Opposite Changes in Glutamatergic and GABAergic Transmission Underlie the Diffuse Hyperexcitability of Synapsin I–Deficient Cortical Networks

Synapsins (Syns) are synaptic vesicle (SV) phosphoproteins that play a role in synaptic transmission and plasticity. Mutation of the SYN1 gene results in an epileptic phenotype in mouse and man, implicating Syn1 in the control of network excitability. We used microelectrode array and patch-clamp recordings to study network activity in primary cortical neurons from wild-type (WT) or Syn1 knockout (KO) mice. SYN1 deletion was associated with increased spontaneous and evoked activities, with more frequent and sustained bursts of action potentials and a high degree of synchronization. Blockade of GABA A receptors with bicuculline attenuated, but did not completely abolish, the differences between WT and Syn1 KO networks in both spontaneous and evoked activities. Patch-clamp recordings on cortical autaptic neurons revealed a reduced amplitude of evoked inhibitory postsynaptic currents (PSCs) and a concomitantly increased amplitude of evoked excitatory PSCs in Syn1 KO neurons, in the absence of changes in miniature PSCs. Cumulative amplitude analysis revealed that these effects were attributable to opposite changes in the size of the readily releasable pool of SVs. The results indicate distinct roles of Syn1 in GABAergic and glutamatergic neurons and provide an explanation for the high susceptibility of Syn1 KO mice to epileptic seizures.

Keywords: epilepsy, GABA/glutamate release, knockout mice, network activity, synapsin

Introduction

In addition to mutations in specific subunits of voltage- and ligand-gated ion channels, a large number of potential genes involved in neural development, synaptogenesis and synaptic transmission and whose mutation may underlie epilepsy have been uncovered (Noebels 2003; Steinlein 2004). Thus, the large protein families operating in synaptic vesicle (SV) trafficking and exo-endocytosis are potentially implicated in epilepsy. Although a large number of these genes have been inactivated in animal models, only few mutants exhibit an epileptic phenotype, namely knockout (KO) mice lacking members of the synapsin (Syn) and SV2 families of SV proteins. Interestingly, a form of familial epilepsy characterized by a non-sense mutation in the SYN1 gene present in all affected family members was reported (Garcia et al. 2004) and a recent case-control study for genetic susceptibility loci in sporadic epilepsy identified SYN2 among the 5 genes whose variations contribute to disease predisposition (Cavalleri et al. 2007).

Syms are major SV-specific phosphoproteins that play multiple roles in synaptic transmission and plasticity (Hilfiker et al. 1999; Baldelli et al. 2006). In mammals, 3 SYN genes (SYN1/SYN2/SYN3) located in chromosomes X, 3 and 22, respectively, generate several differentially spliced isoforms. Syns control SV trafficking between the reserve pool (RP) and the readily releasable pool (RRP) through phosphorylation-dependent interactions with actin and SVs (Ceccaldi et al. 1995; Hosaka et al. 1999; Chi et al. 2001, 2003; Bonanomi et al. 2005; Menegon et al. 2006). Moreover, Syns play a role in the final postdocking steps of exocytosis including SV priming and fusion (Hilfiker et al. 1998, 2005; Humeau et al. 2001; Fassio et al. 2006; Hvalby et al. 2006; Sun et al. 2006; Baldelli et al. 2007).

In excitatory synapses, SYN1 deletion reduced the size of the RP of SVs (Li et al. 1995; Takei et al. 1995; Ryan et al. 1996; Siksou et al. 2007) and altered short-term plasticity (Rosahl et al. 1993, 1995; Gitler et al. 2004; Hvalby et al. 2006; Kielland et al. 2006; Sun et al. 2006). On the other hand, inhibitory synapses lacking Syn1 showed a decrease in the amplitude of evoked inhibitory postsynaptic currents (eIPSCs) due to a decrease in the RRP of SVs (Terada et al. 1999; Baldelli et al. 2007). Distinct effects of SYN deletion on excitatory and inhibitory neurons have been observed in triple Syn1/II/III KO mice, with increased rate of depression, but normal basal transmission in glutamatergic synapses and decreased basal transmission, but normal kinetics of depression in GABAergic (γ-aminobutyric acidergic) synapses (Gitler et al. 2004).

These deficits in synaptic transmission and the high susceptibility to spontaneous and evoked epileptic seizures exhibited by Syn KO mice (Li et al. 1995; Rosahl et al. 1995; Gitler et al., 2004) suggest an involvement of the Syns in the delicate balance between inhibitory and excitatory transmission that controls cortical excitability.

In this study we analyzed the excitability and network behavior of primary cortical neurons from WT and Syn KO mice grown onto microelectrode arrays (MEAs) as well as the electrophysiological properties of excitatory and inhibitory neurons by patch-clamp electrophysiology on cortical autaptic neurons. Mutant networks displayed highly synchronized spontaneous activity with intense bursting of action potentials and a prolonged firing probability after electrical stimulation. These changes were associated with a reduction in eIPSC amplitudes and a concomitant increase in eEPSC amplitudes. The unbalance between inhibitory and excitatory transmissions, by promoting the diffuse hyperexcitability and
synchronized bursting displayed by SynI KO networks, may directly contribute to the pathogenesis of the epileptic phenotype.

**Materials and Methods**

**Cell Cultures**

Offspring of littermates of WT and homozygous SynI KO mice (Chin et al. 1995) were used throughout. All experiments were carried out in accordance with the guidelines established by the European Communities Council (Directive of November 24, 1986) and the National Council on Animal Care and approved by the local Animal Care Committee of the University of Genova and adequate measures were always taken to minimize pain of discomfort. Mice were sacrificed by inhalation of CO₂, and 17- to 18-day embryos (E17–18) were removed immediately by cesarean section. Removal and dissection of cortex, isolation of neurons and culturing procedures were previously described (Chiappalone et al. 2006). Briefly, cerebral cortices were digested in 0.125% Trypsin for 20 min and then mechanically dissociated with a fire-polished Pasteur pipette. No antimitotic drugs were added to prevent glia proliferation.

**Cortical Cultures on MEAs**

Neurons were plated onto poly-D-lysine and laminin-coated MEA at the final density of 1200–2000 cells/mm². The cells were incubated with 1% Glutamax, 2% B-27 supplemented Neurobasal medium (Brewer et al. 1993), penicillin-streptomycin 1% (all from Invitrogen, Carlsbad, CA) in a humidified 5% CO₂ atmosphere at 37 °C. Every week a half-volume medium replacement was performed. Experiments were performed at various ages of in vitro development, ranging from the second to the fifth week after plating.

**Cortical Autaptic Cultures**

Autaptic neurons were prepared as described previously (Bekkers and Stevens 1991) with slight modifications. Briefly, dissociated neurons were plated at very low density (20 cells/mm²) on microdots (40–400 μm in diameter) obtained by spraying a mixture of poly-D-lysine (0.4 mg/ml) and collagen (0.25 mg/ml) on dishes which had been pretreated with 0.15% agaroze. Both glial cells and single autaptic neurons were present under this culture condition. Patch-clamp experiments were performed at 17–23 days in vitro (DIV).

**MEA Recording and Stimulation System**

Multisite extracellular recordings were performed using MEAs made up of 60 planar microelectrodes (TIN/SIN, 30 μm electrode diameter, 200 μm pitch) arranged over an 8 × 8 square grid (Multi Channel Systems [MCS], Reutlingen, Germany). After 1200× amplification (MCS MEA 1060), signals were sampled at 10 kHz using the MCS data acquisition card controlled by the MCS MCRack software for data monitoring, acquisition and storage. After recording, the raw signals obtained from each experiment were processed off-line by using custom software tools specifically developed in MATLAB (The Mathworks, Natick, MA) (see below). Electrical stimuli were delivered by using a commercial general-purpose stimulus generator (MCS STG 1008), signals were present under this culture condition. Patch-clamp experiments were performed at 17–23 days in vitro (DIV).

**MEA Experimental Protocols**

**Development**

Before each recording session, the culture medium was replaced by an extracellular solution containing (in mM): 150 NaCl, 1.3 CaCl₂, 0.7 MgCl₂, 2.8 KCl, 10 glucose, 10 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.4) (Chiappalone et al. 2006). The spontaneous activity was monitored and recorded for 20–30 min, starting 15-20 min after medium change to let the culture adapt to the new environment and reach a stable level of activity (Streit et al. 2001). Experiments were performed at various developmental stages (12-15, 18-20, 24-26, and 31-35 DIV).

**Pharmacology**

Pharmacological studies were performed on 24–35 DIV cultures. Spontaneous activity in physiological solution was recorded for 20 min (control condition). Then, networks were exposed to the GABA<sub>A</sub> receptor antagonist bicuculline (BIC, Tocris, Bristol, UK) added at the final concentration of 30 μM (Keefer and Gramowski 2001; Lin et al. 2002; Gramowski et al. 2004) and the recording session was continued for additional 20 min.

**Electrical Stimulation**

Four of the 60 electrodes of the array were probed with test stimuli for 20 min. Test stimuli were sent sequentially to each selected electrode at 0.2-Hz frequency. In all the experiments, probe pulse amplitude was fixed at 1.5–2.0 V peak-to-peak (Wagenaar et al. 2004). The stimulus pulse was biphasic (positive phase first) and lasted 500 μs with 50% duty cycle. Only electrodes recording electrical activity from the network were chosen as stimulation sites. Recordings of evoked activity were performed on 27-33 DIV cultures.

**Processing Techniques for MEA Recordings**

**Spike Detection**

Peak-to-peak thresholds (WT = 29.7 ± 0.42 μV [calculated for a subset of n = 4 cultures]; SynI KO = 27.9 ± 0.45 μV [calculated for a subset of n = 4 cultures], mean ± SEM, age range 27–33 DIV) were calculated as 8 times the standard deviation of the biological noise and were defined separately for each recording channel before the off-line processing of the experiments. Briefly, a sliding window, sized to contain, at most, one single-unit spike (i.e., 2–3 ms wide) was continuously moved over the recorded signal as long as the difference between the maximum and the minimum within the window was below the peak-to-peak threshold. When the difference exceeded the threshold, a spike was detected and its time-stamp was saved.

** Burst Detection**

Cortical networks show both random spiking activity and bursting behavior (Robinson et al. 1993; Opitz et al. 2002). A burst consists in a fast sequence of spikes, usually occurring simultaneously at many channels (i.e., network burst). Bursts consist of packages of spikes distributed over a range of a few milliseconds which generally last from hundreds of milliseconds up to seconds, and are separated by long quiescent periods. A previously developed algorithm was used to detect the presence of bursts and analyze their features (i.e., duration, rate; Chiappalone et al. 2005). The procedure is based on setting a minimum number of spikes within a single channel burst (i.e., Spikes<sub>burst</sub> ≥ 5) and a maximum interspike interval between consecutive spikes belonging to the same burst (i.e., ISIs<sub>burst</sub> = 200 ms). In addition, the burst detection procedure generated new time series for analysis, the burst event train, containing only the initial spikes of each burst (Cozzi et al. 2006). The channels showing a bursting rate lower than 0.4 burst/min were not included in the analysis.

**Cross-correlation**

The cross-correlogram function (Knox 1981; Salinas and Sejnowski 2001) is constructed from the spike trains recorded from 2 electrodes and measures the frequency at which one electrode (i.e., the “target”) fires as a function of time relative to the firing in the other electrode (i.e., the “reference”). In order to analyze the synchronization level of the networks, we built cross-correlograms on “burst events,” as previously described (Chiappalone et al. 2007). Briefly, given 2 trains (i.e., X and Y), recorded from 2 electrodes of the MEA, we counted the number of events in the Y train within a time frame of ± T (usually set at 150 ms) around the X event, by using bins Δt usually set at 1 ms. The cross-correlation function C<sub>XY</sub>(τ) was obtained by a normalization procedure, according to the following formula,

\[ C_{XY}(\tau) = \frac{1}{\sqrt{N_X N_Y}} \sum_{t=-\infty}^{+\infty} X(t) Y(t-\tau) \]

where \( \Delta t \) indicates the timing of an event in the X train, \( N_X \) and \( N_Y \) are the total number of events in the X and Y train, respectively, and \( \Delta t \) is the...
calculated as the ratio of the integral of a cross-correlation function in the number of bins) and is a measure of the synchronization level.

Responses of primary cortical neurons to high-frequency stimulations on the SV recycling rate in excitatory and inhibitory neurons when the 4 KCl, 10 glucose, 10 HEPES (pH 7.4). No significant effects were found (i.e., \( C_{xy}(t) = C_{xy}(t + \Delta t) \)) (Eytan et al. 2004). In particular, the cross-correlogram coefficient, \( C_{xy}(0) \)

\[
C_{xy}(0) = \frac{\sum_{t=1}^{n} C_{xy}(t)}{n}
\]

represents the area of the cross-correlogram around the zero bin (\( k \) is the number of bins) and is a measure of the synchronization level between the recording channels. From Equation (2), the Coincidence Index (CI) (Jimbo et al. 1999; Tateno and Jimbo 1999) can be calculated as the ratio of the integral of a cross-correlation function in a specified area around zero (i.e., \( C_{xy}(0) \)) to the integral of the total area, according to

\[
CI = \frac{\sum_{t=1}^{n} C_{xy}(t)}{\sum_{t=1}^{n} C_{xy}(t)}
\]

To get qualitative information on how the activity of one electrode \( x \) is correlated to the activity of all the other electrodes \( y \) (with \( y \neq x \)), we defined the mean correlogram, according to the definition below:

\[
C_{xy}(t) = \frac{1}{n-1} \sum_{t=1}^{n} C_{xy}(t)
\]

for \( x \neq y \) and \( 1 \leq t < n \), \( n = 60 \). This function can be plotted in an 8 x 8 square array in order to see, at the same time, how each channel is globally correlated to all the others.

**Poststimulus Time Histogram**

When stimulating currents/voltages are delivered through one electrode of the array at a constant frequency, the network responds by generating a rich repertoire of reverberating electrical activities lasting 100-200 ms (Shahaf and Marom 2001). Thus, to investigate the neuronal activity evoked by stimulation, we computed the poststimulus time histogram (i.e., PSTH), which represents the impulse response of each site of the experimental preparation to the electrical stimulation. The PSTHs for each electrode were calculated by recording the spiking activity over a 600-ms period after each stimulus (i.e., generally 50 stimuli, delivered from one stimulating channel). Then, the number of spikes occurring in each 4-ms bin was calculated to generate a cumulative histogram which was subsequently normalized by the total number of stimuli and the bin size (Rieke et al. 1997).

**Patch-Clamp Recordings, Data Acquisition, and Analysis**

Whole-cell patch-clamp recordings were made from autaptic neurons grown on microislands, as described (Baldelli et al. 2007). Patch electrodes, fabricated from thick borosilicate glass (Hilgenberg, Mannsfeld, Germany), were pulled and fire-polished to a final resistance of 3–4 MΩ. The inhibitory or excitatory nature of the recorded neurons were evoked by depolarizing the cell body to +40 mV for 0.5 ms at 0.1 Hz. Evoked and/or miniature PSCs were recorded in each cell. Miniature and evoked PSCs were acquired at 10–20 kHz sample frequency and filtered at half the acquisition rate with an 8-pole low-pass Bessel filter. Recordings with leak currents >100 pA or series resistance >20 MΩ were discarded. Data acquisition was performed using PatchMaster programs (HEKA Elektronik, Lambrecht, Germany).

The mini-potential current (mPSC) analysis was performed by using the MiniAnalysis program (Synaptosoft, Leonia, NJ). The amplitude and frequency of mPSCs were calculated using a peak detector function using different appropriate threshold amplitude and threshold area for mPSCs and mIPSCs, respectively. PSCs were inspected visually, and only those which were not contaminated by spontaneous activity were considered. To calculate the peak current during an isolated stimulus or a train of stimuli, we first subtracted an averaged trace containing the stimulus artifact and the action potential current, but lacking any discernible synaptic current (i.e., synaptic failures). Such traces were easily identified toward the end of a train of stimuli, when synaptic depression was maximal. These traces were averaged and scaled to the peak Na+ current contaminating the mPSC.

To analyze the paired-pulse ratio (PPR), 2 brief depolarizing pulses (+40 mV for 0.5 ms) were applied to the autaptic neuron at 20- to 800-ms intervals. For each couple of PSCs, PPR was calculated from the equation: PPR = 100 (I2 - I1)/I1, where I1 and I2 are the amplitudes of the mPSCs evoked by the conditioning and test stimuli, respectively (Mallart and Martin 1967). The amplitude of I2 was determined as the difference between the I1 peak and the corresponding value of I1 calculated by monoexponential fitting of the mPSC decay (Jensen et al. 1999). Because of the high intrinsic variability of PPR, the mean PPR was calculated from the responses to at least 3–6 paired-pulse stimulation protocols for each interpulse interval.

**Cumulative ePSC Amplitude Analysis**

The size of the RRP of synchronous release (RRPsyn) and the probability that any given SV in the RRP will be released (Prrel) were calculated using the cumulative amplitude analysis (Schneggenburger et al. 2002). RRPsyn was determined by summing up peak PSC amplitudes during 40 repetitive stimuli applied at a frequency of 40 and 20 Hz for excitatory and inhibitory synapses, respectively. This analysis assumes that depression during the steady-state phase is limited by a constant recycling of SVs and equilibrium occurs between released and recycled SVs (Schneggenburger et al. 1999). The number of data points to include in the linear fitting of the steady-state phase was evaluated by calculating the best linear fit including the maximal number of data points starting from the last data point (i.e., from the 40th sPSC). The cumulative amplitude profiles of the last 20 data points were fitted by linear regression and back-extrapolated to time 0 (see Fig. 9). The intercept with the y-axis gave the RRPsyn and the ratio between the first ePSC and the last ePSC amplitude (I1) and RRPsyn yielded the Prrel. To correctly evaluate the postsynaptic currents evoked by high-frequency trains, the baseline of each ePSC in the train was defined as the final value of the decay phase of the preceding ePSC and the ePSC amplitude calculated by subtracting the baseline value from its peak value.

**Immunocytochemistry**

Cortical autaptic neurons obtained from WT mice were plated onto glass coverslips. Cells were fixed in 4% (w/v) paraformaldehyde at 10–14DIV and double stained using the anti-Syn monoclonal antibody (mAb10.22) (Vaccaro et al. 1997) and polyclonal antibodies against the vesicular GABA transporter or the vesicular glutamate transporter (VGAT or VGluT1; Synaptic Systems GmbH, Göttingen, Germany) followed by Alexa 488- or Alexa 594–conjugated secondary antibodies.
(Invitrogen–Molecular Probes, Milano, Italy). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Milano, Italy). Images were acquired using an IX71 inverted microscope (Olympus, Hamburg, Germany) and an ORCA-ER digital CCD camera (Hamamatsu Photonics, Milano, Italy). Exposure times were kept constant for all the acquisitions. Images were taken by focusing on small micro-islands containing a single autaptic neuron using the appropriate filter sets. Digital images (binning = 1; 1344 × 1024 pixels) were acquired from 3 to 5 independent preparations of WT cortical autaptic neurons.

Statistical Analysis
Data were expressed as means ± SEM. The normal distribution of experimental data was assessed using the Kolmogorov–Smirnov test. As the network data from MEA were not normally distributed, nonparametric statistical tests such as the Mann–Whitney U test or the Kruskal–Wallis multiple comparison tests were used and P values < 0.05 were considered significant. Statistical analysis was carried out by using the Statistica software package (StatSoft, Inc., Tulsa, OK).

Results
Networks of Cortical Neurons from Syn1 KO Mice Display a Diffuse Spontaneous Hyperactivity
Epileptic seizures are circuit phenomena generated by an initially localized hyperexcitability that spreads out along interconnected networks if not properly counterbalanced and spatially restricted by inhibitory mechanisms. To study the cellular bases of the high susceptibility to epileptic seizures of mice lacking Synl, we first analyzed the spontaneous electrical activity of networks of cortical E17 primary neurons prepared from WT and Syn1 KO embryos and plated onto MEA (Fig. 1A). The raster plots of the spiking activity of representative cultures recorded at 31 DIV are shown for WT (Fig. 1B, zoom in the right panel) and Syn1 KO (Fig. 1C, zoom in right panel) networks. The spontaneous activity of the cultures from both genotypes was characterized by a balanced presence of
random spikes and bursts of action potentials. However, SynI KO networks displayed a much higher spiking and bursting activity than WT networks and the majority of their electrical activity took place as part of network-wide collective events, known as "network bursts" (Van Pelt et al. 2004; Vajda et al. 2008). The number of active channels (bursting rate above the threshold of 0.4 burst/min) was always higher in SynI KO than in WT cultures (19.2 ± 2.8 and 27.9 ± 6.2 for WT and SynI cultures, respectively; n = 8 experiments for both genotypes; age range 24–35 DIV).

The Hyperactivity of SynI KO Networks Develops with Network Maturation In Vitro

The spontaneous activity of 29 MEA cultures (14 for WT and 15 for SynI KO cultures) from 6 to 8 independent preparations was monitored under basal conditions at various stages of the in vitro development (12–15 DIV, 18–20 DIV, 24–26 DIV, and 31–35 DIV). All cultures exhibited an activity pattern which changed temporally and strongly depended on the specific genotype (Fig. 2). In WT cultures, the main activity parameters (firing rate, bursting rate and burst duration), very low at early stages (<15 DIV), dramatically increased and peaked at 18–20 DIV, in accordance with previous observations in cortical cultures from embryonic rats (Muramoto et al. 1993; Van Pelt et al. 2005; Chiappalone et al. 2006; Wagenaar et al. 2006). After the peak, the 3 parameters slowly decreased and reached a stable level at later stages of network development (>35 DIV; Fig. 2A–C). SynI KO networks displayed a markedly different developmental profile. The firing rate, bursting rate and burst duration, already very high at early stages, further increased along network development, resulting significantly higher than those monitored in WT cultures over the entire in vitro development (Fig. 2A–C). As the developmental changes in spontaneous activity in WT and SynI KO cultures were opposite, the differences between the 2 genotypes were greatly emphasized upon network maturation. On the other hand, the intraburst firing rate increased with age in WT networks, whereas it gradually decreased during maturation in SynI KO networks (Fig. 2D). Such decrease in the intraburst firing rate observed in SynI KO networks appears to be a consequence of the decrease in spike frequency associated with the tail of long-lasting bursts that characterize mutant networks.

SynI KO Networks Display a High Degree of Burst Synchronization

To further characterize the differences in the spontaneous activity of WT and SynI KO cultures, we examined how the bursting activity was synchronized across the network by computing the cross-correlation function of burst event trains (see Materials and Methods). To this aim, we calculated, for each MEA culture, cross-correlograms of the activity recorded by all the possible pairs of electrodes (59 × 59, excluding autocorrelation) under basal conditions (Selinger et al. 2004). The mean spatial cross-correlograms between each recording channel and all the other channels in the array are depicted over an 8 × 8 grid in the MEA layout for representative WT (Fig. 3A, left) and SynI KO (Fig. 3A, right).

Figure 2. Cortical cultures from SynI KO mice are hyperactive during in vitro development. Primary cortical neurons from WT (gray bars) and SynI KO (black bars) mice were cultured onto MEAs for various DIV and monitored at various developmental stages. Before each recording session, the culture medium was replaced by extracellular solution and the spontaneous activity was recorded for 20–30 min, starting 15–20 min after the medium change. Extracellularly recorded spikes were detected using a threshold-based algorithm described previously (Chiappalone et al. 2006) and bursting activity was identified and analyzed as described in Materials and Methods. Firing rate (A), bursting rate (B), burst duration (C) and intraburst firing rate (D), calculated for each experimental group, are shown as means (±SEM) at the following developmental stages: 12-15, 18-20, 24-26, and 31-35 DIV. Statistical analysis was carried out by using the Mann-Whitney U test for independent samples. **P < 0.01 SynI KO versus WT within each developmental stage; n = 3-5 MEA cultures for each genotype and developmental stage, with a total of 29 independent experiments.
networks at 30 DIV. It is noteworthy that in the SynI KO preparation, not only the number of synchronized channels is higher, but also the level of synchronization reaches its maximum values in most of the bursting channels and the correlation peak is much sharper. To better quantify the differences between the 2 genotypes, we extracted 2 parameters from the cross-correlograms obtained from a subset of representative MEA cultures at 27-33 DIV: the cross-correlation in zero, $C(0)$, and the CI, which are related to the extent of synchronization between pairs of actively bursting channels (see Materials and Methods). The histograms calculated for all bursting pairs (left) and the corresponding box plots (right) for $C(0)$ and CI are shown in Figure 3B and C, respectively. In SynI KO networks, the $C(0)$ histogram is markedly shifted to the right (many channel pairs display high $C(0)$ values), indicating that the correlation peaks generated by the SynI KO cultures are statistically higher than those generated by WT cultures. Similar considerations can be drawn for the CI histogram and its box plot (Fig. 3C), further indicating that bursting dynamics is significantly more synchronized in SynI KO networks than in WT networks.

The Differences between the 2 Genotypes are Attenuated in the Presence of GABA$_A$ Receptor Blockade

The diffuse spontaneous hyperactivity observed in SynI KO networks could be ascribed to an impairment in inhibitory transmission, an increase in excitatory transmission or both. To test these possibilities, we challenged WT and SynI KO networks with the specific GABA$_A$ receptor blocker BIC to remove the influence of inhibitory systems on network excitability (Fig. 4). In case of an isolated impairment in GABAergic transmission, as previously reported in the hippocampus (Terada et al. 1999; Gitler et al. 2004; Baldelli et al. 2007), the activity of WT and SynI KO networks under BIC should become similar whereas, if a change in glutamatergic transmission is associated, SynI KO networks should be still hyperactive also under BIC.

Figure 3. Spontaneous activity in SynI KO networks is highly synchronized. (A) Mean cross-correlation on burst event for representative WT (left) and SynI KO (right) cultures. Empty windows indicate the inactive channels. Scale bars: x-axis, -150/150 ms; y-axis, 0-0.1. (B) Histogram of the $C(0)$ (left) calculated in WT (gray trace) and SynI KO (black trace) cultures and respective box plot of $C(0)$ (right). The cultures from the 2 genotypes are statistically different ($P < 0.05$, Mann-Whitney U test; $n = 3-4$ MEAs for each genotype at 27-33 DIV). (C) CI calculated from same experiments reported in (B) (left) and respective box plot (right). The 2 groups are statistically different ($P < 0.05$, Mann-Whitney U test).
Figure 4. BIC increases the duration but decreases the rate of spontaneous bursting in SynI KO cortical networks. Cultures of primary cortical neurons on MEAs were recorded between the fourth and the fifth week in vitro (24–35 DIV). The spontaneous activity of each culture was first measured for 20 min under basal conditions (physiological solution) and then for another 20 min epoch after the addition of 30 μM BIC. (A) Raster plot of the spontaneous activity recorded from 10 channels of representative WT (top) and SynI KO (bottom) networks kept under control conditions (left) and treated with BIC (right). (B) Scatter plots of burst duration versus bursting rate recorded in each active channel (i.e., channels with a bursting rate > 0.4 burst/min) of MEA cultures from WT (left panels; n = 8 experiments) or SynI KO (right panels; n = 8 experiments) mice in the absence (top panels) or presence (bottom panels) of BIC. The mean number (±SEM) of active channels/MEA culture was 19.25 ± 2.75, 24.5 ± 3.06 under basal and BIC conditions, respectively for WT cultures; 27.88 ± 6.22 and 29.88 ± 6.73 under basal and BIC conditions, respectively for SynI KO cultures. (C–H) Effects of BIC treatment on firing rate (C), bursting rate (D), burst duration (E), intraburst firing rate (F), fraction of spikes in bursts (G) and overall bursting activity (H). Data, calculated as described in Materials and Methods, are shown as means (±SEM) for WT (gray bars) and SynI KO (black bars) cultures. Statistical analysis was carried out by using the Kruskal-Wallis multiple comparison test. *P < 0.05; **P < 0.01 SynI KO versus WT within the same treatment; *P < 0.05; **P < 0.01 BIC versus Basal within genotype; n = 8 experiments for each experimental group.
The treatment of WT networks with BIC produced a clear-cut increase in synchronized bursting activity which was accompanied by a sharp decrease in the random spiking activity (see raster plots shown in Fig. 4A, left panels). In SynI KO networks, the intense bursting present under basal conditions was further enhanced and synchronized (Fig. 4B, upper left and right panels, respectively), with a much higher rate and duration of spontaneous bursts in the latter networks, as described above (see Fig. 2). BIC markedly altered the activity dynamics of both WT and SynI KO networks with respect to basal conditions (Fig. 4B, lower left and right panels, respectively). However, although the difference in bursting rate between the 2 genotypes was greatly attenuated under BIC, the enhancement in burst duration was much higher in SynI KO networks than in WT networks.

The analysis of the main activity parameters performed in a large number of MEA cultures (n = 8 for each genotype; age range 24–35 DIV) is shown in Figure 4C–H. In WT networks, BIC induced a significant increase in burst duration (84.24 ± 4.96 and 188.47 ± 9.14 ms, under basal and BIC conditions, respectively) and in the fraction of spikes in bursts (64.53 ± 2.58% and 75.55 ± 2.15%, under basal and BIC conditions, respectively), whereas no major changes were observed in the firing rate (1.21 ± 0.10 spikes/s and 1.31 ± 0.10 spikes/s under basal and BIC conditions, respectively), bursting rate (5.98 ± 0.47 bursts/min and 5.52 ± 0.34 bursts/min, under basal and BIC conditions, respectively) or intraburst firing rate (1.21 ± 0.10 spikes/s under basal and BIC conditions, respectively). On the other hand, SynI KO networks responded to BIC with a significant increase in burst duration, intraburst firing rate and fraction of spikes in bursts (burst duration, 187.01 ± 11.43 ms and 378.82 ± 31.30 ms; intraburst firing rate, 130.48 ± 4.40 spikes/s and 207.60 ± 0.75 spikes/s; fraction of spikes in burst, 72.24 ± 1.89% and 79.63 ± 1.58%, under basal and BIC conditions, respectively). However, these effects were accompanied by a significant decrease in the firing rate and bursting rate which, starting from very high levels under basal conditions, approached the levels of WT networks treated with BIC (firing rate, 5.13 ± 0.45 spikes/s and 3.33 ± 0.31 spikes/s; bursting rate, 15.42 ± 0.78 and 7.73 ± 0.37 burst/min; under basal and BIC conditions, respectively). As a result, the overall bursting activity (percentage of the time spent in bursting activity) was significantly increased by treatment with BIC in WT networks (0.86 ± 0.12 and 1.87 ± 0.13%, under basal and BIC conditions, respectively), whereas it was not significantly altered in mutant networks (6.14 ± 0.56 and 4.60 ± 0.34%, under basal and BIC conditions, respectively).

**Synapsin I Deletion Increases the Amplitude of eEPSCs and Reduces the Amplitude of eIPSCs**

We next asked whether an altered synaptic function at the level of single neurons underlies the differences in the network firing properties observed in SynI KO mice. However, the high neuronal density conditions of MEA cultures and the strong heterogeneity in synaptic connections makes it difficult to evaluate the excitatory/inhibitory balance at single cell level and to distinguish primary effects of the mutation from secondary activity-dependent plasticity effects. For this reason, we carried out a parallel analysis on excitatory and inhibitory synapses of cortical autaptic neurons obtained from WT and SynI KO mice. Cortical excitatory pyramidal neurons (Fig. 6A1 and B1) and interneurons (Fig. 6A2 and B2), plated in microisland cultures (Segal and Furshpan 1990; Bekkers and Stevens)

**Synapsin KO Networks Display a Prolonged Enhanced Excitability in Response to Low-Frequency Stimulation**

We next analyzed the response over time of WT and SynI KO networks to a train of low-frequency, localized extracellular stimulations applied through a single electrode of the array. The raster plots of the spikes evoked in response to the 50 stimuli in the train occurring within a time window of 600 ms after the stimulus, recorded from 2 selected electrodes from
1991), formed autapses that reached a fully functional maturation after 2 weeks in vitro. This experimental system offers several advantages over brain slices and high-density cultures for quantifying neurotransmitter release. Most important is that the “autaptic” responses originate from the same cell, so that the sources of the incoming synaptic input are homogeneous and defined. As a result, evoked and spontaneous release can be recorded from the same synapses, allowing direct comparisons between these 2 forms of release. Finally, because synaptic transmission is unitary (monosynaptic), the synaptic responses do not depend critically on the properties of the stimulus. Given that SynI deletion has been reported to delay synaptogenesis (Chin et al. 1995; see Ferreira and Rapoport 2002 for review), and that differences in the expression of synapsin isoforms in distinct neuronal populations have been reported (Südhof et al. 1989; Bogen et al. 2006; Kielland et al. 2006; Bragina et al. 2007), we performed an immunocytochemical analysis to verify whether autaptic...
excitatory and inhibitory neurons prepared from WT cortex express SynI to a similar extent (Fig. 6A1 and A2).

WT cortical autaptic neurons stained with antibodies to VGLUT1 or VGAT to identify their transmitter phenotype, were counterstained with anti-SynI antibodies to assess the homo-
geneity in SynI expression in these neuronal populations. SynI was expressed by virtually all WT autaptic excitatory neurons and by the majority of WT autaptic inhibitory neurons. Moreover, no significant genotype-dependent differences were observed between the 2 neuronal populations in the mean
number of inhibitory or excitatory terminals formed at 17 DIV, that is, at the stage of in vitro development chosen for the electrophysiological studies (not shown).

The amplitude of excitatory and inhibitory autaptic currents (Fig. 6C1 and C2) was characterized by a certain level of variability between neurons, which could be ascribed to differences in the size of individual neurons and thereby in the number of autapses per neuron. Therefore, we systematically evaluated the membrane capacitance to yield an estimate of cell size for each individual patched autaptic neuron.

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difference between WT and SynI KO cultures was observed when the size of either neuronal type was compared (Fig. 6D1 and D2).

Autaptic responses were elicited by brief voltage pulses that evoked propagating action potentials (Fig. 6C1 and C2). The absence of SynI affected the synaptic responses of excitatory and inhibitory neurons in an opposite manner. Evoked autaptic EPSCs were found to be significantly enhanced in SynI KO

Figure 6. Lack of SynI enhances eEPSC amplitude and reduces eIPSC amplitude recorded in whole-cell-clamped cortical autaptic neurons. (A) Immunofluorescence images of glutamatergic (A1) and GABAergic (A2) autaptic neurons double stained with polyclonal antibodies against either the vesicular glutamate transporter VGLUT-1 (A1, left panel, green) or the vesicular GABA transporter, VGAT (A2, left panel, green) and with an anti-SynI monoclonal antibody (mAb10.22; A1 and A2, middle panel, red). Merged images are shown in the right panels. Calibration bar 50 and 30 µm for A1 and A2, respectively. (B) Representative phase-contrast images of an autaptic pyramidal neuron (B1) and an autaptic interneuron (B2). Calibration bar 50 and 30 µm for B1 and B2, respectively. (C) Representative traces of autaptic eEPSCs (C1) and eIPSCs (C2) elicited by a short (0.5 ms) depolarizing step from −70 to +40 mV recorded from WT (gray trace) and SynI KO (black trace) neurons. (D) Mean (±SEM) capacitance of excitatory (D1) and inhibitory (D2) autaptic neurons from WT (gray bars) and SynI KO (black bars) mice. (E) Mean (±SEM) amplitude of eEPSCs (E1) and eIPSCs (E2) recorded from WT (gray bars) and SynI KO (black bars) autaptic neurons (glutamatergic neurons, n = 15 and n = 10 for WT and SynI KO, respectively; GABAergic neurons, n = 13 and n = 11 for WT and SynI KO, respectively). *P < 0.05, Mann-Whitney U test for independent samples.
neurons with respect to WT neurons (1155 ± 174, n = 15 and
1693 ± 172, n = 10 for WT and SynI KO neurons, respectively;
P < 0.05; Fig. 6E)). The increase of eEPSC amplitude was not
accompanied by any change in decay time of eEPSC (decay
time constant: 7.59 ± 0.9 and 7.58 ± 0.5 ms for WT [n = 15]
and SynI KO [n = 10] neurons, respectively).

Consistent with previous reports in low-density and autaptic
hippocampal neurons (Gitler et al. 2004; Baldelli et al. 2007),
we found that inhibitory transmission was substantially
impaired in cortical autaptic neurons from SynI KO mice.
Although evoked eIPSCs were very similar in waveform in WT
and SynI KO neurons (decay time constant: τfast 20.7 ± 1.7, τslow
110 ± 5 and τfast 21.9 ± 2, τslow 108 ± 7 ms; for WT [n = 13] and
SynI KO [n = 11] neurons, respectively), the mean amplitude of
IPSCs in SynI KO neurons was reduced by 23.4% of the mean
value determined in WT neurons, (1343 ± 82, n = 13 and 1028 ±
99, n = 11 for WT and SynI KO neurons, respectively; P < 0.05;
Fig. 6E)).

**Synapsin I Deletion Does Not Affect Spontaneous
Neurotransmitter Release and the Quantum Content
at Excitatory and Inhibitory Autapses**

To evaluate whether Syn detetion could affect the glutama-
tergic and GABAergic quantal size, miniature excitatory and
inhibitory PSCs (mEPSCs and mIPSCs) were continuously re-
corded at the soma of voltage-clamped neurons held at
−70 mV (Vh) in Tyrode solution containing 300 nM TTX to block
spontaneous firing activity (see Materials and Methods).

Spontaneous mEPSCs and mIPSCs were present in both types
of neurons and were closely similar in both WT and SynI KO
neurons (Fig. 7A1–A2). The mean mEPSC amplitude (17.20 ±
2.20 and 14.63 ± 2.69 pA, for WT [n = 5] and SynI KO [n = 5]
mice, respectively; P = 0.17; Fig. 7B1) and the mean mEPSC
frequency (4.60 ± 0.85 and 4.77 ± 1.12 Hz, for WT [n = 5] and
SynI KO [n = 5] mice, respectively; P = 0.45; Fig. 7C1) were not
significantly different between the 2 genotypes, indicating no
change in the quantum content and rate of spontaneous
transmitter release. Moreover, no changes were detected in the
mEPSC waveform, as indicated by the analysis of the decay and
rise times (Fig. 7D1–E1).

Similar to excitatory transmission, no significant differences
were observed in the mean amplitude (9.68 ± 0.21 and 9.98 ±
0.28 pA, for WT [n = 5] and SynI KO [n = 5] mice, respectively;
P = 0.30; Fig. 7B2) and frequency (6.76 ± 1.09 and 6.17 ± 0.57
pA, for WT [n = 5] and SynI KO [n = 5] mice, respectively; P =
0.38; Fig. 7C2) of spontaneous mIPSCs, as well as in their rise
decay times (Fig. 7D2–E2).

Taken together, the data suggest that the absence of Syn
had opposite effects on glutamate and GABA release evoked by
single action potentials, without affecting the spontaneous
release of the both neurotransmitters.

**Synapsin I Deletion Enhances Excitatory Paired-Pulse
Facilitation and Does Not Affect Inhibitory Paired-Pulse
Depression in Autaptic Cortical Neurons**

Few data are available on the role played by Syns in paired-pulse
plasticity. Paired-pulse facilitation (PPF) of field EPSPs was
increased in SynI KO mice (Rosahl et al. 1993, 1995), whereas it
was not changed in SynIII or SynII/III KO mice (Feng et al.
2002; Gitler et al. 2004). Although the absence of Syns seems not
to impair PPF, Syn seems to be necessary for the increase in
PPF of EPSPs promoted by the constitutive activation of the
Ras/MAP kinase pathway (Kushner et al. 2005). Finally, we
found no changes in PPR of eIPSCs of patch-clamped
hippocampal neurons of SynI KO (Baldelli et al. 2007).

At glutamatergic synapses, the responses to paired stimuli
separated by intervals ranging from 20 to 800 ms (Fig. 8A1)
were not homogenous. The response to the second stimulus at
20-ms interpulse interval was always smaller than the response
to the first stimulus with a mean paired-pulse depression of
−14.6 and −17.3% for WT and SynI KO neurons, respectively.
At longer interpulse intervals, a facilitating phase appeared at 30-,
40-, 50-, and 80-ms intervals in SynI KO neurons, whereas a
transient facilitation was only seen at 30-ms interval in WT
neurons, with a significant increase in facilitation in SynI KO
neurons with respect to WT neurons (50-ms interpulse interval;
P < 0.05; n = 9 for WT and n = 10 for SynI KO; Fig. 8B1).

When inhibitory synapses were stimulated by 2 consecutive
stimuli administered at interpulse intervals ranging between 20
and 800 ms, the response to the second stimulus was always
smaller than the response to the first stimulus because of syn-
aptic depression (Fig. 8A5 see also Fig. 9). The extent of paired-
pulse depression of autaptic eIPSCs elicited at the various
interpulse intervals was similar in WT and SynI KO neurons
(Fig. 8B5 P > 0.05).

**Opposite Effect of Syn Deletion on the Size of the RRP in
Excitatory and Inhibitory Autapses**

We further examined whether the changes in the amplitude of
eEPSCs and IPSCs observed in the absence of Syn were
attributable to specific changes in the elementary events
responsible for quantal release. To this aim, we used a method
which analyzes the cumulative amplitude profile during high-
frequency trains of stimuli (Schneeggenburger et al. 1999;
Baldelli et al. 2007) and allows to extract the values of release
probability and size of the RRP. Short trains of 40 stimuli were
applied at high frequency (20 and 40 Hz) to excitatory and
inhibitory autaptic neurons from both WT and SynI KO mice.
A significant depression of eEPSCs and eIPSCs became apparent
during the trains in both WT and SynI KO neurons (Fig. 9A1–
A2). Excitatory transmission was characterized by a slower rate
of depression (τ = 280 and 75 ms at 20 and 40 Hz, respectively;
Fig. 9A1–B1) with respect to inhibitory transmission (τ = 37
ms at 20 and 40 Hz, respectively; Fig. 9A1–B2), according
to the higher probability of release which characterizes
GABAergic synapses (Kraushaar and Jonas 2000). The time-
courses of depression for both excitatory and inhibitory
autapses in SynI KO neurons did not significantly differ from
those described in matched WT neurons (Fig. 9A,B).

In both experimental groups, the cumulative profile of
eEPSCs showed a more rapid rise than the cumulative profile of
eIPSCs. This phase was followed by a slower linear increase at
later pulses; this linear phase was less steep in excitatory
autapses than in inhibitory autapses (Fig. 9C1–C2). Because the
slow linear rise is thought to correspond to the equilibrium
between the release-induced depletion and the constant
replenishment of the RRP, back-extrapolation of the linear
portion to time zero yields a rough estimation of total release
minus total replenishment, corresponding to the size of the
RRP of synchronous release (RRPsyn; Schneeggenburger et al.
1999). The results of such analysis are shown in Figure 9D–E.
The RRPsyn for eEPSCs was significantly increased in SynI KO
neurons (4372 ± 578 and 7085 ± 861 pA in WT [n = 6] and SynI
KO [n = 8] neurons respectively; P < 0.05; Fig. 9E) to the same
extent of the mean amplitude of the first EPSC in the train (1155 ± 174 and 1693 ± 172 pA in WT and SynI KO neurons, respectively; *P* < 0.05; Fig. 9D1). On the contrary, the RRPSyn for IPSCs was significantly decreased in SynI KO neurons (2661 ± 233 and 2037 ± 162 pA in WT, *n* = 5) and SynI KO (*n* = 8) neurons, respectively; *P* < 0.05; Fig. 9E2) to the same extent of
the mean amplitude of the first IPSC in the train (1343 ± 82 and 1028 ± 99 pA in WT and SynI KO neurons, respectively; \( P < 0.05 \); Fig. 9D2). SV release probability, \( P_{\text{ves}} \), calculated as the ratio between the first PSC in the train (\( I_1 \)) and RRP_{syn} (see Materials and Methods) was not significantly affected in either excitatory (0.29 ± 0.04 and 0.27 ± 0.04 in WT and SynI KO neurons respectively; \( P = 0.20 \); Fig. 9F1) or inhibitory autapses (0.52 ± 0.05 and 0.53 ± 0.05 in WT and SynI KO neurons, respectively; \( P = 0.44 \); Fig. 9F2).

Discussion

Epileptic seizures are thought to be generated by an initially localized hyperexcitability that spreads into neuronal networks and is not circumscribed by inhibitory mechanisms (Steinlein 2004). The current evidence indicates that the Syns are involved in crucial steps of presynaptic physiology of both excitatory and inhibitory synapses and can therefore participate in the determination of the firing activity of cortical networks. Thus, it is expected that Syn mutations result in changes in synaptic transmission and plasticity, which could be potentially epileptogenic. As a matter of fact, a severe epileptic phenotype is displayed by SynI, SynII, SynI/II, and SynI/II/III KO mice (see Baldelli et al. 2006 for review). To date, SYN are the only SV protein genes whose mutations were found to be associated with human epilepsy (Garcia et al. 2004; Cavalleri et al. 2007).

The loss of Syns was shown to disrupt the RP of SVs and to impair the ability of neurons to release neurotransmitter during sustained high-frequency activity (Rosahl et al. 1995; Gitler et al. 2004; Baldelli et al. 2007). It has been hypothesized that such an impairment predominantly affects inhibitory neurons which are characterized by high frequency, bursting-type firing and therefore are more sensitive to SV depletion. Indeed, a deficit in hippocampal GABAergic neurons has been indeed demonstrated in SynII (Terada et al. 1999; Baldelli et al. 2007), SynIII (Feng et al. 2002), and SynI/II/III (Gitler et al. 2004) KO mice. However, such an impairment may not be sufficient to generate an epileptic phenotype, because SynIII KO mice are not epileptic (Feng et al. 2002).

SynI KO Networks Exhibit Developmentally Regulated Hyperexcitability In Vitro

We have investigated the bases of the seizure propensity by studying the electrical activity of dissociated cultures of primary cortical neurons from WT and SynI KO mice. Neuronal networks develop in vitro as a self-organized system which displays a highly synchronized activity due to lack of external inputs and reverberating connectivity typical of closed systems (Wagenaar et al. 2005; Eytan and Marom 2006). Primary cortical
networks from WT mice grown under normal conditions exhibited a developmental profile of electrical activity similar to that previously reported (Van Pelt et al. 2005; Chiappalone et al. 2006; Wagenaar et al. 2006). Both spiking and bursting activity increased from very low levels at early stages (12–15 DIV) to a peak reached at 18–20 DIV, followed by a lower steady-state in which the 2 patterns of activity coexisted. In contrast, SynI KO cultures displayed a general increase in firing rate, bursting rate and burst duration which became more marked at later stages of network maturation (31–35 DIV), with a progressively larger divergence from the corresponding parameters of WT cultures. The hyperexcitability of SynI KO networks was further confirmed by the sustained and prolonged bursting responses to low-frequency focal electrical stimulation.

Although SynI KO networks appeared already hyperexcitable at early stages of the in vitro development, the progressive build-up of hyperexcitability in parallel with synaptic maturation may depend on the concomitant increase in SynI expression in WT networks (Ferreira et al. 2000), and correspond to the period in which the regulatory activity of SynI comes into play. This developmental pattern is consistent with the late

Figure 9. The changes in the amplitude of eEPSCs and eIPSCs are accompanied by parallel changes in the size of the RRP. The estimation of the RRP size and Pr was carried out by using the cumulative amplitude profile analysis as described in Materials and Methods. (A) Representative recordings during a high-frequency train applied to glutamatergic (A1; 40 stimuli at 40 Hz) or GABAergic (A2; 40 stimuli at 20 Hz) neurons from WT (gray traces) and SynI KO (black traces) mice. (B) Plot of the mean ePSCs amplitude (±SEM) versus time during repetitive stimulations of glutamatergic (B1; 40 stimuli at 40 Hz; 17–23 DIV; n = 6 for WT [gray symbols] and n = 8 SynI KO [black symbols] neurons, respectively) and GABAergic (B2; 40 stimuli at 20 Hz; 17–21 DIV; n = 5 for WT [gray symbols] and n = 8 SynI KO [black symbols] neurons, respectively) neurons. (C) Cumulative eEPSC (C1) and eIPSC (C2) amplitude profiles. To estimate the RRP, data points in the linear range of the curves were fitted by linear regression and back-extrapolated to time 0 (WT, gray symbols; SynI KO, black symbols). (D–F) The amplitude of the first ePSC in the train (D), the RRP size (E) and the calculated Pr (F) are shown as means (±SEM) for glutamatergic (left, D1–F1) and GABAergic (right, D2–F2) autapses from WT (gray bars) and SynI KO (black bars) mice. Statistical analysis was carried out by using the Mann-Whitney U test for independent samples. *P < 0.05 versus WT group (glutamatergic neurons, n = 6 and n = 8 for WT and SynI KO neurons, respectively; GABAergic neurons, n = 5 and n = 8 for WT and SynI KO neurons, respectively).
appearance of the impairment in evoked GABAergic currents observed in primary cultures of hippocampal neurons (Baldelli et al. 2007) and is also reminiscent of the susceptibility of SynI KO mice to epileptic seizures that starts at 2–3 months of postnatal development and becomes progressively more severe with age (Rosahl et al. 1995).

The Response of SynI KO Networks to the Blockade of GABAergic Transmission Reveals Multiple Mechanisms for Hyperexcitability

The electrical activity of WT networks became highly synchronized when blockade of inhibitory synapses was applied, with a significant increase in both burst duration and fraction of spikes in bursts and overall bursting activity (Chiappalone et al. 2007; Pasquale et al. 2008). On the contrary, the spiking and bursting rates of SynI KO networks, very high under basal conditions, were decreased by BIC to approach the bursting rate of WT networks, whereas the BIC-induced increase in burst duration and fraction of spikes in bursts was similar to that of WT cultures. If the impairment caused by SynI deficiency were due to a decrease in GABAergic transmission, as suggested by previous studies (Terada et al. 1999; Baldelli et al. 2007), then there would be little difference between WT and SynI KO networks under BIC, whereas if the impairment were due to an increase in glutamatergic transmission, then SynI KO networks treated with BIC would become even more hyperexcitable. Both conditions seem to occur under BIC, because SynI KO networks tended to approach the overall level of activity of WT cultures, still maintaining a significant hyperexcitability with respect to WT networks as far as firing rate, bursting rate, burst duration and overall bursting activity are concerned.

Thus, the data are consistent with a model in which the absence of SynI leads to a primary imbalance between inhibitory and excitatory systems, thereby generating diffuse spontaneous and stimulation-evoked hyperexcitability. Such an imbalance could be subsequently maintained, and possibly enhanced, by the development of long-term plastic changes in the excitoryatory transmission induced by the intense and repetitive firing.

SynI KO Autaptic Neurons Reveal the Existence of an Impairment in Inhibitory Transmission and a Parallel Enhancement in Excitatory Transmission due to Opposite Changes in the Size of the RRP

To elucidate the molecular basis of this phenomenon at the level of single neurons and to avoid the contribution of secondary effects due to synaptic plasticity processes, we analyzed synaptic transmission in mature cortical inhibitory and excitatory autapses at 17–23 DIV by patch-clamp. Both types of WT autaptic neurons expressed SynI and its immunoreactivity precisely overlapped with the immunoreactivity for the respective vesicular neurotransmitter transporter. The results of these experiments confirmed the suggestions from the network studies by demonstrating an impairment of GABA-mediated IPSCs and a concomitant enhancement of glutamate-mediated eEPSCs in mutant neurons.

The decrease in the mean amplitude of evoked IPSCs was not accompanied by any change in the amplitude of the mIPSCs, indicating the absence of postsynaptic effects and/or alterations in the quantum content. These observations indicate that the reduction in the IPSC amplitude is due to a decreased number of SVs released in response to the action potential. Cumulative amplitude analysis suggested that this effect is the consequence of a decreased size of the RRP, rather than a change in release probability. The fact that paired-pulse depression of GABAergic transmission was not affected by the SynI mutation further confirms the absence of changes in release probability. The impairment in GABAergic transmission in cortical neurons is consistent with previous reports in hippocampal interneurons of both SynI and SynI/II/III KO mice (Terada et al. 1999; Gitler et al. 2004) which was found to be due to a selective decrease in the RRP size (Baldelli et al. 2007) or to a decrease in the number of SVs physically docked to the presynaptic membrane (Gitler et al. 2004). Because it is currently believed that, under physiological conditions, the sizes of RP and RRP are in a relatively constant ratio and the number of docked SVs correlates with the total SV number (Mozhayeva et al. 2002; Moulder et al. 2007), the decrease in the RRP size found in SynI KO neurons may reflect the concomitant, marked depletion of the RP reported by ultrastructural and functional observations in SynI and SynI/II/III KO neurons (Li et al. 1995; Rosahl et al. 1995; Terada et al. 1999; Gitler et al. 2004; Baldelli et al. 2007).

The increase in glutamatergic transmission is an unexpected finding. Glutamatergic neurons exhibit a high variability in the size of their SV pools and have a large percentage of SVs which are resistant to release as compared with GABAergic neurons, suggesting distinct mechanisms of SV trafficking and recruitment for release (Li et al. 1995; Moulder et al. 2007). We found that the increase in the amplitude of eEPSC was not associated with an increase in the mEPSC amplitude. As observed for GABAergic transmission, the lack of changes in mEPSC amplitude indicates that, in the absence of postsynaptic effects and/or changes in the mean quantum content of SVs, the increase in eEPSC amplitude depends on an increased number of SVs released in response to the action potential. This effect seems to be entirely attributable to an increased size of the RRP, rather than to an increase in release probability, as suggested by the cumulative amplitude analysis. An increase in release probability is also ruled out by the increased paired-pulse facilitation in SynI KO glutamatergic neurons. Although an increase in the RRP size in mutant excitatory synapses is not consistent with the very limited changes in the number of docked SVs estimated by electron microscopy in Syn KO glutamatergic terminals (Li et al. 1995; Rosahl et al. 1995; Gitler et al. 2004; Siksou et al. 2007), cumulative amplitude analysis gives a functional and dynamic estimation of the RRP size in contrast with the morphological description of RRP provided by electron microscopy. It is possible that the observed increase in the functional RRP size in mutant glutamatergic terminals could be attributable to the partial disassembly of the RP which may recruit more SVs to the RRP.

Functional Heterogeneity between Excitatory and Inhibitory Neurons may Account for the Differential Effects of the SynI Mutation

The results indicate that SynI differentially affects the physiology of excitatory and inhibitory synapses. The cellular basis for this phenomenon can be several fold. Syns, although present in most conventional chemical synapses, display...
a differential isoform expression pattern (Südhof et al. 1989; Kielland et al. 2006; Bragina et al. 2007).

The Syns have been suggested to be part of the mechanisms clustering SVs and tethering them to the actin cytoskeleton within the RP. In addition, Syns are implicated in the final postdoctoring stages operating exocytosis (SV priming and/or fusion). By virtue of these multiple actions, Syns can affect the size of both RP and RRP. However, various synapses may use distinct SV tethering mechanisms regulating SV trafficking between RP and RRP and/or be differentially sensitive to extracellular messengers (Atwood and Karunanithi 2002). Thus, lack of Syns differentially affects short-term plasticity responses involving the RRP in distinct Syn-positive excitatory synapses in the hippocampus (Hvalby et al. 2006). Lack of SynIII specifically impairs GABAergic terminals, leaving glutamatergic terminals unaffected (Feng et al. 2002), whereas lack of all Syns differentially affects GABAergic and glutamatergic neurons (Gitter et al. 2004). Again, hippocampal excitatory SynIII KO synapses display an increased size of the SV recycling pool and milder depression (Feng et al. 2002), whereas the same synapses experience a more severe depression in SynI/II/III KO mice (Gitter et al. 2004). That the effects of Syn ablation strongly depends on the neuronal type is further indicated by the increase in depolarization-induced dopamine release found in SynI/II/III KO neurons and chromaffin cells (Villanueva et al. 2006; Kile et al. 2007).

In conclusion, we have demonstrated that the ablation of the SYN1 gene is associated with opposite changes in the strength of excitatory and inhibitory synapses which promote a diffuse hyperexcitability of cortical networks. Such a complex imbalance may force neuronal circuits into a state of basal heightened excitability which facilitates the spontaneous or stimulus-evoked onset of epileptic seizures associated with mutations of the SYN1 gene in mice and man.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

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Address correspondence to Fabio Benfenati, MD, Department of Experimental Medicine, University of Genova, Viale Benedetto XV, 3 16132 Genova, Italy. Email: fabio.benfenati@unige.it.

**References**


