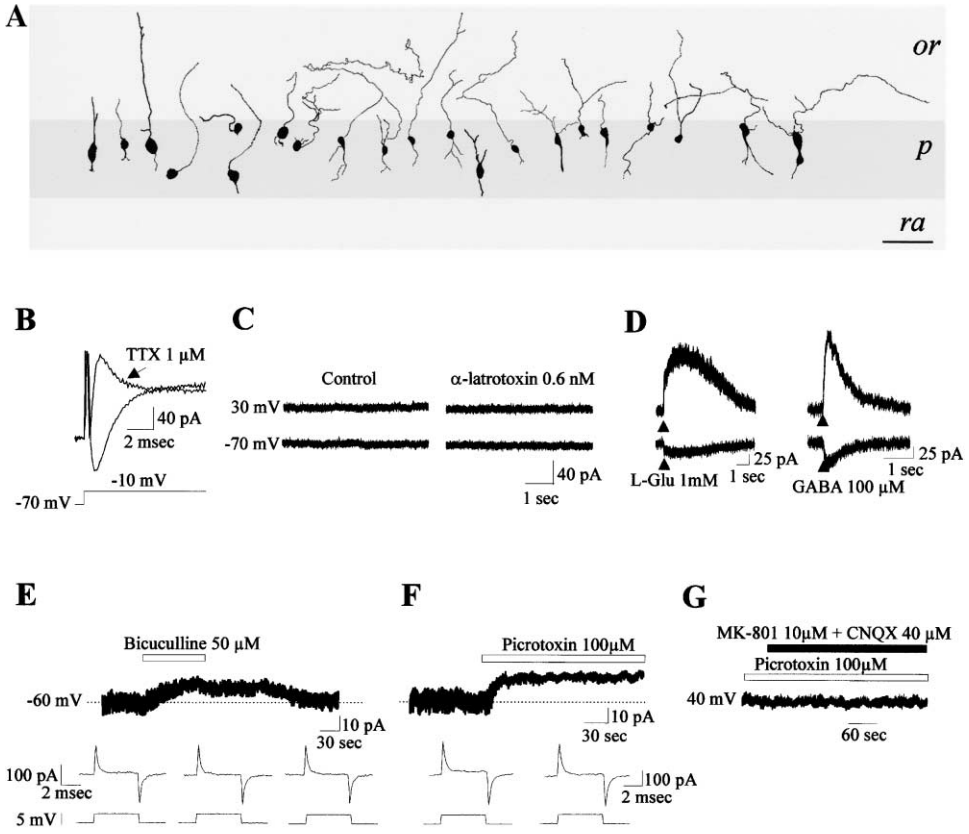

Figure 1. Synaptically Silent Cells Exhibit a Tonic Form of GABAergic but Not Glutamatergic Activation

(A) Camera lucida reconstruction of synaptically silent pyramidal cells injected with biocytin. Or, stratum oriens; p, stratum pyramidale; ra, stratum radiatum. Scale bar, 25 μm.

(B) TTX-sensitive fast inward current evoked by a depolarizing step of 60mV in a P0 synaptically silent pyramidal neuron (Cm, 14 pF).

(C) Pyramidal neuron recorded with a pipette that contained 10 mM Cl^-, (CsGlu solution). Spontaneous events are not observed at −70 and 30mV, even after the bath application of α-latrotoxin (0.6 nM; applied for 90 s).

(D) Left traces: P0 synaptically silent neuron (Cm, 15 pF) recorded with a CsGlu-filled pipette solution in the presence of TTX (1 μM) and bicuculline (50 μM) to block $GABA_A$ receptors. Pressure ejection of glutamate (1 mM, 120 ms, 5 psi) evokes currents at −70 and 30mV. Right traces: another cell (Cm, 10 pF) recorded with CsGlu-filled pipette solution in the presence of TTX, MK-801, and CNQX to block glutamatergic receptors. Pressure ejection of GABA (100 μM, 6 ms, 1 psi) generates currents at −70 and 30mV.

(E) P0 synaptically silent neuron (Cm, 15 pF) recorded at −60mV with a CsCl-filled pipette solution. Bath application of bicuculline (50 μM) generates a small and reversible outward current (the trace is filtered with a low pass band at 300 Hz). Traces depicted below represent the responses of the cell to a 5mV depolarizing pulse applied before bicuculline (left), at the plateau of the tonic current (middle), and after the recovery (right) to show that the serial resistance (Rs) does not change during the recording (Rs, 19 mOhm).

(F) Another synaptically silent neuron (Cm, 12 pF) recorded at E21, in which picrotoxin also generates an outward current (trace is filtered) with no change of Rs (17 mOhm). Note that the current is associated with a large decrease of the basal noise.

(G) Same neuron as before. The membrane potential was held at 40mV, still in the presence of picrotoxin. Bath application of NMDA, AMPA, and kainate receptor antagonists does not generate any current.

high input resistance (1–3 GΩ) and a small capacitance (6–15 pF) and responded to a depolarizing current pulse by a TTX-sensitive fast inward Na^+ current (Figure 1B). Morphological reconstruction of these neurons showed that they had an axon oriented toward the stratum oriens and an anlage of apical but no basal dendrite (Figure 1A) (Tyzio et al., 1999). In contrast, neurons that have a higher capacitance (15–50 pF) have more developed apical dendrites that extend to the neuropile layers (Figure 3A), and exhibit spontaneous GABA-only or GABA and glutamate receptor-mediated PSCs (Tyzio et al., 1999). They also responded to α-latrotoxin (Figure 3C).

Synaptically Silent Neurons Exhibit a Tonic Form of GABAergic but Not Glutamatergic Activation

As shown in Figures 1E and 1F, bicuculline (50 μM, n = 7/11) or picrotoxin (100 μM, n = 4/6) generated an outward current (mean outward current, 0.75 ± 0.16 pA/pF). In contrast, glutamate receptor antagonists, CNQX (40 μM), and MK-801 (10 μM) or APV (50 μM) did not generate a current (Figure 1G; n = 10/10). Therefore, ambient GABA but not glutamate tonically activates neurons that have no synapses.

Electrical Stimulation of Stratum Radiatum Evokes a Slow Current in Synaptically Silent Neurons

We then tested if a current can be generated by electrical stimulation of stratum radiatum, where most GABAergic neurons and glutamatergic fibers are localized (see Introduction). A single stimulation generated a large current with a slow kinetic (mean time to peak, 6.1 ± 0.6 s; mean decay time, 19.1 ± 1.2 s; duration range, between 10 and 30 s) (Figure 2A). This current, which we

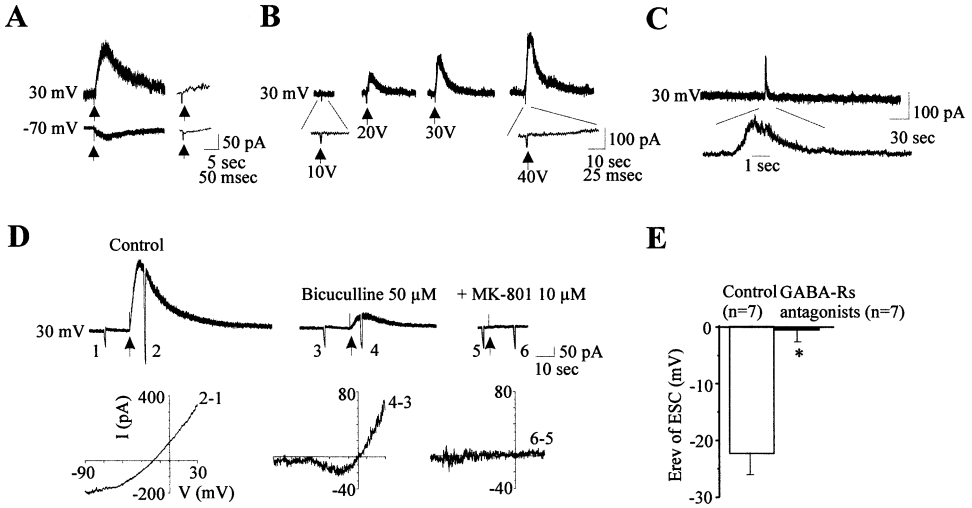

Figure 2. A Single Stimulation of the Stratum Radiatum Evokes an ESC Mediated by $GABA_A$ and NMDA Receptors

(A) P0 synaptically silent cell (Cm, 11 pF) recorded with CsGlu-filled pipette electrode. The electrical stimulation of the stratum radiatum (30V, 40 μs duration, indicated by an arrow) evokes slow currents at −70 and 30mV (left traces, lowest time scale), but not PSCs (right traces, highest time scale).

(B) Another P0 silent pyramidal neuron (Cm, 12 pF) recorded at 30mV. ESC increases with the stimulus intensity. The absence of fast synaptic response in the cell is shown below at higher time scale.

(C) Spontaneous slow event in a synaptically silent neuron (Cm, 14 pF) recorded at 30mV.

(D) ESC recorded in an E20 silent pyramidal neuron (Cm, 13 pF) at 30mV with a CsGlu-filled pipette solution (stimulation artifacts are indicated by arrows). Voltage ramps (from 30 to −100mV, 1 s duration; indicated by numbers) were applied before and at the peak of the ESC. The difference between both ramps gives the I/V relationship (shown below each traces; 2-1, IV curve corresponding to the control ESC; 4-3, IV curve corresponding to ESC evoked 5 min after the application of bicuculline; 6-5, IV curve constructed 5 min after the application of MK-801).

(E) Bar graph showing the mean effect of $GABA_A$ receptor antagonists on the reversal potential of ESC (Erev). The number of cells is indicated in brackets. The asterisk indicates statistically significant difference in the Erev (ANOVA test).

shall refer to as an early slow current (ESC), was inward at −70mV and outward with a higher amplitude at 30mV (with a CsGlu-filled pipette electrode, n = 24). The amplitude of the ESC increased with stimulus intensity (up to 400–500 pA; Figure 2B). ESC was evoked consistently in silent neurons (mean Cm = 13.7 ± 1.3 pF) when the stimulating electrode was positioned in stratum radiatum at a distance of less than 250 μm from the recorded neuron. In contrast, similar stimulations of the pyramidal layer failed to evoke an ESC (n = 14/15; also see below). Since an electrical stimulation can generate an electroporation (Haas et al. 2001), we tested whether the stimulus used to generate an ESC was associated with a penetration of a fluorescent marker in the recorded neuron. Stimuli applied to the somatic layer generated an electroporation, as revealed by the staining of cells by lucifer yellow; this was, however, consistently not associated with an ESC (n = 5/5) (see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/36/6/1051/DC1). In contrast, stimulation of the stratum radiatum that generates an ESC did not induce an electroporation. Therefore, ESC is not mediated by the formation of pores.

If an electrical stimulation could evoke an ESC, it was important to determine whether similar currents were also recorded spontaneously in a slice. We thus performed 30–60 min of recordings from silent neurons without stimulation and observed spontaneous slow currents (52.1 ± 8.7 pA at 30mV, 2–10 s duration, 1–3 events in 30–60 min of recording; n = 7/12 cells) (Figure 2C). The presence of spontaneous ESC provides direct evidence that these events are relevant physiologically and are not due to an artificial action of the electrical stimulation. Thus, in neurons with no functional synapses, spontaneous and evoked currents with unique features are present.

ESCs Are Mediated by $GABA_A$ Receptors with a Small Contribution of NMDA Receptors

Bath application of bicuculline (50 μM) or picrotoxin (100 μM) reduced the ESC amplitude by 85% ± 5% (n = 7), suggesting that most of the current is mediated by $GABA_A$ receptors. An involvement of NMDA receptors is, however, suggested by the following observations: (1) bath application of MK-801 (10 μM), but not CNQX (40 μM), fully blocked the remaining current (Figure 2D); (2) in control conditions, voltage ramps (see Experimental Procedures) generated an I/V curve with a higher slope conductance at positive than at negative membrane potentials and a reversal potential (Erev) at −28.5 ± 2.4mV (n = 24). After $GABA_A$ receptor blockade, Erev was significantly shifted to a more depolarized value (Figures 2D and 2E; $p = 10^{-4}$), and the IV curve displayed a region of negative slope.

To directly demonstrate that the effects of bicuculline or picrotoxin on ESC were due to the blockade of $GABA_A$ receptors, we used an intracellular technique that enables blockage of $GABA_A$ receptors only in the recorded neuron by an intracellular perfusion with CsF (Nelson et al., 1994, Leinekugel et al., 1997). This experiment, as well as an analysis of the contribution of Cl^- to the Erev of $GABA_A$-Rs mediated response, confirmed both the predominant role of $GABA_A$ receptors and the contribution of NMDA receptors in ESC generation (see Supple-

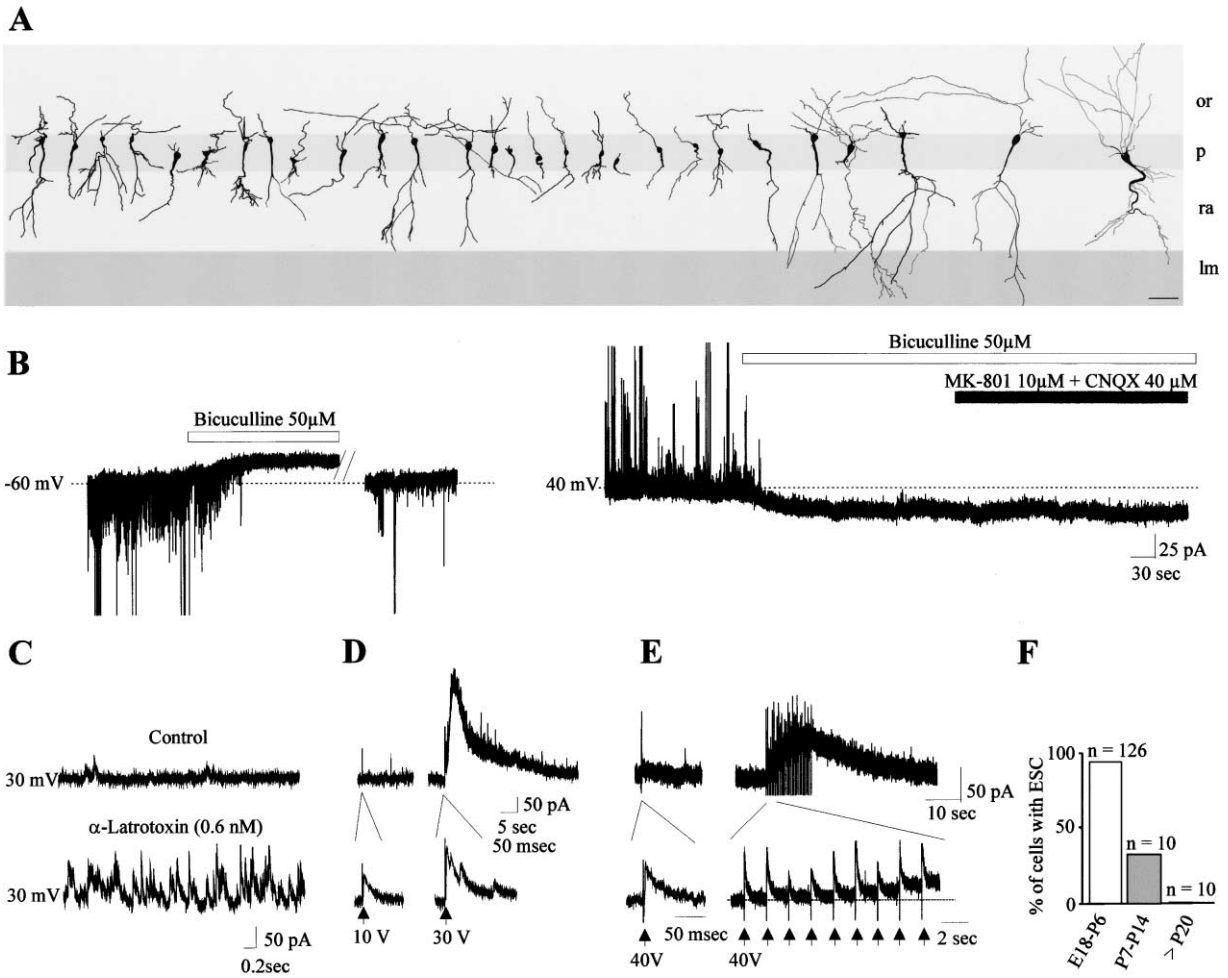

Figure 3. GABA Tonic Current and ESC Are Observed in Synaptically Active Cells

(A) Camera lucida reconstruction of synaptically active pyramidal cells. Scale bar, 25 μm.

(B) Left trace: P0 synaptically active neuron (Cm, 42 pF) recorded at −60mV with a CsCl-filled pipette solution. Bath application of bicuculline blocks the spontaneous fast synaptic activity and generates an outward current associated with a decrease of basal noise. These effects were reversible after the washout of the drug. Right trace: from the same neuron. The membrane potential was held at 40mV and bicuculline was reapplied. This also blocks spontaneous fast synaptic events and generates an inward current associated with a reduction of basal noise. Bath application of MK801 and CNQX does not affect the holding current.

(C) Spontaneous events recorded in a pyramidal neuron (Cm, 25 pF) at 30mV with a pipette filled with CsGlu. Application of α-latrotoxin (same conditions as in Figure 1C) strongly enhances the activity.

(D) P0 pyramidal neuron (Cm, 27 pF) showing that the stimulus intensity required to evoke ESC by a single pulse is higher than that required to evoke a PSC. Traces below show PSCs at a lower time scale.

(E) Traces from another synaptically active cell (Cm, 19 pF) showing that the stimulus intensity required to evoke PSC (40V, 40 μs duration) may induce an ESC when applied repetitively (30 pulses, 400 ms pulse interval).

(F) This histogram depicts the number of pyramidal neurons (expressed in %) that displayed ESC as a function of the age.

mental Figure S2 at http://www.neuron.org/cgi/content/full/36/6/1051/DC1). Therefore, GABA and glutamate released by electrical stimulation diffuse and activate distant neurons that have no functional synapses.

GABA Tonic Currents and Evoked ESCs Are Restricted to Early Developmental Stages

If transmitters diffuse and activate synaptically silent neurons, they should also generate currents in more mature pyramidal cells that have functional synapses. At birth, these are present in the same slices adjacent to silent neurons (Tyzio et al., 1999). Bicuculline generated a current in 14 out of 18 neurons (0.65 ± 0.15 pA/pF), whereas the coapplication of CNQX + MK-801 or APV did not produce any current in 8 out of 9 cells (Figure 3B). Therefore pyramidal neurons with PSCs exhibit a tonic form of GABAergic but not glutamatergic activation.

A single electrical stimulation of the stratum radiatum evoked an ESC in these cells (in 77 out of 81; Cm, 26.7 ± 1.5 pF; n = 77) with an amplitude of up to 600–700 pA (measured at 30mV). ESC was clearly distinguishable from the PSCs by (1) its long kinetic that was in the same range as that observed in synaptically silent cells (5.1 ± 0.4 s and 18.6 ± 1.4 s for rise and decay time, respectively) and (2) by the parameters of stimulation (Figures 3D and 3E). In active cells, ESC was also mediated by the activation of $GABA_A$ and NMDA receptors

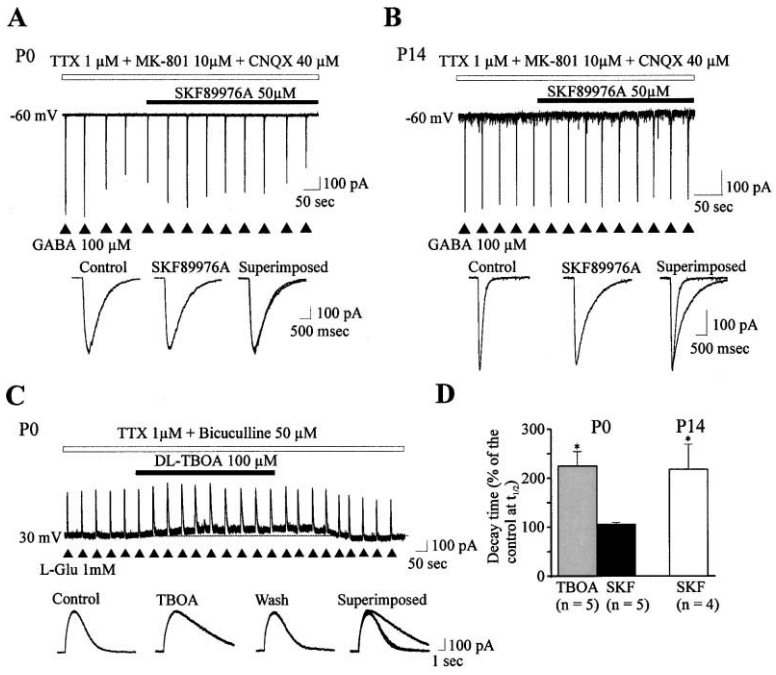

Figure 4. Glutamate but Not GABA Transporters Contribute to the Clearance of Transmitter at Birth

(A) P0 pyramidal neuron (Cm, 10 pF) recorded at −60mV in the presence of TTX and antagonists of glutamate receptors with a pipette filled with CsCl. Top trace: currents evoked by pressure ejection of GABA (100 μM, 6 ms, 1 psi, every 60 s) before and during bath application of the GAT-1 transporter inhibitor SKF89976A (50 μM). Bottom traces: GABA currents at a higher time scale (average of five traces) in control, during SKF89976A, and a superposition of both. Note that SKF89976A has no effect on the GABA current.

(B) P14 pyramidal neuron (Cm, 180 pF) recorded in the same conditions as in (A). Currents evoked by pressure ejection of GABA (5 ms, 1 psi, every 30 s) are prolonged by SKF89976A.

(C) P0 pyramidal neurons (Cm, 25 pF) recorded at 30mV in the presence of TTX and bicuculline. Top trace: currents evoked by pressure ejection of L-glutamate (1 mM, 50 ms, 10 psi, every 60 s) before, during, and after bath application of TBOA (100 μM). Bottom traces: glutamate currents at a higher time scale (average of four traces) in control, during, and after the washout of TBOA and a superposition of the three. TBOA generates an outward current (dashed line represents the baseline) and slows down the decay of the current.

(D) Bar graph showing the effect of transporter inhibitors on the decay time of agonist-mediated currents.

(data not shown). Thus, ESC with similar properties was observed in the pyramidal cells at the three stages of morphological and physiological development described during this period of life (Tyzio et al., 1999). However, ESC is restricted to developing neurons and networks, as it was not evoked in adults (Figure 3F).

Glutamate but Not GABA Transporters Operate at Birth

The observations that neurons were preferentially activated by GABA and to a lesser extent by glutamate could be due to a different efficacy of GABA (GAT-1) and glutamate transporters in the clearance of transmitters at this early stage of development. Immunohistochemical studies suggest that both glial and neuronal GABA and glutamate transporters are present in the hippocampus at birth (Furuta et al., 1997; Ullensvang et al., 1997; Yan et al., 1997; Gadea and Lopez-Colomé, 2001), but their relative efficacy has not been studied. At birth, in the presence of TTX and glutamate receptor antagonists, currents generated by pressure ejections of GABA (100 μM) were not sensitive to SKF899756A (50–100 μM) (Figures 4A and 4D), an irreversible and powerful blocker of GAT-1 (Isaacson et al., 1993; Borden et al., 1994), whereas it prolonged the decay of GABA currents at P14 (Figures 4B and 4D). SKF89976A also had no action on the isolated GABA component of ESC (Figure 5B). At birth, DL-TBOA (100 μM), which blocks the three main glutamate transporters EAAT1-3 (Shimamoto et al., 1998), reversibly prolonged the decay of the current generated by pressure ejections of glutamate (Figures 4C and 4D). TBOA also prolonged the decay of the isolated NMDA component of ESC (Figures 5A and 5B) and reversibly increased its amplitude by 46.2% ± 14.6% (4 out of 5 cells). In addition, TBOA generated a current mediated by NMDA receptors either in the presence (2.3 ± 1.4 pA/pF, n = 5; Figure 4C) or the absence (2.05 ± 0.9 pA/pF, in 3 silent and 2 active neurons) of TTX (Figure 5A). Thus, EAATs but not GAT-1 operate at early stage of development. This would limit the spatial domain of glutamate and facilitate the diffuse action of GABA in CA1.

ESC and Tonic Currents Do Not Require the Activation of Voltage-Dependent Na^+ and Ca^{2+} Channels

We next examined if transmitters that mediate ESC and tonic currents are released through a conventional action potential and a Ca^{2+}-dependent mechanism. Applications of TTX (1 μM) and replacement of Ca^{2+} by an equimolar concentration of Mg^{2+} and addition of Cd^{2+} (200 μM) to block voltage-dependent Ca^{2+} channels fully blocked conventional PSCs but only reduced ESC by 25% ± 8%. As in control conditions, the remaining ESC was mediated by GABA and glutamate receptors (n = 9/9 cells) (Figure 5C). Furthermore, in TTX and Ca^{2+}-free/Cd^{2+} solution, both $GABA_A$ receptors antagonists and TBOA still generated a current (0.82 ± 0.16 pA/pF, n = 7/9 cells, and 2.35 ± 0.5 pA/pF, n = 5/5 cells, with bicuculline and TBOA, respectively, at Vh = 30mV). All together, these data suggest a dual mechanism of GABA and glutamate release that contributes to the generation of ESC: a conventional Ca^{2+}-dependent vesicular release that provides roughly a quarter of the current, and a Na^+- and Ca^{2+}-independent mechanism responsible for most of the current. A contribution of vesicular mechanisms is suggested by currents generated by pressure ejection of an hyperosmotic solution (ACSF + 500 mM sucrose), which stimulates vesicular exocytosis (Rosenmund and Stevens, 1996; Capogna et al. 1997). As

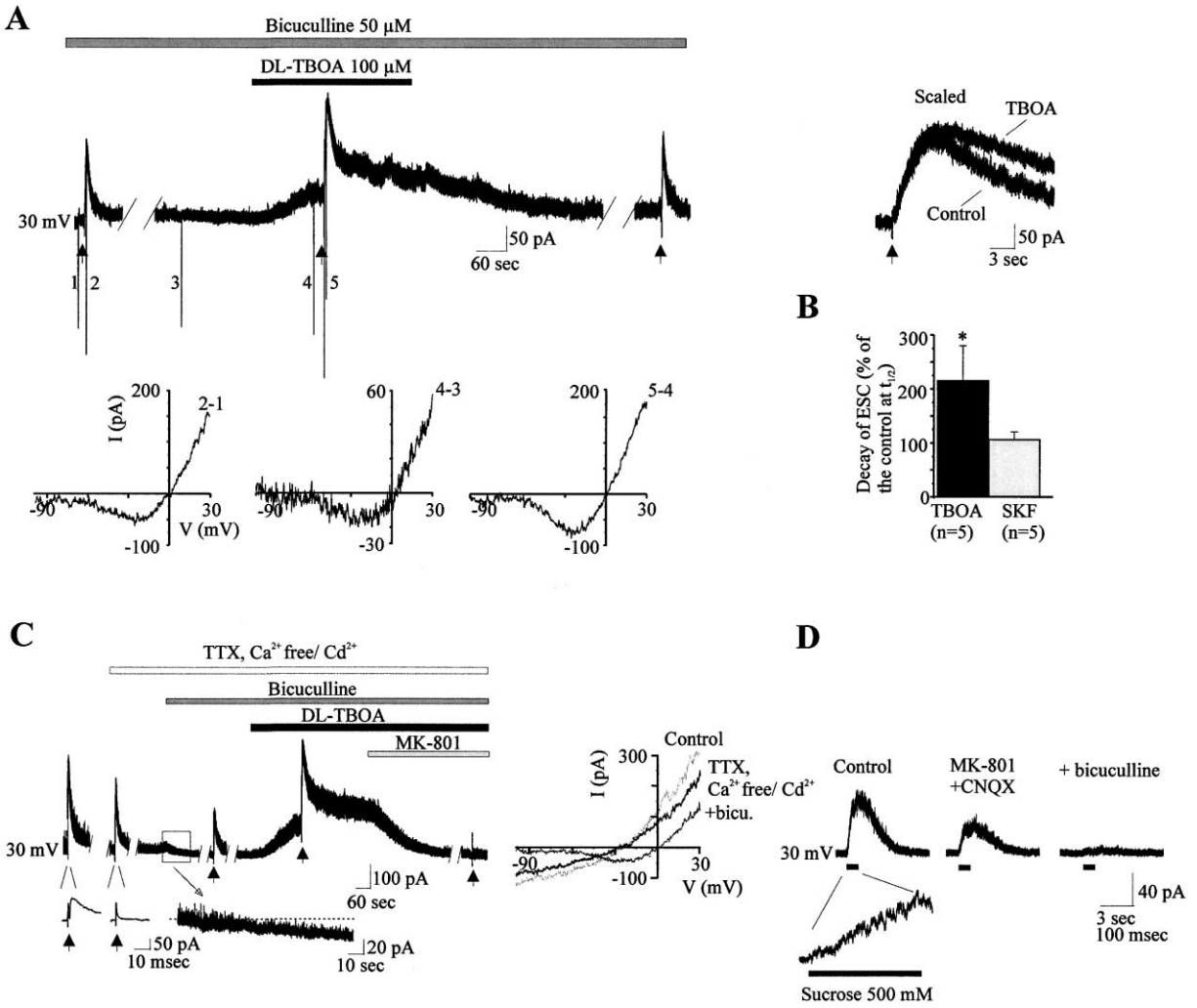

Figure 5. ESC and Tonic Currents Persist in the Absence of Action Potential and Ca^{2+} Influx

(A) P0 synaptically silent pyramidal neuron (Cm, 14 pF) recorded with a CsGlu-filled pipette solution. DL-TBOA potentiates the isolated glutamate component (NMDA) of ESC, slows down its decay, and unmasks a tonic current mediated by NMDA receptors. Individual ramps are indicated by numbers, and IV curves are shown below the traces (2-1, IV relation of the control ESC evoked in the presence of bicuculline; 4-3, IV relation of the current mediated by TBOA; 5-4, IV relation of ESC evoked under TBOA). Note that the IV relationship of the current induced by TBOA displays a region of negative slope, suggesting that it was mediated by NMDA receptors.

(B) Bar graph showing the effects of glutamate and GABA transporter inhibitors on the decay time of the isolated glutamate component of ESC and GABA component of ESC, respectively.

(C) P0 synaptically active pyramidal neuron (Cm, 22 pF) recorded with a CsGlu-filled pipette solution. Left part: TTX and Ca^{2+}-free/Cd^{2+} solution reduces but does not block the dual GABA and NMDA components of ESC nor tonic currents generated by bicuculline and TBOA. Bicuculline still produces an inward current associated with a decrease in the basal noise. TBOA (100 μM) produces an outward current. Both ESC and the current evoked by TBOA are abolished by MK-801. Traces below show at a higher time scale and higher magnification PSCs before and after addition of the voltage-dependent Na^+ and Ca^{2+} channels blockers and the current produced by bicuculline. Right part: I/V relation of ESC in control, in TTX and Ca^{2+}-free/Cd^{2+} solution, and after application of bicuculline (50 μM).

(D) Synaptically silent pyramidal neuron (Cm, 13 pF) recorded at 30mV with a pipette filled with CsGlu. Pressure ejection of sucrose (500 mM, 1 psi, 1 s) generates an outward current that is blocked by $GABA_A$ and glutamate receptors antagonists.

shown in Figure 5D, sucrose generated in silent cells a GABA and glutamate receptor-mediated current with a mean amplitude of 95 ± 12.6 pA (n = 4/7) that lasted 6.4 ± 2.6 s (mean time of sucrose application, 780 ± 200 ms).

A SNARE Complex-Independent Release of Transmitters Contributes to the Generation of ESC and Tonic Currents

We then determined whether a vesicular mechanism underlies the generation of the principal component of the ESC and the tonic currents. Botulinium toxin (BoT/A) cleaves SNAP-25, a SNARE protein, and prevents vesicular exocytosis (Capogna et al., 1997; Schiavo et al., 2000). Incubation of P0 hippocampal slices for 15–24 hr with BoT/A (100 ng/ml) affected neither the gross microscopy of slices nor the morphology of intracellular filled neurons (Figures 6A–6C), but effectively blocked fast synaptic transmission. Thus, after the incubation, large neurons (Cm > 15 pF and an apical dendrite reaching at least the stratum radiatum) had neither spontaneous nor evoked PSCs (28 neurons recorded in 24 slices),

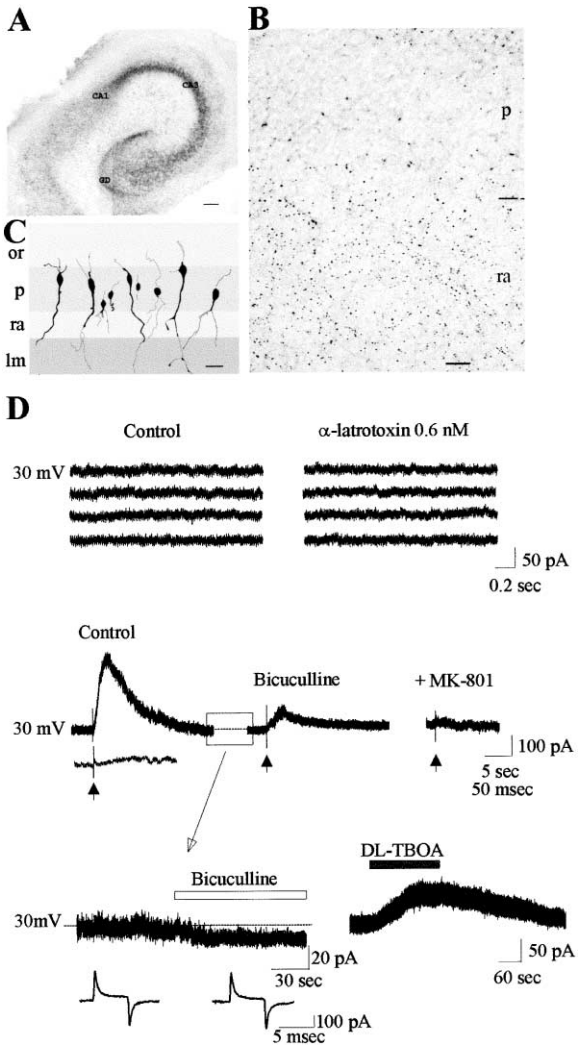

Figure 6. ESC and Tonic Currents Persist in Botulinium Treated Slices

(A) P0 organotypic slice cultured for 24 hr with botulinium toxin (BoT/A, 100 ng/ml) and immunostained with the neuronal marker NeuN. Note that the general organization of the hippocampus is maintained.

(B) Same procedure as before, but the slice was immunostained with synapsin-1. Punctate staining of terminal synaptic boutons is present in the stratum radiatum but not in the stratum pyramidale, as described before in normal hippocampal sections from newborn rats (Tyzio et al., 1999).

(C) Camera lucida reconstruction of biocytin-stained pyramidal neurons from slices treated with botulinium toxin; their shape and morphology were not apparently affected by such treatment.

(D) Recording of a pyramidal neuron (Cm, 26 pF) with a CsGlu-filled pipette solution from a slice treated with botulinium toxin for 15 hr. α-latrotoxin (0.6 nM, applied for 180 s) fails to generate fast synaptic events, indicating that botulinium efficiently blocked the exocytosis. Electrical stimulation evokes ESC (thick trace) but not PSC (thin trace at the highest time scale) that is sensitive to $GABA_A$ and NMDA receptors antagonists. The two traces below showed currents generated by bicuculline and TBOA. The current generated by bicuculline is not asssociated with a change in Rs (14 mOhm before and during the tonic current). The baseline is indicated by a dashed line. Scale bars, 100 μm in (A) and 25 μm in (B) and (C).

even in the presence of latrotoxin (Figure 6D; n = 7). In contrast, electrical stimulation of stratum radiatum evoked an ESC that was blocked by $GABA_A$ and NMDA receptor antagonists. Furthermore, application of $GABA_A$ receptor antagonists and DL-TBOA still generated a current (0.50 ± 0.1 pA/pF, n = 7/17 cells, and 1.34 ± 0.39 pA/pF, n= 6/7 cells, with $GABA_A$ receptors antagonists and TBOA, respectively) (Figure 6D).

A second approach relied on the use of Munc18-1-deficient mice in which the deletion of this protein leads to a complete loss of neurotransmitter secretion from synaptic vesicles (Verhage et al. 2000). Since homozygote mutant mice die at birth, we recorded slices obtained from E18.5 embryos. They have a phenotype easily distinguishable from normal or heterozygote littermates since they are in a tucked position, have a smaller size, are paralyzed, with no spontaneous movement or sensorimotor reflex, and have an external edematous appearance (Figure 7A). Munc18 immunohistochemistry of slices used for electrophysiology confirmed post hoc the absence of this protein in the selected mice (Figure 7B). In the mutant, the morphology of CA1 pyramidal neurons was not altered, with just an anlage of apical dendrite or dendrites reaching the stratum radiatum (Figure 7C). In agreement with Verhage et al. (2000), spontaneous and evoked fast PSCs were never observed, but electrical stimulation evoked an ESC (n = 11 cells; 4 slices), and spontaneous slow currents were also observed in four cells (71.8 ± 21 pA of amplitude, 3.1 ± 0.8 s of duration, 1–3 slow events during 30 min recording; Figures 7D and 7E). In addition, in four out of eight cells, bicuculline generated a current (0.49 ± 0.07 pA/pF), indicating that these neurons exhibit a tonic activity mediated by $GABA_A$ receptors (Figure 7F).

All together, these data suggested that transmitters that mediate ESC and tonic currents have either a nonvesicular origin or are released through a SNARE complex-independent exocytosis.

Discussion

Our results suggest that immature neurons—including neurons that have no operative synapses—communicate by diffusion of GABA and, to a lesser extent, glutamate that generate slow and tonic currents. ESCs are very different from conventional PSCs because of: (1) their several orders of magnitude longer kinetics; (2) their generation by a mechanism that does not involve Ca^{2+} influx and SNARE machinery; and (3) their restriction to an early developmental stage. In adults, currents equivalent to ESCs are not observed, even when transporter inhibitors are applied (data not shown). We suggest that this mechanism of release together with the absence of a functional GAT-1 transporter (in contrast to glutamate transporters) enables diffusion of large concentrations of GABA in the extracellular space and an intercellular communication prior to the formation of synapses. These observations are an additional element in favor of the central role of GABA as the principal excitatory transmitter during early stages of development.

Sequential Functional Expression of Transporters Reflects the Predominant Role of GABA

In resting conditions, antagonists of $GABA_A$ but not glutamate receptors generated a current, suggesting that a tonic release of GABA activates immature pyramidal

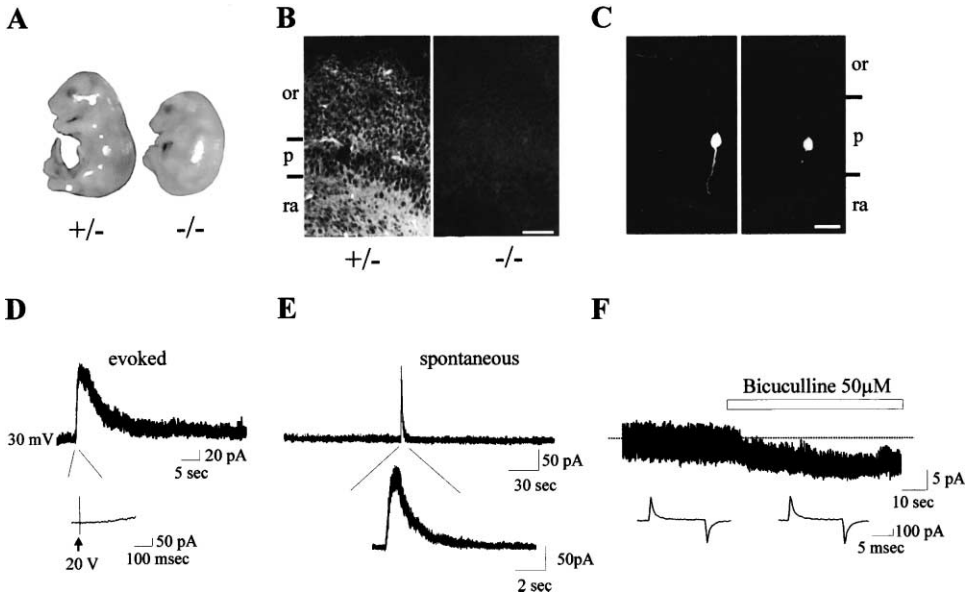

Figure 7. GABA Tonic Current and ESC Are Observed in Munc18-1-Deficient Mice

(A) The characteristic gross morphology of the homozygote Munc18-1 embryo (right) as compared with a normal litermate (left). The size is smaller and it presents a characteristic tucked position.

(B) Munc18 immunohistochemistry on slices from a heterozygote (left) and KO littermates (right). Bar scale, 50 μm.

(C) Confocal pictures of two recorded cells from KO mice filled with microruby. Left: cell with apical dendrite in the stratum radiatum. Right: cell with just an anlage of apical dendrite. Bar scale, 20 μm.

(D–F) Electrophysiological recordings from KO mice. Electrical stimulation (20V, 40 μs duration) evokes ESC but not PSC (D). Spontaneous slow current in a 5 min recording (E). Current produced by bicuculline (F). This is associated with a decrease in the basal noise. Rs does not change (18 mOhm before and during the application of bicuculline).

neurons (but also see Birnir et al. 2000). The differential functional expression of GABA and glutamate transporters in immature animals underlies this preferential contribution of $GABA_A$ receptors in both tonic current and ESC. Indeed, blockade of the principal GABA transporter GAT-1 did not slow the decay of the GABA component of ESC and the currents generated by exogenous application of GABA, suggesting that GAT-1, though expressed in the hippocampus by this period of life (although at moderate levels in the CA1 region) (Yan et al., 1997), does not contribute to the clearance of GABA from the extracellular space. This is age dependent, as SKF89976A strongly prolonged the exogenous GABAergic current in more mature pyramidal cells (P14). These results are in keeping with the fact that tiagabine, another GAT-1 transporter inhibitor, unravels a $GABA_B$ receptor-dependent paired pulse depression in adult but not neonatal CA3 pyramidal neurons (Caillard et al., 1998). Thus, the absence of an efficient extruding system will enable a sufficient concentration of GABA to exert its distal action. In contrast, antagonists of glutamate receptors did not generate a current unless a blocker of transporters was applied, suggesting that an efficient glutamate transport system is operative at an early developmental stage. In keeping with this, glutamate transporters are present in the neonatal hippocampus (specifically EAAT1; Furuta et al., 1997), and DL-TBOA generates a NMDA receptor-mediated current (see also Jabaudon et al. 1999), slows down the decay of exogenous glutamate receptor-mediated current, and increases the amplitude and the duration of the isolated glutamate component of ESC. DL-TBOA is a nontransportable inhibitor that circumvents the problem of heteroexchange (Shimamoto et al., 1998) and does not activate directly NMDA receptors (Jabaudon et al., 1999). Therefore, there is a tonic release of glutamate, but this is efficiently removed from the extracellular space by transporters. Only NMDA receptors contribute to ESC and to the tonic current unmasked by TBOA. Further studies are required to determine whether the activation of NMDA but not AMPA receptors is solely due to differences in affinity for glutamate between these receptors or to other mechanisms, including desensitization or a need for clustering of AMPA receptors that may take place at a later stage.

Source of Transmitter Release

The kinetics of ESC may result from several factors, including the distance between the source and the target, the time course of transmitter release, the coefficient of diffusion, and the receptor properties (Rusakov and Kullmann, 1998). The observations that ESC has a similar kinetics in both silent and active neurons as well as that ESC mediated by NMDA receptors is observed at E18 at a time when glutamate EPSCs are not present (Hennou et al., 2002) suggest that elements that are in the proximity but not in a close contact to the pyramidal neurons constitute a likely source of GABA and glutamate. These elements include GABAergic and glutamatergic growth cones (axonal, dendritic), which are present in embryos (see Introduction) and/or astrocytes

as they release glutamate and GABA that may diffuse and activate neurons (Liu et al., 2000; Parri et al., 2001).

Nonconventional Release of Transmitters Provides a Substantial Component of Communication between Immature Neurons

The following observations suggest that a significant component of ESC and tonic currents originate from transmitters released in a Ca^{2+}- and SNARE-independent manner: (1) both are observed in the presence of voltage-dependent Na^+ and Ca^{2+} channel blockers; (2) both persisted in slices treated with botulinium toxin, which prevents the SNARE complex formation required for the fusion of synaptic vesicles to the membrane (Schiavo et al., 2000); and (3) both were recorded in slices from Munc18-1-deficient mice in which vesicular release has been completely abolished (Verhage et al., 2000). This suggests that these transmitters have either a nonvesicular origin or arise from vesicles that do no use the conventional SNARE machinery for exocytosis.

A nonvesicular release of transmitters has been observed in several preparations and generally is thought to occur via the reversion of transporters (Schwartz, 1987; Taylor and Gordon-Weeks, 1991, Gaspary et al., 1998) or through exchanger (Warr et al., 1999). A reversion of transporters is clearly not involved in the generation of ESC and tonic currents since GAT-1 does not contribute to GABA clearance at this early developmental stage, and glutamate transporter blockade increases the ESCs and unmasks an NMDA tonic current. Release from exchanger, like the cystine/glutamate exchanger, is unlikely since it is not detected in the hippocampus by in situ hybridization (Sato et al., 2002). It may be, however, that other nonvesicular mechanisms may contribute to the currents, such as transmitter leakage like that described for acetylcholine at the neuromuscular junction (Katz and Miledi, 1977) or for ATP in cortical astrocytes through connexin-hemichannel (Cotrina et al., 1998). Nevertheless, there is also evidence in several preparations that Ca^{2+} is not fully required for vesicular exocytosis (Mochida et al., 1998; Tse and Tse, 2000; Zhang and Zhou, 2002). Interestingly, in DRG neurons, tetanus toxin, which cleaves synaptobrevin, did not completely abolish a calcium-independent, voltage-dependent exocytosis (Zhang and Zhou, 2002). This raises the possibility that some vesicles fuse with the plasma membrane without the requirement of Ca^{2+} and SNARE complex formation. Further studies will be required to define the cellular mechanism whereby transmitters that generate ESC and tonic currents are released.

Concluding Remarks

Our data demonstrate that in the CA1 region of the embryonic and newborn hippocampus, the absence of synapses does not necessarily imply an absence of communication between developing neurons through transmitters. Adjacent neurons may interact through the release of transmitters acting in a paracrine manner in the absence of action potential and Ca^{2+} influx. This communication, primarily mediated by GABA, may be particularly important at early stages of development, when networks are still maturing and voltage-dependent Na^+ and Ca^{2+} channels are not fully expressed by neurons. The activation by glutamate occurs only when the release is sufficiently high to overwhelm the action of glutamate transporters. Because GABA is depolarizing during this period of life and promotes a rise in $[Ca^{2+}]_i$ (Leinekugel et al. 1995; Owens and Kriegstein 2002; Ben-Ari, 2001, 2002), the paracrine activation may favor the morphological development of synaptically silent neurons (Barbin et al., 1993) and accelerate the expression of the KCC2 transporters, which maintains low $[Cl^-]_i$ (Rivera et al., 1999; Ganguly et al., 2001). On more mature cells, this could also affect the efficacy of newly formed GABAergic and glutamatergic synapses, which are known to be modulated by a rise in $[Ca^{2+}]_i$ (McLean et al., 1996; Sjostrom and Nelson, 2002).

In a more general perspective, it is interesting to stress that transmitters have been shown to exert a wide range of actions on the developing nervous system at a time when synapses are not present (see for review Nguyen et al., 2001). This has, however, been questioned by the observation that the formation of the major brain structures is normal in mice in which vesicular release is abolished in Munc18-1-deficient mice (Verhage et al., 2000). However, since a substantial part of the release of GABA and glutamate is made by a Ca^{2+}- and SNARE-independent mechanism (see also Flint et al., 1998), transmitters may still play a role at an early stage of maturation, acting at distal sites as diffusible signaling molecules.

Experimental Procedures

Experiments were performed on CA1 pyramidal neurons in hippocampal slices from Wistar rats obtained from embryonic day 18 (E18) to postnatal day 30 (P30). Some experiments were also performed on hippocampal slices from homozygous E18.5 Munc18-1-deficient mice (a kind gift by Dr. T.C. Südhof, University of Texas Southwestern Medical Center, Dallas, Texas) (Verhage et al., 2000)

Electrophysiology

Pregnant Wistar rats at E18–E22 days of gestation were anaesthetized with chloral hydrate (7%), and embryos were exposed by cesarean. Animals were decapitated and brains rapidly removed and placed in oxygenated ice-cooled artificial cerebrospinal fluid (ACSF) with the following composition: 126 mM NaCl, 3.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM $NaHPO_4$, 10 mM glucose (95% O_2 and 5% CO_2 [pH 7.4]). The hippocampus was isolated, and transverse slices (350–400 μm) were obtained with a vibratome and kept in oxygenated ACSF at room temperature at least 1 hr before use. Individual slices were then transferred to the recording chamber where they were fully submerged and superfused with ACSF at 30°C–32°C at a rate of 2–3 ml/min. Postnatal rats were also anaesthetized with chloral hydrate, and a similar procedure as for embryos was used.

A similar procedure was used for heterozygous pregnant female mice (C57BL/6) at E18.5 days of gestation. In homozygous Munc18-1-deficient embryos, cells with $Cm > 15$ pF had neither spontaneous nor evoked by electrical stimulation fast synaptic events, confirming the lack of synaptic release. Adjacent slices to that selected for physiology were stained with Munc18 antibodies to confirm post hoc the absence of Munc18-1 protein.

CA1 pyramidal cells were recorded under visual control with an axioscop Zeiss or a Leica DM LFS microscope using patch-clamp technique in the whole-cell configuration. Microelectrodes had a resistance of 5–10 mOhm and were filled, in the majority of the experiments, with a solution of the following composition: 120 mM CsGlu, 10 mM CsCl, 0.4 mM $CaCl_2$, 1.1 mM EGTA, 10 mM HEPES, 4 mM Mg^{2+}ATP, 0.3 mM Na^+GTP (pH 7.25); 270–280 mOsm. Some

experiments were performed with a CsCl- or CsF-filled pipette solution of the following composition: 120 mM CsCl, 10 mM CsGlu, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 10 mM HEPES, 4 mM Mg^{2+}ATP, 0.3 mM Na^+GTP or 120 mM CsF, 10 mM CsCl, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 10 mM HEPES, 4 mM Mg^{2+}ATP, 0.3 mM Na^+GTP. Biocytine (0.5%–0.8%) or microruby was added to these solutions. Liquid junction potentials for all solutions were measured (Neher, 1992), and all voltages reported are corrected values. Slices were stimulated by a bipolar twisted nichrome electrode or in some experiments by a theta patch pipette filled with ACSF placed in the stratum radiatum of CA1 at the border of the stratum pyramidale. Stimulus intensity ranged between 10–90V of amplitude and 40 μs duration (bipolar twisted nichrome electrode) or 10–30V and 200 μs (patch pipette electrode).

Whole-cell measurements in voltage-clamp or current-clamp mode were filtered at 3 kHz using an EPC-9 amplifier (HEKA). All data were digitized (1–2 kHz) with a Labmaster (Axon Instruments) interface card to a personal computer and analyzed with Acquis 1 program (G. Sadoc, Biologic). To obtain the current voltage-curve (I/V), cells were maintained at 30mV, and voltage ramps were applied for 0.5–1 s from 30 to −100mV or to −75mV. In some experiments, voltage ramps were applied from −75 to 30mV. The IV curves of the stimulation-evoked slow current or NMDA-evoked current were constructed by subtracting the ramp response in control from that during the maximal current response. The capacitance and series resistances were monitored by the EPC-9 pulse fit software. The series resistance ranged between 10 and 30 mOhm and was not allowed to vary more than 20%.

Organotypic Slice Culture

Organotypic slices where prepared from hippocampi of P0 male Wistar rats according to the procedure described by Stoppini et al. (1991) with minor changes. Briefly, pups were cryoanesthetized and decapitated. Hippocampal sections, 400 μM thick, were prepared using a vibratome and placed onto Millicell-CM culture inserts (0.4 μm, five slices per insert; Millipore) with 1 ml defined medium: Neurobasal (GIBCO), B27 (GIBCO), L-Glutamine (3 mM). For botulinium toxin treatment, 100 ng/ml of BoT/A was added to the media, and 10 μl of the toxin (100 ng/ml) was applied directly on the slices. Cultures were maintained for 12–24 hr in a cell culture incubator at 37°C and then transferred to the recording chamber for electrophysiology. Vesicular exocytosis was considered to be blocked by botulinium toxin when cells with Cm > 15 pF have neither spontaneous nor evoked by electrical stimulation or α-latrotroxin (0.6 nM, applied for 90–180 s) fast synaptic events.

Immunocytochemistry

Slices where fixed by immersion for 1 hr at room temperature in 4% paraformaldehyde. After rinsing and a 30 min incubation in 2% normal goat serum in PBS containing 0.3% Triton X-100, the slices were incubated overnight at 4°C with monoclonal antibodies to NeuN (1/10000, Sigma) or synaptophysine (1:500, Chemicon) or polyclonal antibodies to Synapsin-1 (1:1000, Chemicon) or Munc18 (1:1000; Synaptic systems, Göttingen, Germany). Antigen-antibody complexes were revealed using the avidin-biotin-peroxydase procedure (ABC, Vectastain Elite; Vector Labs) or fluorochrome-conjugated secondary antibodies (Jackson Immunoresearch).To reveal biocytine-injected cells, slices were incubated overnight at 4°C in avidin-biotin-peroxydase and then processed as described before. Stained cells were reconstructed using a camera lucida. In some cases, microruby was used to fill recorded cells that were directly observed in a confocal Olympus microscope.

Statistical Analysis

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

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References

Barbin, G., Pollard, H., Gaiarsa, J.L., and Ben-Ari, Y. (1993). Involvement of $GABA_A$ receptors in the outgrowth of cultured hippocampal neurons. Neurosci. Lett. *152*, 150–154.

Ben-Ari, Y. (2001). Developing networks play a similar melody. Trends Neurosci. *24*, 353–360.

Ben-Ari, Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. Nature Rev. Neurosci. *3*, 728–738.

Birnir, B., Everitt, A.B., Lim, M.S.F., and Gage, P.W. (2000). Spontaneously opening $GABA_A$ channels in CA1 pyramidal neurones of rat hippocampus. J. Membr. Biol. *174*, 21–29.

Borden, L.A., Murali-Dhar, T.G., Smith, K.E., Weinshank, R.L., Branchek, T.A., and Gluchowski, C. (1994). Tiagabine, SKF89976A, CI *966*, NNC-711 are selective for the cloned GABA transporter GAT-1. Eur. J. Pharmacol. *269*, 219–224.

Caillard, O., McLean, H., Ben-Ari, Y., and Gaiarsa, J.L. (1998). Ontogenesis of presynaptic $GABA_B$ receptor-mediated inhibition in the CA3 region of the hippocampus. J. Neurophysiol. *79*, 1341–1348.

Capogna, M., McKinney, A., O'Connor, V., Gähwiler, B., and Thompson, S.M. (1997). Ca^{2+} and Sr^{2+} partially rescue synaptic transmission in hippocampal cultures treated with botulinium toxin A and C, but not tetanus toxin. J. Neurosci. *17*, 7190–7202.

Cotrina, M.L., Lin, J.H.C., Alves-Rodrigues, A., Liu, S., Li, J., Azmi-Ghadimi, H., Kang, J., Naus, C.C.G., and Nedergaard, M. (1998). Connexins regulate calcium signaling by controlling ATP release. Proc. Natl. Acad. Sci. USA *95*, 15735–15740.

Diabira, D., Hennou, S., Chevassus-au-Louis, N., Ben-Ari, Y., and Gozlan, H., (1999). Late embryonic expression of AMPA receptor function in the CA1 region of the intact hippocampus in vitro. Eur. J. Neurosci. *11*, 4015–4023.

Dupuy, S.T., and Houser, C.R. (1996). Prominent expression of two forms of glutamate decarboxylase in the embryonic and early postnatal rat hippocampal formation. J. Neurosci. *16*, 6919–6932.

Flint, A.C., Liu, X., and Kriegstein, A.R. (1998). Nonsynaptic glycine receptor activation during early neocortical development. Neuron *20*, 43–53.

Furuta, A., Rothstein, J.D., and Martin, L.J. (1997). Glutamate transporter protein subtypes are expressed differentially during rat CNS development. J. Neurosci. *17*, 8363–8375.

Gadea, A., and Lopez-Colomé, A.M. (2001). Glial transporters for glutamate, glycine, and GABA: II. GABA transporters. J. Neurosci. Res. *63*, 461–468.

Ganguly, K., Schinder, A.F., Wong, S.T., and Poo, M.M. (2001). GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell *105*, 521–532.

Gao, X.B., and Van den Pol, A.N. (2000). GABA release from mouse axonal growth cones. J. Physiol. *523*, 629–637.

Gaspary, H.L., Wang, W., and Richerson, G.B. (1998). Carrier mediated GABA release activates GABA receptors on hippocampal neurons. J. Neurophysiol. *80*, 270–281.

Haas, K., Sin, W.C., Jahaherian, A., Li, Z., and Cline, H.T. (2001). Single cell electroporation for gene transfer in vivo. Neuron *29*, 583–591.

Hall, Z.W., and Sanes, J.R. (1993). Synaptic structure and development: the neuromuscular junction. Cell Suppl. *72*, 99–121.

Hennou, S., Khalilov, I., Ben-Ari, Y., and Gozlan, H. (2002). Early sequential formation of functional $GABA_A$ and glutamatergic synapses on CA1 interneurones of the rat fœtal hippocampus. Eur. J. Neurosci. *16*, 197–208.

Isaacson, J.S., Solis, J.M., and Nicoll, R.A. (1993). Local and diffuse synaptic actions of GABA in the hippocampus. Neuron *10*, 165–175.

Jabaudon, D., Shimamoto, K., Yasuda-Kamatani, Y., Scanziani, M.,

Gähwiler, B., and Gerber, U. (1999). Inhibition of uptake unmasks rapid extracellular turnover of glutamate of nonvesicular origin. Proc. Natl. Acad. Sci. USA *96*, 8733–8738.

Katz, B., and Miledi, R. (1977). Transmitter leakage from motor nerve endings. Proc. R. Soc. London B Biol. Sci. *196*, 59–72.

Komuro, H., and Rakic, P. (1993). Modulation of neuronal migration by NMDA receptors. Science *260*, 95–97.

Leinekugel, X., Tseeb, V., Ben-Ari, Y., and Bregestovski, P. (1995). Synaptic $GABA_A$ activation induces Ca^{2+} rise in pyramidal cells and interneurons from rat neonatal hippocampal slices. J. Physiol. *487*, 319–329.

Leinekugel, X., Medina, I., Khalilov, I., Ben-Ari, Y., and Khazipov, R. (1997). Ca^{2+} oscillations mediated by the synergistic excitatory actions of $GABA_A$ and NMDA receptors in the neonatal hippocampus. Neuron *18*, 243–255.

Liu, Q.Y., Schaffner, A.E., Chang, Y.H., Maric, D., and Barker, J.L. (2000). Persistent activation of $GABA_A$ receptor/Cl^- channels by astrocyte-derived GABA in cultured embryonic rat hippocampal neurons. J. Neurophysiol. *84*, 1392–1403.

Lo Turco, J.J., Blanton, M.G., and Kriegstein, A.R. (1991). Initial expression and endogenous activation of NMDA channels in early neocortical development. J. Neurosci. *11*, 792–799.

Lo Turco, J.J., Owens, D.F., Heath, M.J., Davis, M.B., and Kriegstein, A.R. (1995). GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron *15*, 1287–1298.

McLean, H., Caillard, O., Ben-Ari, Y., and Gaiarsa, J.L. (1996). Bidirectional plasticity expressed by GABAergic synapses in the neonatal rat hippocampus. J. Physiol. *496*, 471–477.

Mochida, S., Yokoyama, C.T., Kim, D.K., Itoh, K., and Catterall, W.A. (1998). Evidence for a voltage-dependent enhancement of neurotransmitter release mediated via the synaptic protein interaction site of N-type Ca^{2+} channels. Proc. Natl. Acad. Sci. USA *95*, 14523–14528.

Neher, E. (1992). Correction of liquid junction potentials in patch-clamp experiments. Methods Enzymol. *207*, 123–131.

Nelson, S., Toth, L., Sheth, B., and Sur, M. (1994). Orientation selectivity of cortical neurons during intracellular blockade of inhibition. Science *265*, 774–777.

Nguyen, L., Rigo, J.M., Rocher, V., Belachew, S., Malgrange, B., Rogister, B., Leprince, P., and Moonen, G. (2001). Neurotransmitters as early signals for central nervous system development. Cell Tissue Res. *305*, 187–202.

Owens, D.F., and Kriegstein, A.R. (2002). Is there more GABA than synaptic inhibition? Nature Rev. Neurosci. *3*, 715–726.

Parri, H.R., Gould, T.H., and Crunelli, V. (2001). Spontaneous astrocytic Ca^{2+} oscillations in situ drive NMDAR-mediated neuronal excitation. Nat. Neurosci. *4*, 803–812.

Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M., and Kaila, K. (1999). The K^+/Cl^- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature *397*, 251–255.

Rosenmund, C., and Stevens, C.F. (1996). Definition of the readily releasable pool of vesicles at hippocampal synapses. Neuron *16*, 1197–1207.

Rozenberg, F., Robain, O., Jardin, L., and Ben-Ari, Y. (1989). Distribution of GABAergic neurons in late fetal and early postnatal rat hippocampus. Brain Res. Dev. Brain Res. *50*, 177–187.

Rusakov, D.A., and Kullmann, D.M. (1998). Extrasynaptic glutamate diffusion in the hippocampus: ultrastructural constraints, uptake and receptor activation. J. Neurosci. *18*, 3158–3170.

Sato, H., Tamba, M., Okuno, S., Sato, K., Keino-Masu, K., Masu, M., and Bannai, S. (2002). Distribution of cystine/glutamate exchange transporter, system Xc^-, in the mouse brain. J. Neurosci. *22*, 8028–8033.

Schiavo, G., Matteoli, M., and Montecucco, C. (2000). Neurotoxins affecting neuroexocytosis. Physiol. Rev. *80*, 717–766.

Schwartz, E.A. (1987). Depolarization without calcium can release gamma-aminobutyric acid from a retinal neuron. Science *238*, 350–355.

Shimamoto, R., Lebrun, B., Yasuda-Kamatani, Y., Sakaitani, M., Shigeri, Y., Yumoto, N., and Nakayjima, T. (1998). DL-threo-beta-benzoloxyaspartate, a potent blocker of excitatory amino acid transporters. Mol. Pharmacol. *53*, 195–201.

Sjostrom, J., and Nelson, S.B. (2002). Spike timing, calcium signals and synaptic plasticity. Curr. Opin. Neurobiol. *12*, 305–314.

Soeda, H., Tatsumi, H., and Katayama, Y. (1997). Neurotransmitter release from growth cones of rat dorsal root ganglion neurons in culture. Neuroscience *77*, 1187–1189.

Stoppini, L., Buchs, P.A., and Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods *37*, 173–182.

Südhof, T.C. (2001). α-latrotoxin and its receptors: neurexins and CIRL/latrophilins. Annu. Rev. Neurosci. *24*, 933–962.

Super, H., and Soriano, E. (1994). The organization of the embryonic and early postnatal murine hippocampus. II. Development of entorhinal, commissural, and septal connections studied with the lipophilic tracer DiI. J. Comp. Neurol. *344*, 101–120.

Taylor, J., and Gordon-Weeks, P.R. (1991). Calcium independent GABA release from growth cones: role of GABA transport. J. Neurochem. *56*, 273–280.

Tse, F.W., and Tse, A. (2000). Stimulation of Ca^{2+}-independent exocytosis in rat pituitary gonadotrophs by G-protein. J. Physiol. *526*, 99–108.

Tyzio, R., Represa, A., Jorquera, I., Ben-Ari, Y., Gozlan, H., and Aniksztejn, L. (1999). The establishement of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. J. Neurosci. *19*, 10372–10382.

Ullensvang, K., Lehre, K.P., Storm-Mathisen, J., and Danbolt, N.C. (1997). Differential developmental expression of the two rat brain glutamate transporter proteins GLAST and GLT. Eur. J. Neurosci. *9*, 1646–1655.

Valeyev, A.Y., Cruciani, R.A., Lange, G.D., Smallwood, V.S., and Barker, J.L. (1993). Cl^- channels are randomly activated by continuous GABA secretion in cultured embryonic rat hippocampal neurons. Neurosci. Lett. *155*, 199–203.

Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., Van Den Berg, T.K., Missler, M., et al. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science *287*, 864–867.

Warr, O., Takahashi, M., and Attwell, D. (1999). Modulation of extracellular glutamate concentration in rat brain slices by cystine-glutamate exchange. J. Physiol. *514*, 783–793.

Xie, Z., and Poo, M.M. (1986). Initial events in the formation of neuromuscular synapse. Proc. Natl. Acad. Sci. USA *83*, 7069–7073.

Yan, X.X., Cariaga, W.A., and Ribak, C.E. (1997). Immunoreactivity for GABA plasma membrane transporter, GAT-1, in the developing rat cerebral cortex: transient presence in the somata of neocortical and hippocampal neurons. Brain Res. Dev. Brain Res. *99*, 1–9.

Zhang, C., and Zhou, Z. (2002). Ca^{2+}-independent but voltage-dependent secretion in mammalian dorsal root ganglion cells. Nat. Neurosci. *5*, 425–430.