Riluzole-Induced Oscillations in Spinal Networks

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Yvon C, Czarnecki A, Streit J. Riluzole-induced oscillations in spinal networks. J Neurophysiol 97: 3607–3620, 2007. First published March 7, 2007; doi:10.1152/jn.00924.2006. We previously showed in dissociated cultures of fetal rat spinal cord that disinhibition-induced bursting is based on intrinsic spiking, network recruitment, and a network refractory period after the bursts. A persistent sodium current (\(I_{\text{NaP}}\)) underlies intrinsic spiking, which, by recurrent excitation, generates the bursting activity. Although full blockade of \(I_{\text{NaP}}\) disrupts such bursting, the present study shows that partial blockade of \(I_{\text{NaP}}\) with low doses of riluzole maintains bursting activity with unchanged burst rate and burst duration. More important, low doses of riluzole turned bursts composed of persistent activity into bursts composed of oscillatory activity at around 5 Hz. In a search for the mechanisms underlying the generation of such intraburst oscillations, we found that activity-dependent synaptic depression was not changed with low doses of riluzole. On the other hand, low doses of riluzole strongly increased spike-frequency adaptation and led to early depolarization block when bursts were simulated by injecting long current pulses into single neurons in the absence of fast synaptic transmission. Phenytoin is another \(I_{\text{NaP}}\) blocker. When applied in doses that reduced intrinsic activity by 80–90%, as did low doses of riluzole, it had no effect either on spike-frequency adaptation or on depolarization block. Nor did phenytoin induce intraburst oscillations after disinhibition. A theoretical model incorporating a depolarization block mechanism could reproduce the generation of intraburst oscillations at the network level. From these findings we conclude that riluzole-induced intraburst oscillations are a network-driven phenomenon whose major accommodation mechanism is depolarization block arising from strong sodium channel inactivation.

INTRODUCTION

Network oscillations of different frequency ranges are ubiquitous in the CNS. They are believed to be important in such key functions or states as locomotion (Grillner 2003), breathing (Rekling and Feldman 1998), sleep (Steriade 2001), and epileptogenesis (McNamara 1994). Whereas some studies suggest a critical role of single-cell properties in the generation of such oscillatory activity (Silva et al. 1991; Sipila et al. 2005; Tresch and Kiehn 2000), other work focuses on a network-driven mechanism, based on repetitive activation of the network through recurrent excitatory connections. Such network-driven rhythms were previously described in various preparations as in cortical (Sanchez-Vives and McCormick 2000), hippocampal (Prida and Sanchez-Andres 1999; Staley et al. 1998), and brain stem slices (Rekling and Feldman 1998), as well as in cortical (Jimbo and Robinson 2000) and spinal slice cultures (Ballerini and Galante 1998; Tschelter et al. 2001). They require, first, intrinsically spiking neurons that are able to recruit the network and, second, one or several accommodation mechanisms to quiet the network (Darbon et al. 2002b, 2003). Network-driven rhythms do not necessarily require a specific network architecture in terms of excitatory and inhibitory connections because rhythmic activity can reliably appear after pharmacological blockade of fast synaptic inhibition (disinhibition). In addition, they can even be produced in simple networks with no preexisting anatomical specialization as in dissociated cultures of retinal neurons (Harris et al. 2002), cortical neurons (Kamioka et al. 1996; Robinson et al. 1993; Segev et al. 2001), and spinal neurons (Droge et al. 1986; Streit et al. 2001). To better assess the basis of oscillatory activity in complex, structured networks in vivo under physiological and pathophysiological conditions, it is therefore essential to gain understanding of the diverse mechanisms capable of generating oscillations in such simply structured networks.

We previously described the major mechanisms underlying disinhibition-induced rhythms in dissociated and organotypic slice cultures of fetal rat spinal cord. In these preparations, we showed that the network refractory period after a burst results from a suppression of network excitability caused by an up-regulation of the Na/K electrogenic pump during the burst (Darbon et al. 2003). We found that intracellular calcium levels are not critically involved in rhythm generation (Darbon et al. 2002a). Furthermore, the persistent sodium current (\(I_{\text{NaP}}\)) and the hyperpolarization-activated cationic current (\(I_h\)) together were shown to be involved in the generation of intrinsic spiking and disinhibition-induced bursting, with \(I_{\text{NaP}}\) being the critical component (Darbo et al. 2004). Yet one remarkable difference between disinhibited rhythms in organotypic and dissociated spinal cultures remains to be cleared up. Whereas in dissociated cultures bursts show a decreasing but continuous level of activity, in organotypic cultures these bursts consist of oscillatory activity with decreasing frequency (from about 8 to 1 Hz). Similar intraburst oscillations were observed during disinhibition-induced bursting in the isolated spinal cord of the rat (Braacci et al. 1996). Spontaneous episodes composed of oscillatory activity (about 1 Hz) were also described in the isolated embryonic chick spinal cord (Landmesser and O’Donovan 1984). These oscillations were originally hypothesized to be attributed to a “fast” form of frequency-dependent synaptic depression (Senn et al. 1996; Streit et al. 1992; Tabak et al. 2000). Meanwhile, other mechanisms underlying the generation of oscillatory activity in excitatory networks have been proposed. In several theoretical models, for example, it was shown that spike-frequency adaptation is essential for the synchronization of network oscillations during periods of repetitive firing (Crook et al. 1998; Fuhrmann et al. 2002; van Vreeswijk and Hansel 2001). In the hippocampus in vivo a
mechanism of depolarization block of spike generation in interneurons was suggested for the emergence of oscillatory activity during epileptic afterdischarges (2–6 Hz) (Bragin et al. 1997). Last, a strong depolarization block was shown to accompany termination of epileptiform activity waves in disinhibited neocortical slices (Pinto et al. 2005).

In this study, we investigate the mechanisms underlying the generation of intraburst oscillations in spinal cultures under disinhibition. We take advantage of our finding that the sodium channel blocker riluzole, at low micromolar doses, turns bursts of persistent activity into bursts of oscillatory activity in dissociated cultures. This unexpected finding offers us the unique opportunity to directly compare these two burst structures in the same preparation. Therefore here we use these riluzole-induced oscillations as a model to investigate the underlying mechanisms. In particular, we focus on activity-dependent synaptic depression, spike-frequency adaptation, and depolarization block.

**METHODS**

**Culture preparation and recordings**

All cultures were prepared from spinal cords of rats at embryonic age 14 (E14). The cultures were prepared as described previously (Streit et al. 2001; Tschelter et al. 2001). The embryos were delivered by caesarian section from deeply anesthetized animals (0.4 ml pentobarbital, administered intramuscularly) and killed by decapitation. After the delivery of the embryos, the mother was killed by intracardiac injection of pentobarbital. Animal care was in accordance with guidelines approved by Swiss local authorities. The backs of the embryos were isolated from their limbs and viscera and cut into 225-μm-thick transverse slices with a tissue chopper.

For the dissociated cultures, slices of all regions of the spinal cord without dorsal root ganglia were exposed to a 0.3% trypsin solution for 3 min at 37°C. They were then mechanically dissociated by forcing them through fine-tipped pipettes several times. The cells were plated on multielectrode arrays (MEAs) and glass coverslips at a density of about 5,000 and 2,500 cells/mm², respectively. MEAs were produced as described previously (Tschelter et al. 2001) and coated for 1 h with diluted (1:50) Matrigel (Falcon/Biocat, Becton Dickinson, Basel, Switzerland). The cells were restricted to an area around the electrodes (30 mm²) using cloning glass cylinders attached to the MEAs or coverslips with silicone sealant. The cells were maintained in culture dishes containing between 75 and 150 μl of nutrient medium and incubated in a 5% CO₂-containing atmosphere at 36.5°C for ≤12 wk. The medium consisted of serum-free Neurobasal medium (Gibco BRL, Life Technologies, Basel, Switzerland) supplemented with B27 and Glutamax (both Gibco BRL). Half of the medium was changed weekly.

For the organotypic cultures, spinal cord slices with their dorsal root ganglia attached were fixed on MEAs using reconstituted chicken plasma (Cocalico Biologicals, Reamstown, PA) coagulated by thrombin (Sigma, Fluka Chemie, Buchs, Switzerland). These cultures were maintained in sterile plastic tubes containing 3.5 ml of nutrient bin (Sigma, Fluka Chemie, Buchs, Switzerland). These cultures were changed weekly.

**MEA recording and analysis**

MEAs consisted of 68 electrodes, laid out either in the form of a rectangle (dissociated cultures) or in the form of a hexagon (organotypic cultures). The platinum electrodes had a dimension of 40 × 40 μm and were spaced 160 μm apart (center to center; e.g., Fig. 1A). Channels (i.e., electrodes) showing activity (usually 10–40) were selected by eye and their recording digitized at 6 kHz, visualized, and analyzed.
stored on hard disc using custom-made virtual instruments within LabVIEW (National Instruments, Ennetbaden, Switzerland), as described previously (Streit et al. 2001). Detection of the extracellularly recorded action potentials and further analysis were done off-line with the software package IGOR (WaveMetrics, Lake Oswego, OR) as described previously (Tschetter et al. 2001). The detected signals were fast-voltage transients (<4 ms), which correspond to single action potentials in neurons or axons (single-unit activity). These transients often appeared in clusters (multunit activity) originating from closely timed action potentials of several neurons or axons seen by one electrode. When they appeared at >250 Hz (= upper limit of temporal resolution of the detector), they could not be clearly separated from each other and therefore such activity was set by definition to 333 Hz (Tschetter et al. 2001). No attempt was made to sort spikes seen by one electrode. The selectivity of event detection was assessed using recordings obtained in the presence of tetrodotoxin (TTX, 1.5 μM) as a zero reference. The processed data were displayed as event raster plots, binned color-coded raster plots, and/or network activity plots. Event raster plots show the time markers of detected activity of each selected channel (e.g., Fig. 1C). Color-coded raster plots show the activity of each selected channel summed within a sliding time window of 30 ms, shifted by 1-ms steps, in the form of a rainbow color code (e.g., Fig. 2B). Network activity plots show the total activity of all selected channels summed within a sliding window of 30 ms, shifted by 1-ms steps (e.g., Fig. 1C). For the experiments investigating intrinsic activity (= asynchronous activity persisting after pharmacological blockade of fast synaptic transmission), only channels showing a frequency >0.1 Hz were considered as intrinsically active. This lower boundary corresponded to the maximal frequency observed in 22 dissociated cultures (i.e., 770 channels) investigated under TTX and corroborated data obtained from current-clamp recordings.

Whole cell patch-clamp recording and analysis

Intracellular voltage measurements were obtained from individual neurons in cultures on MEAs and glass coverslips using the whole cell patch-clamp technique (Hamill et al. 1981) with an Axoclamp 2B amplifier (Axon Instruments, Union City, CA). The patch pipettes were filled with a solution containing (in mM): K-glucolate, 100; KCl, 20; HEPES, 10; Mg-ATP, 4; Na2-GTP, 0.3; Na2-phosphocreatine, 10 (pH 7.3 with KOH). The electrodes had a resistance of 4–7 MΩ. No series resistance compensation was applied. Native resting membrane potentials were in the range of −40 to −70 mV. Cells with a potential less negative than −40 mV were discarded. For the measurements of sodium currents in voltage clamp, the patch pipettes were filled with Cs-based solution containing (in mM): CsCl, 120; HEPES, 10; Mg-ATP, 4; Na2-GTP, 0.3; Na2-phosphocreatine, 10 (pH 7.3 with CsOH), to minimize potassium currents. The bath solution was replaced by a 0 Ca–3 Mg solution containing (in mM): NaCl, 145; KCl, 4; MgCl2, 3; HEPES, 5; Na-pyruvate, 2; glucose at pH 7.4, to suppress synaptic transmission and calcium currents. Fast Na+ currents were elicited in neurons clamped at −60 mV with a depolarizing pulse of 500 ms at −20 mV after a 250-ms preconditioning pulse at −105 mV. The latter pulse was used to completely remove fast inactivation of the Na+ channels. Series resistance was compensated.
by 60–80%. The recordings were digitized at 6–10 kHz, visualized, and stored on computer using custom-made virtual instruments within LabVIEW (National Instruments) or pClamp software (Axon Instruments) when no simultaneous MEA recordings were made. The signals were analyzed off-line using custom-made programs in IGOR (WaveMetrics) and Clampfit software (Axon Instruments). The input resistance of the cells was calculated by fitting the steady-state current–voltage response curves from 10 hyperpolarizing voltage steps of various amplitudes made in the absence of fast synaptic transmission. Synaptic potentials were evoked by injecting short suprathreshold current pulses of 50 ms into one cell and recording in a second nearby cell. Alternatively, synaptic potentials were evoked by biphasic monopolar voltage pulses addressed to MEA electrodes (duration of 0.5 ms and amplitudes of 0.5 to 3 V). Monosynaptic transmission between two neurons was distinguished from polysynaptic transmission by their constant and short latencies. The predominant frequency of stimulation was 0.2 Hz, but for short periods of 20 s, the frequency was increased to 1–10 Hz (= test frequencies). A minimal period of 60–120 s between different test frequencies was maintained. The stimulation protocol was run automatically by a custom-made virtual instrument within LabVIEW (National Instruments). The depression of excitatory postsynaptic potentials (EPSPs) was determined by taking the average maximal rising slope of the last five to 20 EPSPs at a given test frequency and by normalizing this average to the average maximal rising slope before the train (i.e., at 0.2 Hz). Spike-frequency adaptation, spike height, and spike maximal depolarization rate were measured by injecting a series of 3-s current pulses of different magnitudes (20–200 pA) into the neurons, with pauses of 30 s between pulses of different magnitude. Action potentials were detected by setting a threshold voltage where the rate of change of membrane potential exceeded 10 mV/ms. Spike height was measured from the threshold voltage to the peak amplitude.

**Chemicals**

All drugs were bath applied. The following agents were used: d-APV (d-2-amino-5-phosphonopentanoic acid), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), phenytoin (5,5-diphenyl-2,4-imidazolidinedione), strychnine, tetrodotoxin (TTX) (all Sigma–Aldrich, Buchs, Switzerland), and bicuculline (1:5:9:R(—)-bicuculline methochloride, from Tocris, Anawa Trading SA, Wangen, Switzerland). Solutions of phenytoin and strychnin were made up in NaOH (final concentration of 8 µM) and DMSO (dimethylsulfoxide, final concentration of 0.003%), respectively. At the final concentration used in the experiments, DMSO and NaOH had no significant effect on intrinsic activity and bursting.

**Modeling**

The model is a generalization of the leaky integrate-and-fire model [referred to as the phenomenological Spike Response Model (SRMₚ) in Gerstner and Kistler (2002)], incorporating two additional simplified spike-frequency–dependent adaptation components as well as a Na/K pump component.

**MODEL OF ADAPTING NEURON.** The subthreshold dynamics of the membrane potential in a given neuron i obey

\[
V_i(t) = \eta(t - \hat{t}_i) + \sum_{j \in \mathcal{D}_{ij}} e_{ij}[t - (\hat{t}_j + d_{ij})] - \gamma(t)
\]

with \(e_{ij}(t) = w_{ij}e^{-t/\tau_m}\) where the kernel \(\eta(t)\) is the spike afterpotential; \(e_{ij}[t - (\hat{t}_j + d_{ij})]\) is the time course of the EPSP of neuron i evoked by the firing of presynaptic neuron j at time \(\hat{t}_j + d_{ij}\); and \(d_{ij}\) is the propagation delay. \(\hat{t}_i\) denotes the set of the neurons presynaptic to neuron \(i\), \(F_i\) is the set of all firing times of neuron \(j\), and \(\tau_m\) is the last firing time of neuron \(i\). The kernel \(\eta(t - \hat{t}_i)\) takes the value \(-1\) if \(0 \leq t - \hat{t}_i < 1\) and \(0\) otherwise. \(\gamma(t)\) is the Na/K pump component; \(w_{ij}\) is the strength of the synapse connecting presynaptic neuron \(j\) and postsynaptic neuron \(i\) and has Gaussian distribution \(N[w_{ij} / (\ell_{ij}^2/2)]\), where \(w_{ij}^2\) is drawn from a uniform distribution between 0 and \(w_{\text{max}}\). \(w_{\text{max}}\) is chosen so that a small fraction (roughly 10%) of the monosynaptic connections are strong enough to excite the presynaptic cell. \(\tau_m\) is the membrane time constant.

When EPSPs accumulate and drive the membrane voltage \(V_i\) across the dynamic threshold \(\theta_i(t)\), the neuron i initiates a spike and activates the postsynaptic neurons with various delays. If we call \(\theta_i^c\) the time of occurrence of the 4th spike of neuron \(i\), the value of \(V_i(\theta_i^c)\) is set to \(V_{\text{spike}}\). In what follows, let us define \(V_i(t)\) as the voltage dynamics excluding the voltage discontinuities arising from spikes. To account for the relative refractory period as well as for spike-frequency adaptation, \(\theta_i(t)\) has the following time course after a spike is emitted at time \(\hat{t}_i\):

\[
\theta_i(t - \hat{t}_i) = \theta_i^c + \theta, \exp[-(t - \hat{t}_i)/\tau_r] + \alpha_i(t) + \beta_i(t)
\]

where \(\theta_i^c\) is the reference threshold, \(\theta_i\) is a fixed value accounting for the relative refractory period, \(\tau_r\) is the relative refractory time constant, \(\alpha_i(t)\) is the first adaptation component, and \(\beta_i(t)\) is the second adaptation component. The reference thresholds among neurons are initially distributed according to the function \(N(1, (0.3)^2)\). When the voltage \(V_i(t) \geq \theta_i^c\)

\[
\alpha_i(t) = a \int_{\hat{t}_i}^{t} V_i(s) ds \\
\beta_i(t) = b \int_{\hat{t}_i}^{t} V_i(s) ds
\]

where \(a\) and \(b\) are constants. \(\theta_i^c\) is the last time the voltage crossed \(\theta_i\), and \(\hat{t}_i\) is the voltage dynamics excluding the spike trains. When \(V_i(t) < \theta_i^c\), the first adaptation \(\alpha_i\) decreases exponentially with a slow time constant \(\tau_r\), in an attempt to take the slow recovery from slow Na⁺ channel inactivation into account. The second adaptation \(\beta_i\) decreases exponentially with a fast time constant \(\tau_r\) to account for the removal of fast inactivation of Na⁺ channels. Whenever a spike is emitted, the Na/K pump component \(\gamma(t)\) is incremented by a constant \(c_r\). When \(V_i(t) < \theta_i^c\), \(\gamma(t)\) decreases exponentially with a slow time constant \(\tau_r\).

**NETWORK ARCHITECTURE.** The network is a one-dimensional chain of \(N = 200\) interacting excitatory neurons. The proportion of connectivity of the network (100% being a fully connected network) ranges between 10 and 20% and is estimated from experimental findings. The probability \(p_{ij}\) of connection between two neurons \(i\) and \(j\) follows a normal distribution \(N(0.5, \sqrt{N}/3)\). The “edge effect,” which is the cutoff of connections at the edges of the network, is dealt by mirroring the cut connections into the network. The connection delays are a function of the distance between two connected cells and are given by \(d_{ij} = d_{\text{max}} [1 - \exp(-2(i - j)/N)]\) ms, where \(d_{\text{max}}\) is the maximal delay between two connected cells in the network. Activity in the network is initiated by inserting a given percentage of intrinsically active cells into the network. Neurons with the lowest thresholds \(\theta_i^c\) are selected for this task. In such cells, when the voltage is below \(\theta_i^c/10\), it gradually increases until \(\theta_i^c\) is reached. The intrinsic firing frequency varies among cells with an average value corresponding to experimental findings.

The state of activity of all neurons is updated in parallel in discrete 1-ms timesteps. The network activity is monitored by plotting the number of active cells at each timestep. All simulations are done in IGOR (WaveMetrics).

**Statistics**

Averages values are reported as means ± SD, where \(n\) is the number of cultures. Statistics are based on paired or unpaired Student’s t-tests. For group comparisons with small sample sizes, the nonparametric Wilcoxon signed-rank and Mann–Whitney U tests
were used. Values of $P < 0.05$ were considered significant. A correlation was considered significant when its coefficient $r$ was significantly different from 0 ($t$-statistics, $P < 0.05$).

RESULTS

Results of this study are based on the analysis of 124 dissociated cultures. From these, 70 were investigated by MEAs, 42 by whole cell patch-clamp, and 12 by a combination of both methods. Thirteen organotypic cultures were additionally investigated by MEAs for comparative experiments.

Effects of low doses of riluzole on disinhibition-induced bursting in dissociated cultures

In dissociated and organotypic slice cultures of fetal rat spinal cord, rhythmic synchronous bursting develops spontaneously when glycine and $\gamma$-aminobutyric acid type A (GABA$_A$) receptors are blocked by strychnine and bicuculline, respectively (Ballerini and Galante 1998; Streit 1993; Streit et al. 2001; Tscherter et al. 2001). In a previous study, we showed that the full blockade of the persistent sodium current (INaP) with riluzole (5–20 $\mu$M) suppressed or strongly impaired such bursting by silencing intrinsic spiking neurons (Darbon et al. 2004). In the present study, we found in contrast that lower doses of riluzole (1–2 $\mu$M; low riluzole) had on average no effects on burst rate and burst duration in 15 cultures recorded with MEAs (7.1 ± 2.1/min in control vs. 7.2 ± 3.0/min with low riluzole and 1.8 ± 0.6 vs. 1.5 ± 0.8 s, respectively; Fig. 2A). On the other hand, low riluzole made the rhythm more variable and strongly changed the burst structure. The coefficients of variation (CVs) of both burst rate and burst duration were significantly increased with low riluzole (44.5 ± 15.2 vs. 28.4 ± 7.2%, $P = 0.002$, and 20.4 ± 8.1 vs. 9.7 ± 3.7%, $P = 0.005$, respectively). Low riluzole decreased the correlation usually found between the burst duration and the duration of the preceding interval (Streit et al. 2001). Indeed, the correlation coefficient $r$ decreased from 0.57 ± 0.2 to 0.24 ± 0.3 ($n = 15$, $P = 0.001$). However, it remained significantly different from zero ($P = 0.028$). The activity in the interval between the bursts was not changed with low riluzole (1.1 ± 0.9 vs. 1.5 ± 1.8 events/s). This latter result contrasts with whole cell patch-clamp recordings showing that low riluzole significantly depolarized the neurons in the intervals (from $-64.0 \pm 4.2$ to $-59.5 \pm 4.1$ mV, $n = 7$, $P < 0.001$).

Interestingly, although low riluzole had on average no effects on either burst rate or burst duration, it significantly decreased the activity within the bursts by roughly 40% (96.1 ± 48.0 vs. 162.1 ± 54.4 events/s, $P = 0.0003$). This decrease in burst activity resulted from a particular burst structure. As shown previously in disinhibited dissociated cultures (Streit et al. 2001), bursts consist of an initial wavefront spreading over the whole network, followed by a consistent network activation that gradually declines as a result of lower firing rates and activity extinction at some MEA electrodes (Fig. 2, A$_3$ and B, left). After addition of low riluzole, the time for the initial wavefront to reach maximal activation of the network was not significantly reduced (120.3 ± 33.0 ms in control vs. 111.5 ± 39.6 ms with riluzole, $n = 12$, $P = 0.58$). Similarly, the initial network recruitment (= the percentage of maximal activation of the electrodes; see METHODS) was not changed (95.9 ± 5.4 vs. 91.9 ± 8.4%). With low riluzole, however, the burst onset was followed by oscillations of activity (Fig. 2A$_2$, right). Indeed, clearly distinguishable oscillations (three to 14 within the bursts) were observed in 11 out of 15 cultures treated with low riluzole. In these cultures, the oscillation frequency gradually declined from around 4–7 to 2–4 Hz during the bursts (average: 4.5 ± 1.1 Hz). In the remaining four cultures, bursts were shorter and consisted of only one to three blurred oscillations at low frequency (2–3 Hz; data not shown). All selected electrodes were activated at each oscillation, suggesting that the activity propagated in the whole network each time (Fig. 2B, right). However, this did not rule out the possibility of firing interruption in a few individual neurons (e.g., whole cell recording in Fig. 2A$_2$, right) because spikes generated by different neurons could be seen by a single MEA electrode. To compare the relative contribution of the individual MEA electrodes to network activity in control and after low riluzole, we plotted the mean activity seen by the individual electrodes during 10 min in control versus low riluzole. A strong positive correlation was found ($r = 0.92$, $n = 10$; see Fig. 2C). This latter result strongly suggests that the spatial activity distribution was not changed with low riluzole.

Effects of low doses of riluzole on synaptic depression

The oscillations induced by riluzole in dissociated cultures resembled those described previously in organotypic cultures during disinhibition-induced bursting (Tscherter et al. 2001). In the latter system, oscillations were proposed to be based on synaptic depression (Senn et al. 1996; Streit 1993). Therefore we next investigated the effect of low riluzole on synaptic depression in dissociated cultures.

The effectiveness of synaptic transmission was investigated at different frequencies of stimulation, as reported previously for organotypic cultures (Streit et al. 1992). A first attempt to investigate synaptic transmission between pairs of neurons was done in 12 double-patch current-clamp experiments. In 12 pairs, we found no evidence of monosynaptic responses, suggesting a functional connectivity of $<20\%$ in dissociated cultures. These results do not confirm previous findings in mouse dissociated cultures where approximately one half of the cell pairs tested were connected (Nelson et al. 1981). Because of the low likelihood of finding connected pairs in the cultures, synaptic responses were then evoked by extracellular MEA stimulation and recorded in single cells. The advantage of such a method was that 68 different, fixed stimulation sites (= the number of electrodes on the MEA) could be tested in the same preparation. The disadvantage was that multiple cells could be activated by the extracellular stimulation, thus allowing the summation of the activity of several stimulated cells on the same postsynaptic cell. Nevertheless, this method significantly improved the chance of finding monosynaptic potentials.

We were constrained to evoke EPSPs under disinhibition (during the intervals between the bursts) because the high level of spontaneous activity in spinal cultures under control conditions would have strongly interfered with the external stimulation. Still, in this condition, one has to check carefully whether the bursting itself does not interfere with the measurements of synaptic transmission. For example, synaptic transmission could be depressed after the bursts and slowly recover.
during the interburst intervals. In this situation, the total activity during a given burst would determine the degree of depression in the following interval. Because burst activity was decreased by about 40% with low riluzole, a reliable comparison between EPSPs evoked under disinhibition and after addition of low riluzole would therefore have been compromised. Consequently, we first investigated the effect of disinhibited bursting on monosynaptic EPSPs evoked at 1 Hz during the intervals (Fig. 3A). As shown in the inset of Fig. 3A, the first evoked EPSP in the interval was very similar to the last evoked EPSP in the interval. Indeed, in a group of eight experiments, we found that the maximal rising slope of the first EPSPs in the intervals, normalized to the maximal rising slope of the first EPSPs in the intervals, was not statistically different (+3.6 ± 26.4%; see Fig. 3B). These findings exclude a mechanism of slow recovery from synaptic depression during the interburst intervals and confirmed our previous results obtained with spontaneous excitatory postsynaptic currents (EPSCs) (Darbon et al. 2002b).

Next, we varied the frequency of stimulation. A depression of the EPSP maximal rising slope for stimulus frequencies between 0.2 and 10 Hz was found in seven of eight experiments. The mean maximal rising slopes of the EPSPs at 5 and 10 Hz, normalized to the control values at 0.2 Hz, were 0.54 ± 0.32% (n = 7, P = 0.018) and 0.34 ± 0.29% (n = 6, P = 0.027), respectively (Fig. 3C). The mean latency, measured from the stimulus artifact to the EPSP onset, increased by 14% from 0.2 to 5 Hz, although the difference was not significant (7.6 ± 2.5 ms vs. 8.7 ± 2.4 ms, n = 7, P = 0.36). Interestingly, these results were comparable to those reported previously for organotypic cultures of the spinal cord (Streit et al. 1992), suggesting a similar depression pattern in both dissociated and organotypic cultures. More important, after addition of low riluzole the mean EPSP maximal rising slopes at the tested frequencies were not significantly different from control (Fig. 3D). In a group of six experiments, the mean EPSP maximal rising slopes at 0.2 and 5 Hz with low riluzole, normalized to control values, were 107.7 ± 28 and 105.2 ± 36%, respectively (see also Fig. 3E). The normalized mean delays at 0.2 and 5 Hz were 96.6 ± 5.4% and 98.3 ± 23.2%, respectively. Taken together, these results suggest that activity-dependent synaptic depression is not the key parameter underlying intraburst oscillations in spinal cultures.

**Role of intrinsic activity in the generation of intraburst oscillations**

We next tested the hypothesis that oscillations are based on low rates of intrinsic activity in the network. For this we first compared intrinsic activity under riluzole in dissociated cultures to that in organotypic cultures. Intrinsic activity was assessed with MEA recordings after full blockade of fast synaptic transmission (with bicuculline 20 μM, strychnine 1 μM, APV 50 μM, and CNQX 10 μM; see example in Fig. 4A) as described previously (Streit et al. 2001). As shown in Fig. 4B, low riluzole indeed decreased intrinsic activity by 80% (from 5.1 ± 4.2 to 1.1 ± 1.3 events/s, n = 6, P < 0.03). The proportion of active channels (the channels with event frequency >0.1 Hz; see METHODS) decreased by 58% (from 74.4 ± 15.8 to 33.5 ± 22.6%, P < 0.03). Interestingly, these low values with riluzole were not significantly different from those obtained in seven control organotypic cultures (Fig. 4, B and C). We were aware that these absolute values were certainly overestimated in both cultures because several axons/cells could be seen by one MEA electrode. This overestimation could be even higher for organotypic cultures, if we reasonably assume that these have a higher density of cells. However, the aim here was more to attain rough differences between cultures rather than exact values. In whole cell patch-clamp recordings in the absence of fast synaptic transmission, low riluzole had no effects on the membrane potential and input resistance (−55.3 ± 3.3 vs. −55.3 ± 3.3 mV, P = 0.89; 334.6 ± 67.5 vs. 342.4 ± 77.7 MΩ, P = 0.56, n = 6).

We next reduced intrinsic activity in dissociated cultures with low doses of phenytoin, another I_{NaP} blocker (Chao and Alzheimer 1995; Lampl et al. 1998; Segal and Douglas 1997), to see whether we could also induce intraburst oscillations. The doses were adjusted so that the reduction of intrinsic activity in the absence of synaptic transmission corresponded roughly to the reduction seen with low riluzole. As a result, we found that...
phenytoin 20 μM (low phenytoin) decreased intrinsic activity and the proportion of active channels by 89% (from 4.7 ± 4.1 to 0.6 ± 0.8 events/s, n = 5, P < 0.05) and 83% (from 62.5 ± 19.3 to 11.7 ± 14.2%, P < 0.05), respectively (Fig. 4, B and C). In whole cell patch-clamp recordings, as for low riluzole, low phenytoin had no effects on the membrane potential and input resistance (56.2 ± 3.6 vs. 55.3 ± 3.3 mV, P = 0.16; 327.4 ± 73.0 vs. 342.4 ± 77.7 MΩ, P = 0.83, n = 6).

Under disinhibition, low phenytoin significantly decreased the burst rate by 42.9 ± 24.1% (P < 0.05) in five of six dissociated cultures. In the remaining culture, the burst rate was increased by 35.6%. As with low riluzole, low phenytoin significantly increased the CV period (from 35.1 ± 10.0 to 51.5 ± 16.1%, n = 6, P < 0.05). However, in contrast to low riluzole, low phenytoin significantly decreased the burst duration (−59.5 ± 8.3%, n = 6, P < 0.03) and failed to induce intraburst oscillations (Fig. 5). Moreover, the activity in the bursts remained unchanged (−5.4 ± 8.5%). Lower doses of phenytoin (5 and 10 μM) did not change the pattern of activity within the bursts, whereas higher doses (50 μM) suppressed the bursting (n = 3, data not shown). Taken together, these results suggest that a reduction of intrinsic activity (by partial blockade of $I_{\text{NaP}}$) makes the rhythm more irregular, but is not responsible for the generation of intraburst oscillations in dissociated cultures.

Effects of low doses of riluzole and phenytoin on spike-frequency adaptation and depolarization block

We next sought to find out whether spike-frequency adaptation and early depolarization block may underlie rhythm generation (Fuhrmann et al. 2002). We thus investigated the effects of low riluzole and low phenytoin on repetitive firing in a total of 21 spinal neurons in the absence of fast synaptic transmission. As shown in Fig. 6A, neurons responded with repetitive firing to the injection of depolarizing pulses (ranging from 30 to 200 pA). The time course of the instantaneous firing rate was best fitted with a double exponential, separating early from later phases of adaptation (Fig. 6A, right). To “mimic” the bursts induced by disinhibition, 3-s current steps eliciting similar initial instantaneous firing frequencies (IFFs) as those measured at the onset of the disinhibited bursts were chosen. In

FIG. 4. Effects of low doses of riluzole [2-amino-6-(trifluoromethoxy)benzothiazole] and phenytoin (5,5-diphenyl-2,4-imidazolidinedione) on intrinsic activity and percentage of active electrodes in dissociated cultures in comparison to organotypic slice cultures. A: example of raster plots of intrinsic activity in control [bicuculline 20 μM, strychnine 1 μM, d-2-amino-5-phosphonovaleric acid (d-APV) 50 μM, and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 10 μM] and after addition of riluzole (2 μM) in a dissociated culture. Mean values of intrinsic activity (B) and percentage of active channels (C) in control (n = 11 cultures), after addition of riluzole (n = 6) or phenytoin (20 μM, n = 5) and in control organotypic slice cultures (n = 7). Only channels showing an activity >0.1 Hz were considered as intrinsically active. Control cultures for riluzole or phenytoin experiments were pooled. Data are means ± SD. *P < 0.05.

FIG. 5. Effects of low doses of phenytoin on disinhibition-induced bursting in a spinal-dissociated culture. Network activity plot during disinhibition (top) and after addition of 20 μM phenytoin (bottom). An enlarged burst is also shown in each condition. Note that contrary to riluzole, phenytoin failed to induce intraburst oscillations but significantly shortened the duration of the bursts.
eight disinhibited cultures, we found that the mean initial IFF at the burst onset was 53.9 ± 25.2 Hz. The mean initial IFFs at the burst onset after addition of low riluzole or low phenytoin were not significantly different from this control value (47.2 ± 18.4 Hz, n = 8, P = 0.61 and 51.3 ± 20.6 Hz, n = 5, P = 0.35, respectively). These results suggest that the initial burst wave fronts activating the network had similar strength in all three conditions. They confirmed our findings in MEA recordings showing similar network activation times and network recruitment at the burst onset during disinhibited and low riluzole bursting (see first section).

With low riluzole, repetitive firing elicited by the injection of the “burst-mimicking” current was blocked in 10 of 14 cells (see Fig. 6B₁). This firing block occurred between 0.05 and 1.25 s (mean: 0.46 ± 0.38 s) after the onset of current injection. In the remaining four cells, the firing lasted until the end of the 3-s current pulse. Conversely, low phenytoin blocked the firing in only one of five cells. In this cell, the block occurred 1.59 s after the onset of current injection. We next focused our analysis on the first 150–250 ms after the onset of current injection. This time window corresponded to the period of the infraburst oscillations induced by low riluzole. The adaptation occurring during this interval of time (= early adaptation) was estimated by calculating the difference between the initial IFF and the IFF at the sixth interval. As shown in Fig. 6B₂, we found that low riluzole significantly increased early adaptation by 26.5 ± 9.9% compared with control (n = 14, P < 0.0001), whereas low phenytoin had no effect (+1.5 ± 9.5%, n = 5, P = 0.69). During the same interval of time, the spike height with low riluzole significantly decreased by 26.7 ± 23.1% (P < 0.001) compared with control. As for early spike-frequency adaptation, spike height with low phenytoin remained unchanged (−3.9 ± 10.6%, P = 0.50). We investigated the effect of low riluzole and low phenytoin on the activation of the fast Na⁺ current (Fig. 7). Low riluzole slightly reduced the peak amplitude of the fast Na⁺ current (−10 ± 6%, n = 8, P = 0.01; see Fig. 7, A and C), whereas low phenytoin had no significant effect on the fast Na⁺ current (+12 ± 14.5%, n = 8, P = 0.10; see Fig. 7, B and C). Taken together, these results suggest that the rapid drop of network activity after the burst onset with low riluzole (see first oscillation of the network activity plot in Fig. 2A₂, right) arises from pronounced Na⁺ channel inactivation leading to early depolarization block in several neurons. To see whether the time characteristics of the process of depolarization block and recovery were in the range of the infraburst oscillations, we then investigated the recovery from inactivation of Na⁺ conductances with low riluzole in eight cells in the absence of synaptic

![Image](https://via.placeholder.com/150.png?text=Image)
transmission. This was done by “mimicking” intraburst oscillations by injecting four successive depolarizing pulses of current (ranging from 100 to 300 pA) of 150-ms duration at 5 Hz. As shown in Fig. 8A, the first pulse elicited few spikes followed by a depolarization block. More important, the following pulses were still able to elicit spikes, although these were fewer (Fig. 8B). These results are in good agreement with MEA recordings showing that the network activity was decreased between the first and the second intraburst oscillation (Fig. 2A, right). Besides, the spike maximum rate of depolarization and spike height were significantly decreased between the first and the second pulses (−22.5 ± 13.4%, \( P < 0.05 \), and −22.6 ± 15.6%, \( P < 0.05 \), respectively; Fig. 8C). Altogether, these results suggest that recovery from depolarization block between two intraburst oscillation waves is sufficient to allow spikes to be regenerated.

In line with the hypothesis of depolarization block, data of six cells showed that spike-frequency and spike height during low riluzole bursting, normalized to control values during disinhibited bursting, were decreased by 24.5 ± 19.9% (\( P < 0.05 \)) and 13.0 ± 11.8% (\( P < 0.05 \)), respectively, 120 ms after the burst onset (Fig. 9). In these cells, a positive correlation between the IFF and the maximum rate of depolarization during the action potentials was found in the bursts (\( r = 0.74 \pm 0.17, P < 0.001 \)). This result suggests that inactivation of Na⁺ channels mainly controls the decrease in firing frequency during disinhibited bursts. A loss of this correlation is expected if activity-dependent synaptic depression or an increase in outward currents controls the firing frequency during riluzole-induced oscillations. However, this correlation remained strong after addition of low doses of riluzole (\( r = 0.62 \pm 0.18, P < 0.001 \)). Taken together, these findings suggest that inactivation of Na⁺ channels rather than other mechanisms mainly controls the decrease of the firing frequency during riluzole-induced oscillations.

**Modeling**

To further investigate the role of depolarization block in the generation of network oscillations, we simulated a random excitatory network of model neurons, incorporating a depolarization block mechanism. Synaptic connectivity and synaptic delays were estimated from the patch-clamp experiments. The percentage and the activity of the intrinsically spiking cells inserted into the network were set to match the spontaneous low-rate asynchronous background activity observed in the MEA experiments (see data in Fig. 4). The major accommodation mechanisms involved in disinhibited bursting that we described previously in dissociated cultures—the upregulation of Na/K pump (Darbon et al. 2003) and slow Na⁺ inactivation (Darbon et al. 2004) during the bursts—were included in the model in a simplified form. Briefly, Na⁺ slow inactivation was simulated by progressively increasing the threshold of the cell by the voltage-dependent parameter \( \alpha \), provided that the cell was sufficiently depolarized. The Na/K pump was simulated by decrementing the voltage of the cell by the pump parameter \( \gamma \) whenever the cell emitted a spike (see METHODS). Both \( \alpha \) and \( \gamma \)
had a slow relaxation time. The depolarization block was achieved by the parameter $\beta$, computed like $\alpha$, but with a faster relaxation time (see Methods and Table 1).

In the absence of synaptic transmission, depolarizing the model neurons with 500-ms-long suprathreshold voltage steps elicited repetitive firing with adaptation, in agreement with experimental findings (Fig. 10A$_1$, left). Note, however, that no attempt was made to reproduce the decrease in spike height seen in the experiments. During the pulse, $\alpha$ and $\gamma$ progressively built up, then slowly relaxed after the pulse (Fig. 10A$_2$, left). To achieve repetitive firing throughout the pulse, $\beta$ was made minimal by setting a low value to factor $b$ (Table 1). In the presence of synaptic transmission, and although individual neurons in the network were not intrinsic bursting cells, asynchronous activity evolved into a synchronous network bursting for sufficiently high synaptic coupling strength $w_{\text{max}}$ (Fig. 10B$_1$). During such bursts, individual neurons responded with a depolarizing plateau of declining amplitude with numerous spikes riding on it. An afterhyperpolarization after the bursts was observed in several neurons and reflected the buildup of $\gamma$ (upregulation of the Na/K pump) during the bursts (see Fig. 10B$_2$). $\gamma$ was mainly responsible for setting the length of the interburst intervals. These results are in good agreements with those reported for disinhibited bursting in spinal cultures. Nevertheless, the simulated rhythm was highly regular and no correlation between burst duration and preceding interval was found. This result reflects the deterministic nature of the model. Such rhythm could nonetheless be compared with the fast and highly regular rhythm induced by $N$-methyl-d-aspartate in disinhibited dissociated cultures for which no correlation was reported (Legrand et al. 2004).

Strengthening the adaptation parameter $\beta$ by increasing factor $b$ led to early depolarization block in stimulated neurons in the absence of synaptic transmission (Fig. 10A$_1$, right). Such conditions were intended to simulate the depolarization block observed in synaptically isolated neurons with low riluzole (see Fig. 6B$_1$). Interestingly, at the network level the depolarization block turned bursts of persistent activity into bursts of oscillatory activity (Fig. 10B$_1$, right). The values for the pump parameter $\gamma$ were lower compared with control condition because of the lower firing rate during the oscillatory bursts (compare left and right panels in Fig. 10B$_1$). To maintain the burst rate similar to control levels, the percentage and the activity of intrinsically spiking cells had to be strongly reduced. These results were in good agreement with those obtained from MEA recordings with low riluzole (see Fig. 4).

Taken together, these qualitative results clearly support the hypothesis developed from MEA and whole cell recordings that depolarization block is a critical factor for generating intraburst oscillations in disinhibited random networks.

**Discussion**

The principal finding of this study is that low doses of the sodium channel blocker riluzole turn bursts of persistent activity into bursts of oscillatory activity in spinal-dissociated cultures under disinhibition. Riluzole-induced oscillations are a network-driven phenomenon dependent on strong spike-frequency adaptation leading to early depolarization block in several neurons. Such a phenomenon thus appears to be a very elementary form of synchronized network discharge, which is useful for understanding the basic mechanisms responsible for the generation of oscillatory activities in hyperexcitable networks under physiological and pathophysiological conditions in the CNS.

**Comparing low riluzole rhythm with disinhibited rhythm**

We previously showed that the disinhibited rhythm in cultures of spinal neurons is based on intrinsic spiking in roughly 30–50% of the neurons, network recruitment by recurrent excitation through glutamatergic synaptic transmission, and a network refractory period after the bursts (Darbon et al. 2002b; Streit et al. 2001). We showed that the upregulation of the Na/K electrogenic pump during the burst is responsible for the refractory period after the burst by its hyperpolarizing action (Darbon et al. 2003). We also demonstrated that the blockade of $I_{\text{NaP}}$ by riluzole ($10–20 \mu M$) suppresses the bursting by silencing the intrinsic spiking cells and by suppressing network recruitment. From the latter findings, we concluded that $I_{\text{NaP}}$ underlies intrinsic spiking, which, by recurrent excitation, generates the bursting activity (Darbon et al. 2004). Finally, we proposed that slow inactivation of $I_{\text{NaK}}$ by inducing spike-frequency adaptation during prolonged depolarization, is the major mechanism leading to burst termination at the network level (Darbon et al. 2004).

In the present study, partial blockade of $I_{\text{NaP}}$ with low concentrations of riluzole (low riluzole; 1–2 $\mu M$), although profoundly changing the burst structure, had no effect on the burst rate, on the burst duration, or on the asynchronous activity in the intervals. These results are somewhat surprising if one considers that low riluzole decreased intrinsic activity by roughly 80% in the absence of fast synaptic transmission in MEA recordings (Fig. 4). Indeed, such a dramatic reduction in the source of intrinsic activity should a priori slow down the rhythm and decrease the asynchronous activity in the interburst intervals. This discrepancy, however, can be explained if one takes the bursting mechanisms into account. As a consequence of the intraburst oscillations induced by low riluzole, the activity within the bursts decreased by about 40%. In such a case, the Na/K pump is expected to be less upregulated during the bursts than in a control. Consequently, less hyperpolarization is expected during the interburst intervals. This is indeed what we observed in whole cell patch-clamp experiments, in which low riluzole depolarized the cells by about 5 mV in the intervals. This value corresponds to a twofold reduction of the hyperpolarization normally produced by the upregulation of

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Disinhibited Bursting</th>
<th>Low Riluzole Bursting</th>
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<tr>
<td>$N_c$</td>
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<td>200</td>
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<tr>
<td>$\xi_\text{max}$</td>
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<td>$w_{\text{max}}$</td>
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<td>$V_{\text{spike}}$</td>
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<td>$T_m$</td>
<td>20 ms</td>
<td>20 ms</td>
</tr>
<tr>
<td>$a$</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>$\tau_\alpha$</td>
<td>0.0003</td>
<td>0.0003</td>
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<tr>
<td>$\tau_\beta$</td>
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<td>200 ms</td>
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<tr>
<td>$b$</td>
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</tr>
<tr>
<td>$\tau_\pi$</td>
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<td>3 ms</td>
</tr>
<tr>
<td>$c$</td>
<td>0.0002</td>
<td>0.0002</td>
</tr>
<tr>
<td>$\tau_p$</td>
<td>1.200 ms</td>
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the Na/K pump during disinhibited bursting (Darbon et al. 2002b, 2003). This reduction may be even larger in the intrinsically spiking cells if one considers the direct hyperpolarizing action of riluzole on these cells (Darbon et al. 2004). The reduction of intrinsic spiking with low riluzole was therefore fully counterbalanced by the depolarization of the cells during the intervals as a result of the lower Na/K pump activity during the bursts. Consequently, the asynchronous activity during the intervals and the burst rate were maintained at control levels. It is interesting to note that with low riluzole the asynchronous activity during the interburst intervals was comparable to the intrinsic activity measured in the absence of fast synaptic transmission (1.5 ± 1.8 vs. 1.1 ± 1.3 events/s). In control conditions, in contrast, this asynchronous activity was on average five times smaller than the intrinsic activity. Altogether, these findings suggest that the Na/K pump was only weakly upregulated during low riluzole bursting. This hypothesis is supported by the computational model (see pump parameter γ in Fig. 10B2).

Population oscillations

The oscillations within disinhibited bursts with low riluzole were a network-driven phenomenon because synaptically isolated neurons clearly lacked intrinsic oscillatory properties (Fig. 6). The frequency of these oscillations gradually decreased during the bursts from around 4–7 to 2–4 Hz. Such frequency was not correlated with the length of the preceding intervals (personal observations), suggesting that the underlying mechanisms had fast kinetics and fully recovered during the interburst intervals. Single neurons usually contributed with several spikes to each network oscillation (Fig. 9). The spikes were not synchronized among neurons, but the firing rate of the neurons displayed synchronous modulation during the bursts.

There are obvious similarities between these riluzole-induced intraburst oscillations and the intraburst oscillations seen under disinhibition in organotypic slice cultures of fetal rat spinal cord (Tschetter et al. 2001) as well as in the isolated spinal cord of the newborn rat (Bracci et al. 1996). In organotypic slice cultures, riluzole intensifies the oscillations (personal observations), thus strengthening our view of a common mechanism underlying this phenomenon in different culture systems. In both culture systems and in the isolated spinal cord, the oscillation frequency decreases from around 5–8 to 1–3 Hz during the bursts. In the isolated spinal cord, the frequency was shown to be insensitive to large changes in the membrane potential of the cells, suggesting that the oscillations have a network origin (Bracci et al. 1996). In this preparation, the oscillations were proposed to be based on the activation of the Na/K pump (Ballarini et al. 1997). Nevertheless, the oscillations were shown later to reappear at lower frequency after initial suppression during blockade of the pump (Rozzo et al. 2002). In spinal slice cultures, blocking the Na/K pump initially causes a fusion of bursts with ongoing oscillations and only in a late phase the disruption of oscillations (Darbon et al. 2003). Thus the Na/K pump, although it is involved in the generation of oscillations, does not seem to be critical for it. In the isolated embryonic chick spinal cord, network-driven spontaneous episodes are also composed of oscillatory activity at around 1 Hz and are proposed to be based on synaptic depression (Landmesser and O’Donovan 1984; O’Donovan 1999). It is interesting to note that, at this early stage of development, the classical inhibitory neurotransmitters GABA and glycine are functionally excitatory (Sernagor et al. 1995; Wu et al. 1992) and that both glutamate receptor types are transiently overexpressed (Jaqwec et al. 1995; Kalb et al. 1992). Early developing spinal networks are therefore in a hyperexcitable state that may resemble, to some extent, the state after disinhibition.
hition. In other parts of the CNS, for example in the neocortex, hyperexcitable networks also share the tendency to oscillate during periods of intense recurrent synaptic activity (Castro-Alamancos and Rigos 2002; Steriade and Contreras 1998). Therefore oscillations in the frequency band of 1–14 Hz, occurring during periods of intense recurrent excitation, seem to be a fundamental property of hyperexcitable networks that does not necessarily depend on a specific cellular or network origin.

Activity-dependent synaptic depression

Activity-dependent synaptic depression with fast kinetics was previously proposed to underlie intraburst oscillations in organotypic slice cultures of fetal rat spinal cord as well as in the embryonic chick spinal cord (Streit 1993; Tabak et al. 2000). In the rat spinal cord, depression was suggested to underlie the oscillations appearing during blockade of the Na/K pump (Rozzo et al. 2002). In spinal cord slice cultures, this depression hypothesis is based on the following findings. First, synaptic efficacy between dorsal root ganglion cells and motoneurons was shown to be strongly depressed in a frequency-dependent way with stimulation >1 Hz (Streit et al. 1992). Second, a similar pattern of depression of EPSP amplitudes was found within disinhibited bursts (Streit 1993). Finally, in a mean-field model, Senn and coworkers (1996) demonstrated that, in a purely excitatory network with random connections, the frequency of oscillations is modulated by network connectivity and by the recovery time constant of synaptic depression. Therefore we investigated activity-dependent depression in dissociated cultures and tested whether this was increased after addition of low riluzole.

Riluzole was previously found to have multiple effects on ion channels, in particular inhibition of voltage-gated Na+ channels (Benoit and Escande 1991; Darbon et al. 2004; Urbani and Belluzzi 2000; Zona et al. 1998). At low micromolar doses similar to those used in the present study, riluzole was shown to increase paired-pulse EPSC depression in dissociated hippocampal cultures (Prakriya and Mennerick 2000) and decrease kainate-induced currents in spinal motor neurons in culture (Albo et al. 2004).

Unexpectedly, we found that synaptic depression in dissociated cultures is comparable to that reported in organotypic slice cultures (Streit et al. 1992). Why then are there no intraburst oscillations in dissociated cultures? We propose that, although synapses show strong activity-dependent depression, incoming synaptic potentials during disinhibited bursts remain strong enough, resulting from high synaptic weights and sufficient network connectivity, so as to maintain a continuous firing rate. Importantly, activity-dependent synaptic depression remained unchanged after addition of low riluzole. This result suggests that synaptic depression is not the key parameter underlying intraburst oscillations in dissociated cultures in the presence of low riluzole.

Low intrinsic activity

We previously showed in spinal cultures that intrinsic spiking cells are highly excitable cells that are rapidly recruited into bursting and respond with high spike rates during the bursts (Yvon et al. 2005). Decreasing the fraction of such cells could prevent a high, self-sustained level of recurrent activity during the bursts and thus promote oscillatory activity. Furthermore, Latham et al. (2000) showed in a theoretical study that by reducing the number of intrinsically active cells networks switch from a steady firing to a bursting mode.

We previously showed in spinal cultures that \( I_{\text{NaP}} \) is the main source of intrinsic spiking (Darbon et al. 2004). Riluzole (10–20 \( \mu \)M) fully blocks \( I_{\text{NaP}} \) and therefore silences the intrinsic spiking. In the present study, we showed that low riluzole (1–2 \( \mu \)M) reduces the fraction and the activity of intrinsic spiking cells in dissociated cultures by roughly 60 and 80%, respectively, indicating that \( I_{\text{NaP}} \) is only partially blocked. For comparison, in cortical and mesencephalic trigeminal neurons, riluzole 2 \( \mu \)M was shown to reduce \( I_{\text{NaP}} \) by about 50% (Urbani and Belluzzi 2000; Wu et al. 2005). In a recent study, Kuo et al. (2006) reported a similar decrease in motoneurons in culture. Interestingly, the level of intrinsic activity in the dissociated cultures with low riluzole was comparable to the level in organotypic slice cultures under control conditions (Fig. 4). The reason for a lower intrinsic activity in the slice cultures is not known at present. By reducing intrinsic activity with low phenytoin (20 \( \mu \)M) to similar levels as with low riluzole, we tested whether low intrinsic activity by itself was responsible for the generation of intraburst oscillations in spinal cultures. It is interesting to note that, in dissociated cultures of cortical neurons and hippocampal neurons, about 30 \( \mu \)M phenytoin produces half-block of \( I_{\text{NaP}} \) (Chao and Alzheimer 1995; Segal and Douglas 1997). These findings suggest that low riluzole and low phenytoin decreased \( I_{\text{NaP}} \) by a similar amount in our cultures. In contrast to low riluzole, however, we found that phenytoin failed to induce intraburst oscillations, suggesting that low intrinsic activity by partial blockade of \( I_{\text{NaP}} \) is not responsible for the generation of intraburst oscillations in spinal cultures. The major difference between the effects of phenytoin and riluzole on the cellular level is that riluzole increases spike-frequency adaptation and thereby causes an earlier depolarization block. In contrast, phenytoin has only a minor effect on spike-frequency adaptation (see Fig. 6). This finding led us to the assumption that early depolarization block is a critical factor for the generation of oscillations.

Repetitive firing and depolarization block

In the hippocampus in vivo a mechanism of depolarization block of spike generation in interneurons was suggested to underlie the emergence of oscillatory activity during epileptic afterdischarges (2–6 Hz) (Bragin et al. 1997). A strong depolarization block was also shown to accompany termination of epileptiform activity waves in disinhibited neocortical slices (Pinto et al. 2005). We previously showed in disinhibited dissociated cultures that about 25% of the neurons present a strong depolarized plateau during disinhibited bursts (Darbon et al. 2002b). These “plateau” cells receive strong excitatory inputs that rapidly inactivate Na+ channels, leading to a cessation of firing (depolarization block) within the first 100 to 200 ms of the bursts. A possible cause of intraburst oscillations in spinal cultures could be a higher fraction of such “plateau” cells. In dissociated cultures, this could be the result of more pronounced Na+ inactivation arising from low riluzole and, in organotypic cultures, from higher network connectivity.
with the former hypothesis, we showed previously in dissociated cultures that riluzole (10–20 μM) leads to cessation of repetitive firing in current-injected neurons in the absence of fast synaptic transmission (Darbon et al. 2004). In the present study, we found that low riluzole had similar effects when “mimicking” disinhibited bursts with current injection. A reduction or suppression of repetitive firing by similar doses of riluzole was previously shown in other types of neurons and was attributed to the action of riluzole on $I_{\text{NaP}}$ (Centonze et al. 1998; Urbani and Belluzzi 2000). However, in contrast to these and other recent findings (Harvey et al. 2006; Kuo et al. 2006), the effects of riluzole in our study were likely attributable to the interaction of riluzole with the fast Na$^+$ current. Several lines of evidence support this suggestion. First, low riluzole strongly increased the early phase of spike-frequency adaptation and led to a cessation of firing in both intrinsically and nonintrinsically spiking cells. We previously showed that the latter cells contain no $I_{\text{NaP}}$ (Darbon et al. 2004); therefore these effects do not depend on $I_{\text{NaP}}$. Second, low riluzole led to a strong decrease in spike amplitude during repetitive firing, suggesting that Na$^+$ channels rapidly and strongly inactivated. Third, low phenytoin had no such effect on the time course of spike-frequency adaptation, whereas it only slightly decreased spike amplitude toward the end of the current pulses. This was also true for a higher drug concentration (50 μM; personal observation), consistent with previous findings in hypoglossal motoneurons (Zeng et al. 2005). Finally, possible effects of riluzole on high-voltage–activated Ca$^{2+}$ and K$^+$ channels occur at higher concentrations than those used in the present study (Huang et al. 1997; Zona et al. 1998). The action of riluzole on the fast Na$^+$ was extensively studied. Low concentrations of riluzole were shown to stabilize Na$^+$ channels in the inactivated state, with more efficient block at high stimulation frequencies (Benoit and Escande 1991; Hebert et al. 1994; Song et al. 1997). In addition to its effect on $I_{\text{NaP}}$, phenytoin was also shown to affect the transient Na$^+$ current (Kuo and Bean 1994; Lampl et al. 1998). However, this drug has a lower affinity than does riluzole for the inactivated state (Kuo and Bean 1994). When studied with trains of action potentials, block by phenytoin is more pronounced late in the train (Adler et al. 1986; McLean and Macdonald 1983). This effect was also reflected in our experiments by the slight decrease in spike amplitude toward the end of the current pulse.

In summary, we propose the following mechanisms for the generation of intraburst oscillations in spinal cultures: 1) The strong depolarization of neurons during disinhibited bursting progressively inactivates Na$^+$ channels, particularly in the “plateau” cells that are presumably the best connected to the network. The proportion of such cells is significantly increased with low riluzole in dissociated cultures, whereas it may be naturally high as the result of higher network connectivity in organotypic slice cultures. This reduces the amplitude of action potentials leading eventually to a depolarization block in several cells. 2) This phenomenon, if widespread enough among neurons, decreases recurrent excitation to a level where it cannot be self-sustained in the network. As a result, recurrent excitation terminates and the neurons repolarize. 3) During the repolarization phase, Na$^+$ channel inactivation is removed, thus allowing neurons to resume their firing. 4) When a critical number of neurons that fire is reached, neurons are again able to trigger a new intraburst oscillation by recurrent excitation. 5) This process will continue until accommodation mechanisms such as slow inactivation of $I_{\text{Na}}$ reach definite thresholds so as to terminate the bursts.

We hypothesize that similar mechanisms could underlie, in part, the generation of oscillatory activity in hyperexcitable networks of various origins during periods of intense recurrent activity.

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