RHYTHM GENERATION IN SPINAL CULTURES: IS IT THE NEURON OR THE NETWORK?

Jürg Streit, Anne Tscherter, and Pascal Darbon

Institute of Physiology, University of Bern, Bern, Switzerland

from

Advances in Network Electrophysiology Using Multi-Electrode Arrays. Taketani, M. and Baudry, M. New York, Springer, 2005, ***in press***

Table of contents:

1. RHYTHM GENERATON IN NEURAL NETWORKS *IN VITRO*: WORTH STUDYING?

1.1. Rhythm generation in intact preparations

1.1.1. Rhythms as an important feature of CNS function.

Neural networks of many regions of the CNS are able to generate synchronized rhythmic activity. In humans rhythmic cortical activity has been recorded for years with electroencephalography (EEG). The various frequency bands which are observed in these recordings are associated with different states of consciousness (Steriade, 2001). In the hippocampus, rhythmic activity has been related to long term potentiation and memory functions (Vertes and Kocsis, 1997). The release of neuropeptides in the hypothalamus is controlled through rhythmically active neural networks (Kwiecien and Hammond, 1998). Finally, repetitive muscle contractions which occur during respiration, locomotion, or scratching are controlled by rhythmically active neural networks in the brainstem and the spinal cord (Grillner *et al*., 1998; Rekling and Feldman, 1998). All these examples show on the one hand that rhythmic activity in neural networks underlies many of the specific CNS functions and on the other hand they suggest that the capability for rhythm generation must be a fundamental property of neural networks. In principle there are two ways a network generates rhythms: It may be based on a well defined circuit, which is usually composed of excitatory and inhibitory cells, or rhythm generation depends mainly on the cellular properties of the neurons in the network while the circuit structure is of lesser importance. A mechanism of the second type is thought to underlie population bursting: A network is activated through the positive feedback of recurrent excitation and silenced by one or several accommodation mechanisms. It is the aim of this chapter to present some results concerning the mechanisms involved in rhythm generation, which occurs in networks of cultured spinal neurons. Such networks are at least partially grown *in vitro*: In the case of the dissociated cultures they are entirely re-grown from randomly seeded cells. In the case of organotypic slice cultures they develop out of a premature network in the fetal spinal cord. Both types of networks therefore have different levels of circuit structure. Comparing the types of rhythmic activity generated in these networks will reveal some emergent properties of "random" neural networks for rhythm generation.

What are random neural networks? We define them as networks of dissociated neurons, in contrast to networks in slice cultures, acute slices and intact spinal cord, which have increasing levels of complexity of circuit structure grown *in vivo.* To evaluate the findings gained in culture in terms of their relevance for rhythm generation in vivo, we first briefly review some of the most important findings about rhythm generation and fictive locomotion in the intact spinal cord.

1.1.2 Fictive locomotion in the cat spinal cord

The neuronal system generating the stereotypic movements characteristic for locomotion is composed of three parts: first, of a supraspinal part that is responsible for initiating locomotion and for maintaining a certain degree of drive, second of the spinal networks that generate motor patterns, and third, of sensory feedback that adapts the motor pattern to external events. More than 100 years ago, Sherrington (Sherrington, 1898) observed that cats and other mammals can perform locomotor movements of the legs after a complete transection of the spinal cord. He proposed that this activity may be produced in mammals by a chain of reflexes, requiring afferent inputs for its maintenance. However, in 1911 Brown observed locomotor movements in cats after spinal section even when the dorsal roots were cut bilaterally (Brown, 1911). With these experiments he demonstrated that neuronal networks in the spinal cord deprived of sensory inputs and supraspinal influences can generate a co-ordinated rhythmic motor output. Such rhythmic alternating activity in the motoneuron pools of flexor and extensor muscles and also on opposite sides of the isolated spinal cord is called fictive locomotion. It is now clear that the autonomous spinal networks providing this activity – later called central pattern generators (CPGs) – are found in all vertebrates, probably including humans (Dietz *et al*., 1998).

The CPGs have been activated experimentally in three different ways: first, by stimulation of sensory afferents, second, by supraspinal stimulation, and third, by pharmacological activation. Sensory stimuli can trigger the CPGs in high decerebrated cats, because locomotion can simply be initiated by moving the treadmill belt on which the cat is standing (suspended with a harness). In addition, tonic stimulation of the dorsal roots can evoke locomotion (for review see (Barbeau *et al*., 1999). Spinal CPGs can also be activated by descending reticulospinal pathways. In high decerebrate

cats, locomotion can be initiated by electrical stimulation of the mesencephalic locomotor region (MLR) (Shik and Orlovsky, 1976). The speed of locomotion as well as the preferred gaits (walking, trotting or galloping) can be adjusted by modifying the strength of the stimulation or the speed of the treadmill. Lundberg and Jankowska (Jankowska *et al.*, 1967) were the first to show that rhythms can also be evoked pharmacologically by the application of L-dopa. This dopamine precursor can activate fictive locomotion in the paralyzed spinal cat. Many other neurotransmitters have been shown to either activate or modulate CPG rhythms in a state dependent way (for review see (Rossignol *et al.*, 2001).

From the early experiments in the cat the half-center model was proposed to describe CPG function. In this model rhythm generation and alternation are explained by the reciprocal inhibition of two half-centers (for the left and the right side or for flexors and extensors) through crossing inhibitory axons. Later it was shown that rhythm generation but not alternation persisted in the presence of blockers of inhibitory synaptic transmission. These and other experiments led to the new hypothesis of coupled oscillators (Grillner and Zangger, 1979). According to this hypothesis, which is still favoured, CPGs are composed of several oscillator networks, which are functionally independent in terms of rhythm generation. Pattern generation by such networks results from the appropriate phase coupling among the oscillator networks.

1.1.3 The lamprey model

One problem in the cat experiments is the difficulty to get direct experimental access to the CPGs in the spinal cord. Therefore, other preparations like the spinal cord of the turtle (Mortin and Stein, 1989) or embryonic preparations like that of the tadpole (Dale, 1995), or the zebrafish embryo (Fetcho and O'Malley, 1995) were developed to obtain deeper insight into the cellular basis of CPGs. An ideal preparation is the lamprey, a primitive vertebrate with a flat spinal cord and brain stem , which can be maintained in vitro for several days. The lamprey swims by producing an undulating wave based on the alternating activation of motor units on the left and the right side of each segment along the body. Fictive swimming can be evoked in the isolated spinal cord of the lamprey by excitatory amino acids like glutamate or N-methyl-D-aspartic acid (NMDA). The CPGs in the lamprey spinal cord have therefore been investigated and analyzed in great detail. Furthermore, the results from these studies have been used to design computer models of the lamprey CPG, the performance of which could be compared to experimental findings. Much of this work – experimental and computational – has been done in the group of S. Grillner and is reviewed in numerous papers (Grillner *et al.*, 1991; Grillner *et al.*, 1998; Grillner, 2003). Here we will mention just two of the new findings revealed in this model. First, it was shown that the CPG networks are composed of excitatory interneurons, which use glutamate as neurotransmitter and project to the ipsilateral side and inhibitory interneurons, which use glycine and project to the contralateral side. Second, a cellular pacemaker mechanism was found which is based on Ca^{2+}- inflow through NMDA channels as a depolarizing mechanism and the subsequent activation of Ca^{2+} dependent K^{+} currents as the hyperpolarizing mechanism. Other channels, like voltage dependent Ca^{2+} channels, contribute to this pacemaker mechanism. The resulting rhythm can be modulated by various agents including 5-hydroxytryptamine (5-HT).

1.1.4. Development of pattern generators: The chick spinal cord

The chick spinal cord is the preparation mainly been used to investigate the development of CPGs. The isolated spinal cord of the chick embryo displays spontaneous episodes of rhythmic activity. Such spontaneous activity is a characteristic feature of developing circuits in many parts of the CNS. It is remarkably similar in tissues as diverse as the hippocampus, the retina and the spinal cord (O'Donovan and Rinzel, 1997). In the retina this activity is known to be important for the formation and refinement of neuronal projections. In the spinal cord, however, little is known about its role in development. Nevertheless, some effort has been taken mainly by the group of Michael O'Donovan to reveal the mechanisms involved in such embryonic rhythm generation (O'Donovan, 1999). They found that these rhythms can be mediated not only by glutamate receptors (of the (+/-)-α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid hydrobromide (AMPA) and NMDA types) but also by glycine and γ-aminobutyric acid (GABA) A receptors. These normally inhibitory receptors have excitatory effects at the embryonic age because the chloride equilibrium potential lies above threshold. This seems to be a general principle in development, which was first discovered in hippocampal networks (Ben-Ari, 2001). Later, the same phenomenon was demonstrated in the developing rat spinal cord, where rhythm generation first depends on cholinergic, then on GABA

A/glycine/NMDA and finally mainly on AMPA receptors (Milner and Landmesser, 1999). The spontaneous rhythms seen in the spinal cord of the chick embryo have been modelled on the computer based on the two parameters of hyperexcitability and activity dependent depression (Tabak *et al*., 2001). Since theses rhythms share many properties with the bursting induced in spinal cultures by disinhibition, we will discuss this model more extensively later in this chapter.

1.1.5 CPGs in rodent spinal cord (rat and mouse)

The isolated spinal cord of the neonatal rat, introduced in 1987 by Kudo and Yamada, has now become a standard preparation to study mammalian CPGs. The rat is quite immature at birth and a rapid maturation of motor behaviour takes place during the first two postnatal weeks. Although rats younger than postnatal day 12 are unable to walk because of postural weakness (Westerga and Gramsbergen, 1990) their CPGs seem to function, because they can already swim a few hours after birth (Bekoff and Trainer, 1979). The CPGs in the neonatal rat spinal cord proved to share many of the properties previously described in the cat and in the lamprey spinal cord. Rhythmic activity that alternates between the two sides of the spinal cord (and between ipsilateral flexors and extensors) could be activated by various neurotransmitters as well as by supraspinal or afferent stimulation (Atsuta *et al*., 1990; Cazalets *et al*., 1992; Kiehn and Butt, 2003; Kudo and Yamada, 1987; Magnuson and Trinder, 1997; Marchetti *et al*., 2001). The most robust rhythms are induced by a combination of NMDA and 5-HT. The general scheme of lamprey CPGs with ipsilateral projecting excitatory glutamatergic and commissural inhibitory glycinergic interneurons seems to be maintained in the rat, although the situation is certainly more complicated (Beato and Nistri, 1999). New approaches for a detailed characterization of the mammalian locomotor CPG have recently been introduced by combining genetic tools with electrophysiology and anatomy in the isolated mouse spinal cord (Kiehn and Butt, 2003). Using such methods Kullander *et al*. (Kullander *et al*., 2003) showed that the regulation of commissural crossing of axons of interneurons in the spinal cord by Eph receptor and ligand molecules is crucial for the development of alternating patterns of activity. It is also clear that rhythm generation and alternation are different functions, since alternation but not rhythmic activity is suppressed by midsagital transections or by pharmacological block of glycinergic inhibition ((Cowley and Schmidt, 1995; Kremer and Lev-Tov, 1997). Thus the model of the coupled oscillator network is also well suited for the rat spinal cord. As in the lamprey, NMDA induces membrane potential oscillations in interneurons and motoneurons of the rat spinal cord. Such oscillations, in combination with electrical coupling by gap junctions, can induce rhythmic activity (Tresch and Kiehn, 2000). Rhythms can also be induced by high K^+, zero Mg^{2+} or disinhibition by a combination of the glycinergic blocker strychnine and the GABA A blocker bicuculline (Bracci *et al*., 1996; Bracci *et al*., 1998). While high K^+ induces alternating fictive locomotion patterns similar to those induced by NMDA and 5-HT, zero Mg^{2+} evokes rhythms with unstable phase shifts between left and right. Disinhibiton leads to episodes of synchronous rhythmic activity on both sides of the spinal cord which are similar to the spontaneous rhythms described in the embryonic chick (O'Donovan *et al*., 1998a).

1.2. Acute and cultured slices

1.2.1 Pattern generators in slices?

A widely used approach to combine *in vivo* grown neuronal networks with good experimental access to the levels of the network and individual cells is the use of acute slice preparations. However, while this is a widely used preparation in other areas of the CNS like the brain stem, where pattern generators for respiration are investigated (Rekling and Feldman, 1998), very few papers report on locomotor CPG functions in acute mammalian spinal slices (the only example is a recent paper by (Demir *et al*., 2002). In contrast, acute slices of the spinal cord are quite often used to study sensory neurons in the dorsal horns, showing that although the preparation is technically quite demanding, this is not the only reason for its rare use. What then is the reason for this lack in motor studies in slices? To answer this question, we must take a deeper look into the localization of the CPGs in the rat and mouse spinal cord. While there is no doubt that in the lamprey and the chick that the CPGs are distributed along the entire spinal cord, the localization in the rat spinal cord is still debated. The CPGs for the hindlimbs have been proposed to be restricted to the segments between T13 and L2 whereas the lower lumbar segments are passively driven by these CPGs (Cazalets *et al*., 1995). However other groups found the capacity for rhythm generation in all lumbar and even in sacral segments (Cowley and Schmidt, 1997; Kjaerulff and Kiehn, 1996; Kremer and Lev-Tov, 1997; Nakayama *et al*., 1999)

suggesting that locomotor rhythm generation is a more distributed spinal property. The basis for such discrepancies is probably the variations in the sensitivity of different segments to the agents used to induce the rhythms (Kiehn and Kjaerulff, 1998). The CPG of the upper limbs seems to be located at C5 to T1 while the thoracic segments T3 to T10 are driven by either of the two CPGs (Ballion *et al.*, 2001). The most important question with regard to the maintenance of CPGs in slices is what the minimal size of a CPG is. In the rat this was found to be at least two segments (Ballion *et al.*, 2001). Given that a 400 μm thick slice of the spinal cord of the neonatal rat contains about half a segment, it is not expected to contain a fully functional CPG. Nevertheless alternating rhythmic activity was found in such transverse slices of neonatal rats (Demir *et al.*, 2002). The reason for this discrepancy remains to be elucidated. In our hands, few preliminary trials with transverse slices on multielectrode arrays (MEAs) showed asynchronous activity, but no rhythms. Rhythms could be induced by disinhibition in longitudinal slices of the ventral horns (Tscherter, 2002).

1.2.2 Slice cultures

The first attempt to maintain intact spinal slices (explants) in culture goes back to the 1960's. Crain and his group first reported on this new method (Crain and Peterson, 1967). They investigated and described the patterns of "bioelectrical" activity that spontaneously arise in this preparation. Slice cultures are usually made from fetal tissue, in our lab at embryonic age 14 (E14, one week before birth), and are kept in culture for up to 4 weeks. The development in culture therefore covers the last week of fetal development and the first weeks of postnatal development. Nevertheless it is of course not clear to what extent development *in vitro* follows development *in vivo*. This question can not be answered in general but must be kept in mind for each phenomenon and parameter investigated. We will therefore discuss it in the context of the specific findings presented in this chapter. As a reference we briefly present some key points of the development *in vivo* here.

Spontaneous activity of spinal motoneurons can be recorded in ventral roots as early as E13.5. These spontaneous bursts are synchronized and mediated between E13.5 and E15.5 by cholinergic and glycinergic synaptic transmission (Nishimaru *et al.*, 1996; Ren and Greer, 2003). Later (E16.5 – E17.5) the spontaneous activity results from the combination of synaptic drive acting via non NMDA glutamatergic, nicotinic acetylcholine, glycine, and GABA A receptors. Finally, at late stages (E18.5 – E 21.5) the glutamate system acting via non NMDA receptors is the major drive for rhythm generation. The alternation between the left and right ventral roots is established between E16.5 and E18.5 (Kudo and Nishimaru, 1998). The commissural axons responsible for excitatory coupling and thus synchronization between both sides of the spinal cord at early stages and for the inhibitory coupling and thus alternation at late stages are GABAergic. Later, they are successively replaced by glycinergic projections (Kudo and Nishimaru, 1998). Thus the switch from excitatory to inhibitory effects of GABA (and glycine) seems to be crucial for the switch from synchronous activity to alternating activity. Rhythmic activity can be evoked by bath application of 5-HT at E14.5 and by NMDA at E16.5 (Iizuka *et al.*, 1998; Ozaki *et al.*, 1996), at a stage when, interestingly, most of the descending projections are not yet functional. The 5-HT containing projections, for example, reach the lumbar cord at E15 – E16 (Schmidt and Jordan, 2000). The first postnatal week is characterized by changes in the electrical properties of the motoneurons (decrease in input resistance, increase in maximal firing rate), by a refinement of the reflex circuits and by myelination (for review see (Vinay *et al.*, 2000).

In organotypic cultures of rat spinal slices, we have previously shown that a functional reflex arc between dorsal root ganglion cells and co-cultured skeletal muscle develops (Spenger *et al.*, 1991; Streit *et al.*, 1991). Myelination starts in the third week in culture. Disinhibition induces bursts of synchronized activity in the whole slice. The activity within the bursts usually oscillates at 4 – 5 Hz {Streit, 1993 #23}. Such oscillations usually start shortly after the onset of the bursts and slow down during the bursts. Similar patterns of bursting appear in the isolated spinal cord of the neonatal rat following disinhibition (Bracci *et al.*, 1996; Cowley and Schmidt, 1995). Disinhibiton induced bursting is driven through recurrent excitation via glutamate receptors, mainly of the non NMDA type (Legrand *et al.*, 2004). This finding shows that the developmental switch of the GABA and glycine system from excitatory to inhibitory effects did occur in the slice cultures. We have proposed that the oscillations within the bursts are based on activity dependent synaptic depression, which occurs in the cultures as well as in the isolated spinal cord (Pinco and Levtov, 1993; Streit *et al.*, 1992). In a computational study we have shown that, indeed, depression leads to network oscillations in the

observed frequency range (Senn *et al*., 1996). All these findings suggest that the slice cultures maintain important properties of rhythm generation of the *in vivo* spinal circuits. However, CPGs do not fully develop in slice cultures. NMDA and 5-HT are ineffective in evoking rhythms (Ballerini *et al*., 1999; Streit, 1996) and the rhythmic activity that is induced by high K^+ / low Mg^{2+} is always synchronous in the left and the right side in spite of the inhibitory effects of the GABA / glycine system. Thus, fictive locomotion patterns cannot be evoked. Recent findings have shown that the alternation depends on the eph ligand/receptor system which prevents axons of excitatory interneurons from crossing to the other side of the spinal cord (Kullander *et al*., 2003). In the light of this study it may well be that too many excitatory axons that cross the midline develop in the slice. Furthermore there seems to be a homeostatic regulation of circuits producing spontaneous activity: Long term blockade of spontaneous activity in the cultures leads to a suppression of GABAergic inhibitory synapses (Galante *et al*., 2000).

1.3 Random networks

1.3.1 Networks of dissociated spinal neurons

Neural networks can form entirely *in vitro* from dissociated and randomly seeded neurons in culture. While molecular cues of individual cells as well as activity-dependent mechanisms may still structure such networks we call them random networks because the network architecture is randomized at day 0 in culture. It was recognized early that such cultures develop patterns of spontaneous activity. In the 1970s the group of P. G. Nelson described the receptors underlying such spontaneous activity in cultures of dissociated cells of the mouse spinal cord.(Ransom *et al*., 1977). They found that GABAergic circuits develop earlier than the glutamatergic circuits and that the patterns of activity observed at different *in vitro* ages corresponded to the ratio of GABAergic to glutamatergic transmission. Later some of these results were confirmed in cultures of the rat (O'Brien and Fischbach, 1986). At about the same time G. Gross and his group started to grow dissociated mouse neurons on MEAs and to extracellularly record activity simultaneously from many points in the network (Gross *et al*., 1982). Based on such data they investigated how several receptors and ion channels contribute to generate the activity patterns of the network. They found that, as in slice cultures and the isolated spinal cord, rhythmic bursting could be induced either by high K^+ / low Mg^{2+} or by disinhibition with bicuculline and strychnine. More recently they showed that such rhythms become highly regular when all except the NMDA receptors are blocked (Keefer *et al*., 2001).

Looking for the source of spontaneous activity in cultured networks Latham *et al*. (2000) discovered that cultures of dissociated mouse spinal cord contains a percentage of intrinsically spiking neurons. In a theoretical study they showed how the number of intrinsically firing cells can determine whether spontaneous activity in the cultures is steady or bursting (Latham *et al*., 2000a). They could confirm their theoretical predictions by varying the number of intrinsic spikers by means of the culturing conditions (Latham *et al*., 2000b).

1.3.2 Emergent properties of random networks

The finding that at least some of the rhythms observed in networks in the intact spinal cord can be reproduced by random cultures, suggests that the network architecture is not a critical issue for such rhythm generation. This view is even strengthened by the fact that similar rhythms as described above are found in networks of neurons from other areas of the CNS like the cortex or the hypothalamus (Muller and Swandulla, 1995; Robinson *et al*., 1993). This finding shows that this type of rhythm generation is not specific for networks of spinal neurons. Two possible hypotheses may explain such a general mechanism for rhythm generation. First, all these networks contain a class of robust pacemaker cells, which drive the network, or, second, rhythm generation may be an emergent property of neural networks, which does not require a specific network architecture. Emergent properties of networks are properties, which are not immediately evident from the behaviour of the individual neurons (Faingold, 2004). In the remaining part of this chapter we will present some evidence for the second hypothesis. Furthermore we will present some insight into the mechanisms involved in emergent properties from our studies of organotypic and dissociated cultures of mouse and rat spinal cord, combining MEA with whole cell recordings.

Recent studies are just starting to reveal the enormous complexity of the circuits of interneurons in the spinal cord in terms of specific cell types (Kiehn and Kullander, 2004). In the context of these findings

one may ask whether it makes sense to investigate rhythm generation in such artificial systems as cell cultures. We think that with this complexity in mind it is even more important to know which functions of the network emerge from the properties of their components. Such knowledge serves as a basis on which the more complex functions requiring specific network architecture can be understood.

2. SLICE CULTURES OF SPINAL CORD: WHERE ARE THE PACEMAKERS?

2.1 Collective network behaviour revealed by MEA recordings

2.1.1 Slice cultures on MEAs

To analize the ensemble activity of neuronal networks it is crucial to record from many points of the network at once. Several methods have been developed to enable such multisite recording. They are either based on voltage sensitive or calcium sensitive dyes or extracellular electrodes. Dyes which are sensitive to voltage or calcium, produce activity- dependent light signals, which can be detected by a camera or an array of photodiodes (Darbon *et al*., 2002a). They usually have a good spatial but a limited temporal resolution. Extracellular electrodes measure potential differences between the recording electrode and the ground. Such transients are produced by the current flow which is due to changes in membrane conductance of the individual cells. They can be measured by needle electrodes, which are moved close to the cells with micromanipulators or, in a technically easier procedure, by electrodes, which are incorporated into the substrate of the cell culture. Such multielectrode arrays (MEAs) have been used to record from cell cultures since the 1970s. Their spatial and temporal resolution depends on technical parameters such as the number of electrodes that can be packed into the array and the limitations of the analog to digital converter (A/D) card and the computer that acquires the data. Since the speed and the memory capacity of average lab computers has increased dramatically in the last ten years, the handling of large amounts of data and thus the recording with high temporal and spatial resolution with MEAs became possible for many labs.

The MEAs used for our studies were developed in the Institute of Microsystems of the Ecole Polytechnique Fédérale de Lausanne (EPFL) and are now commercially available by Ayanda Biosytems, Lausanne, Switzerland. They are produced using standard photolithographic methods for details see (Heuschkel, 2001). They are composed of a glass substrate (700 μm thick, 21 x 21 mm) indium tin oxide (ITO) electrodes (100 nm thick, 40 x 40 μm) and leads and a SU.8 polymer insulation layer (5 μm thick). In some of the arrays the electrodes are additionally covered by a layer of platinum. The recording site is composed of 68 electrodes arranged in several configurations (hexagon, rectangle or four zones) with a inter-electrode spacing of 200 μm. The electrodes have an impedance of 300 kohm (Platinum electrodes) up to 1 Mohm (ITO electrodes) at 1 kHz in normal extracellular solution.

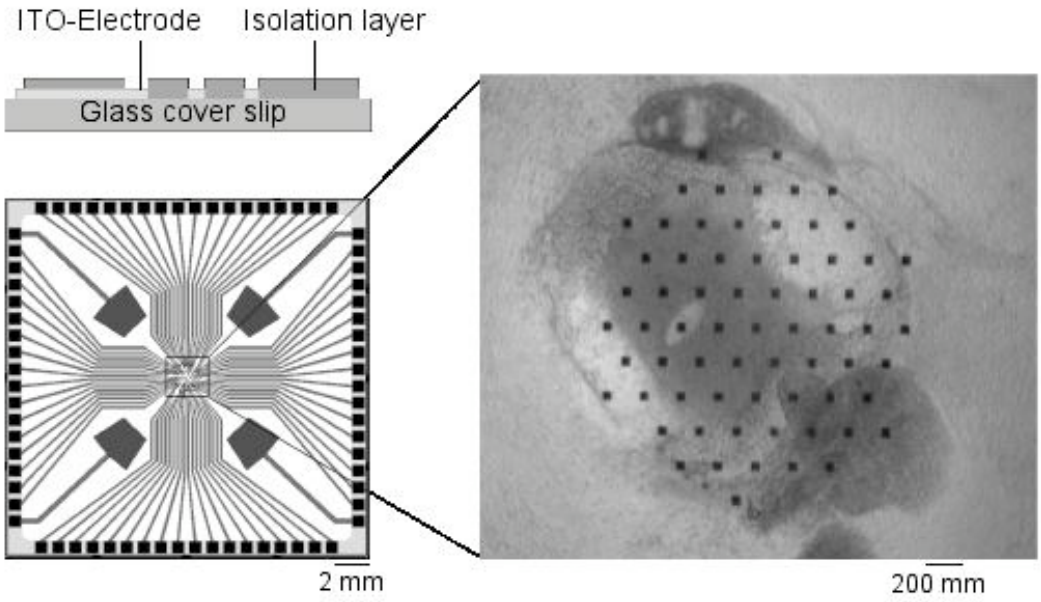


Figure 1: Spinal slice culture on MEA. Left side: cross-section and layout of the MEA chip. Note the four large ground electrodes, Right side: spinal slice culture on a hexagonal layout of black platinum electrodes after 14 days *in vitro*.

Slices of spinal cord from embryonic rats or mice at E14 were fixed on the MEAs (see Fig. 1) with coagulated chicken plasma and kept in plastic tubes which were placed in rotating drums in incubators for up to five weeks. The rotation caused an alternating exposure of the cultures to air (containing 5% CO_2 to maintain the pH at 7.4) and a nutrient medium. The medium was Dulbeccos MEM with glutamax, 10% fetal calf serum and nerve growth factor. More details about the cultures are given in Tscherter *et al*. (2001).

2.1.2. Signals recorded by MEAs

For the experiments, slice cultures with an age of 10 – 20 days *in vitro* (DIV) were used. The MEA with the culture was placed into a plexiglass chamber, mounted on an inverted microscope and superfused with a bath solution of the following composition (mM): NaCl 145, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 5, Na pyruvate 2, glucose 5 at pH 7.4. The bath solution was exchanged every 10 - 15 min during the experiments, which usually lasted for five to eight hours. Recordings were made at room temperature, in the absence of solution flow.

Each electrode was AC-coupled to an individual custom-made preamplifier and amplifier. The amplified signals were digitized at a rate of 6 kHz with 12 bit resolution and stored on hard disc for later off-line analysis. The A/D card was controlled by a custom-made Labview® program. Three different signals were usually recorded by the electrodes (see Tscherter et al., 2001): fast, medium and slow. The fast transients (see Fig. 2), lasting less than 4 ms, correspond to single action potentials in neuronal somata and axons (single unit activity). They often appear in clusters (multiunit activity), which probably originate from closely timed action potentials of several neurons seen by one electrode. The medium transients lasted for 100 – 500 ms and probably correspond to local field potentials which are caused by synaptically induced strong depolarization in large groups of neurons. The slow signals last for several (5 – 20) seconds and are probably caused by changes in the composition or volume of the interstitial space.

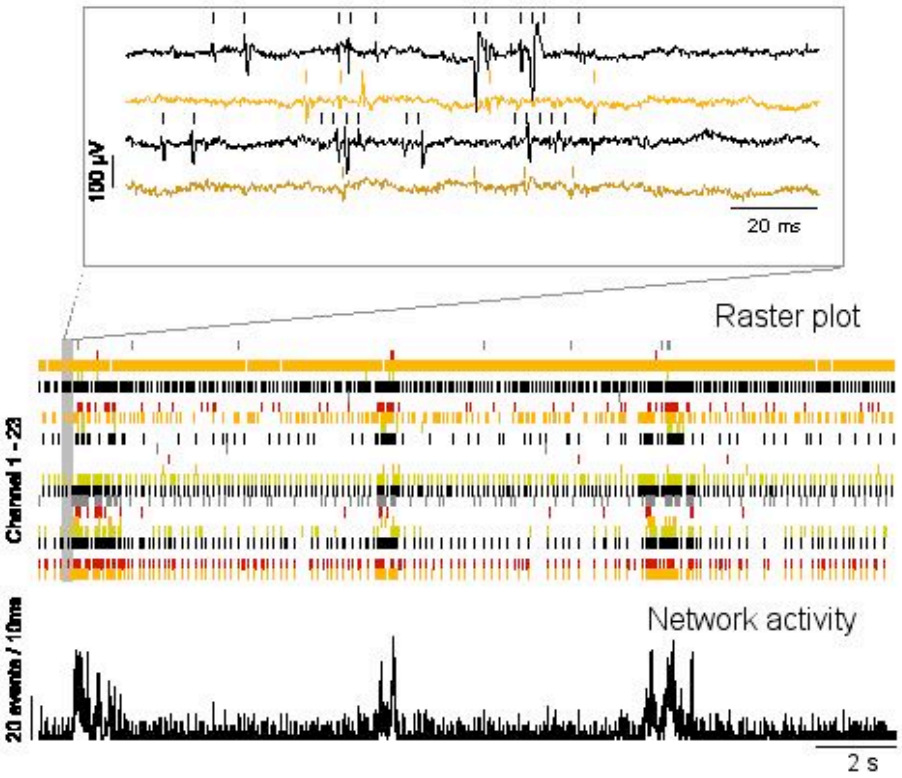


Figure 2: Spike detection. The upper graph shows original traces from four electrodes, from which the fast transients (spikes) were detected and indicated by time markers. Middle graph: The time markers of all electrodes (events) are displayed in a raster plot. Lower trace: All detected events in the network are counted in 10 ms bins and displayed as network activity plot.

Only the fast signals were considered for further analysis. The first step of such analysis was the detection of these signals. No attempt was made to sort the spikes seen at one electrode. Spike detection was based on the computation of the standard deviation of the original traces. A threshold was set at three times the mean standard deviation, above which signals were detected. For single units detection was easy and resulted in so called events, which were directly related to the underlying spikes (see Fig. 2). For multiunit activity, detection of individual spikes was not possible due to the overlap of the signals. For such activity, an event rate of 333 Hz was defined. The selectivity of spike detection was assured in each experiment by using recordings obtained in the presence of the sodium channel blocker TTX as a zero reference. The detected events were plotted versus time for each electrode (raster plot, see Fig. 2). Counting the number of events in bins of 10 ms resulted in plots of the network activity versus time.(Fig 2). The methods of spike detection and presentation are described in more detail in Tscherter *et al*., (2001).

2.1.3 Spontaneous activity in slice cultures

All cultures show a high amount of spontaneous activity. As shown in Fig. 3 most of this activity spreads in the slice leading to simultaneous activity at many or even most of the electrodes. Such "waves" of network activation (see also Tscherter *et al*. 2001) last for about 100 ms and often appear repetitively at frequencies around 4 – 5 Hz (see Fig. 3). During such waves, activity is most prominent in the ventral parts of the slice, around the central fissure. These areas are activated during virtually every wave, whereas the more dorsal parts of the slice are less active and are not reached in all waves. Between the waves sporadic activity, which is restricted to one or a few electrodes (asynchronous background activity) appears. Activation of the slice during the waves is based on glutamatergic synaptic transmission through AMPA / kainate and NMDA receptors (Legrand *et al*., 2004), since they are completely blocked by a combination of blockers of these receptors (6-cyano-7-

nitroquinoxaline-2-3-dione (CNQX) and (+/-)-2-amino-5-phosphonopentanoic acid (APV)). However, after block of excitatory synaptic transmission asynchronous activity is still recorded at several electrodes.

In vivo, the output of the networks of interneurons in the ventral horns goes first to the motoneurons and then to the skeletal muscle. In slice cultures a similar output forms when skeletal muscle is co-cultured with the spinal slices. We have shown previously that such muscle fibres are indeed innervated by spinal neurons and that the patterns of muscle contractions follows the patterns of activity in the spinal slices (Streit *et al.* 1991, Streit, 1996, Tscherter *et al.*, 2001). Combining MEA recordings with an optical device to record muscle contractions, both the electrical and the mechanical activity of muscle fibres can be measured together with the activity in the neuronal networks of the ventral horns. From such recordings it is clear that muscle contractions correlate with the population bursts in the spinal networks. (see Fig. 3). Therefore correlated network activity seems to be necessary to activate the motoneurons. In this way these *in vitro* networks even produce a simple form of behavior.

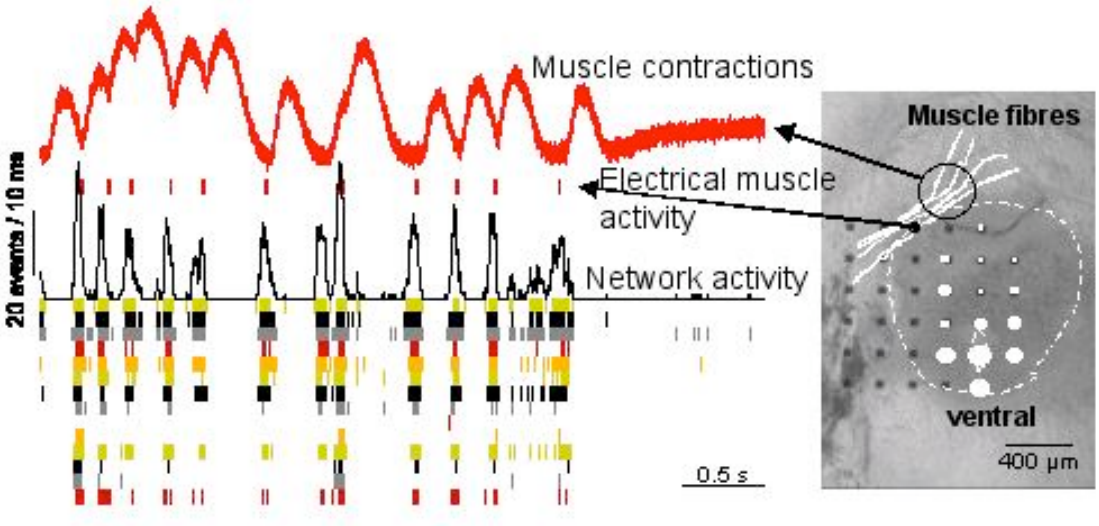


Figure 3. Spontaneous activity in spinal network and in co-cultured muscle. The raster and the network activity plot (black) show the synchronous network activity (population bursts). The distribution of activity in the slice is visualized by the yellow points on the left. The size of the points is proportional to the activity at the electrodes. The population bursts in the spinal network induce contractions in co-cultured skeletal muscle fibres. The red event markers show the electrical activity and the red trace the contractions of the muscle (measured by an optical device).

2.2 How are the rhythms induced?

2.2.1. Fast oscillations

As mentioned before the patterns of spontaneous activity are organized into short population bursts (waves) which often appear in short trains of 4 – 5 bursts following each other with intervals of around 200 ms. In some cultures they form persistent, highly regular oscillations of network activity at 4 – 5 Hz which last for several hours. In principle such oscillations can appear in slice cultures of the rat and the mouse and also in cultures of dissociated spinal neurons. Therefore, like the other rhythms described in this chapter, the oscillations do not depend on a highly specific network architecture, which needs be preserved. Nevertheless the normal pattern of spontaneous activity seen in the cultures is quite irregular with only short episodes of oscillations (see Fig. 3).

In slice cultures of the rat spinal cord, similar oscillations as described above reliably appear under disinhibition. As pointed out in more detail in the next subchapter, disinhibition means the pharmacological removal of synaptic inhibition from the network. Since the most prominent fast inhibitory neurotransmitters in the spinal cord are GABA and glycine, disinhibition is achieved by

combining the blocker of GABA A receptors, bicuculline, and the glycin receptor antagonist strychnine. In the slice cultures of rat spinal cord, disinhibition leads to a pattern of spontaneous activity that consists of long episodes (or bursts) of high network activity followed by silent intervals. During such bursts, activity is usually high at the beginning, then drops to a lower level of sustained activity for several hundreds of milliseconds and finally starts to oscillate for another several seconds (see Tscherter *et al.*, 2001). The period of the oscillations get longer towards the end of the episodes. A very similar pattern of "intra-burst" oscillations is seen in the isolated spinal cord of the neonatal rat after disinhibition (Bracci *et al.*, 1996) and in the chick embryo (O'Donovan *et al.*, 1998b). However, they are usually not found in slice cultures of the embryonic mouse and in cultures of dissociated spinal neurons after disinhibition, while, as mentioned before, they sometimes appear as innate rhythms (without pharmacology) in these preparations. The reasons for these discrepancies are not known.

We have previously proposed that the oscillations are based on repetitive network activation through recurrent excitation. The use-dependent fast depression of excitatory synaptic transmission acts as an accommodation mechanism. Such depression has been found in slice cultures of the rat (Streit *et al.*, 1992) as well as in the isolated spinal cord of the neonatal rat and the chick embryo (Pinco and Levtov, 1993; Tabak and O'Donovan, 1998). A computer model with the main parameters of recurrent excitation and use-dependent synaptic depression reproduces oscillations at 4 – 5 Hz (Senn *et al.*, 1996). It remains to be shown whether the differences in the generation of oscillations between the preparations can be explained by differences in synaptic depression.

2.2.2 Disinhibition – induced slow bursting

As mentioned before, disinhibition by bicuculline and strychnine reliably induces a slow bursting in slice cultures as well as in cultures of dissociated spinal neurons. This pattern is characterized by long-lasting 1-20 s episodes of high activity in the whole network (bursts) followed by silent intervals with low and asynchronous activity. The activity during the bursts is either persistent (in dissociated cultures and mouse slice cultures) or at least towards the end of the bursts oscillating as described in the preceding subchapter. Persistent activity decreases during the bursts with a rapid decay at the beginning, a subsequent plateau phase with slow decay and usually a second rapid decay at the end. On the one hand, this decay is a network effect since it reflects the decaying number of spikes in the network which causes a decreasing synaptic input to the individual cells. On the other hand it is also caused by spike frequency adaptation in indiviual neurons as described later in this chapter. During disinhibition-induced bursting the resting membrane potential of the neurons is hyperpolarized compared to the innate spontaneousactivity by more than 10 mV. This hyperpolarization is due to an increase in the activity of the electrogenic Na /K pump caused by the high level of activity (Darbon *et al.*, 2003). Usually the intervals between the bursts are too short for a visible recovery from such up-regulation of the pump. Therefore the membrane potential during the intervals is stable in many cells. Nevertheless, when the intervals are long (for example due to a partial block of excitatory transmission by CNQX), the recovery from hyperpolarization becomes evident (Darbon *et al.*, 2003).

Two more things can be learned from these disinhibition-induced patterns: First, in all cultures GABA and glycine act as inhibitory neurotransmitters since they do not support but rather suppress rhythms. This shows that the developmental switch of GABA and glycine from an excitatory to an inhibitory system has occurred during *in vitro* development. Second, synaptic inhibition does not usually prevent population bursting, since the latter appears both in the presence and in the absence of functional synaptic inhibition. Nevertheless synaptic inhibition partly contributes to the termination of the population bursts since they are much shorter with functional synaptic inhibition than without.

2.2.3 Regular fast bursting

Disinhibition-induced bursts appear more frequently and more regularly when NMDA is added (see Fig. 4 and Legrand *et al.*, 2004). This typical pattern of regular fast bursting is additionally characterized by a high level of background activity during the interburst intervals, a slowing of burst onsets and a decrease in burst amplitudes. The same rhythms are induced when disinhibition is combined with elevated K^+ in the bath solution, when disinhibition is reduced using low concentrations of bicuculline and strychnine, or when elevated K^+ is combined with 0 Mg^{2+} in the bath solution (Streit *et al.*, 2001). Transiently such rhythms also appear when the Na/K pump is blocked by

strophanthidin (Darbon *et al*., 2003). All these precedures lead to a depolarization of the neurons in the network. We therefore propose that these regular fast rhythms are produced by recurrent excitation, as described before, but in the presence of a general depolarization of the network. In terms of frequency and shape, regular fast rhythms are similar to the fictive locomotion patterns observed in isolated spinal cord preparations. However, slice cultures lack the important feature of alternation between left and right, showing that only rhythm generating networks (unit oscillators) but no complete pattern generators have developed in culture.

2.3 Where are the pacemakers?

2.3.1 burst sources

Some attempts have been made to localize the pattern generator networks in the transverse plane of the intact spinal cord. The methods used include lesion studies, staining with dyes like sulforhodamine that are taken up by neurons in an activity-dependent way (Kjaerulff *et al*., 1994), and calcium imaging (Demir *et al*., 2002; McPherson *et al*., 1997). The outcome of these studies, although not entirely conclusive due to methodological limitations, point to a localization of the patten generator networks around the central canal. Intracellular recordings from this area using sharp electrodes or patch clamp have indeed shown that a high percentage of the neurons are rhythmically modulated during fictive locomotion (Butt *et al*., 2002). However these methods did not allow them to distinguish between cells that drive the rhythms and those that are driven by the rhythms. Therefore the question whether somewhere in the spinal cord there are one or several pacemakers for rhythm generation like the sinus node in the heart, or whether rhythm generation is an emergent property of the pattern generating network, remained unsolved.

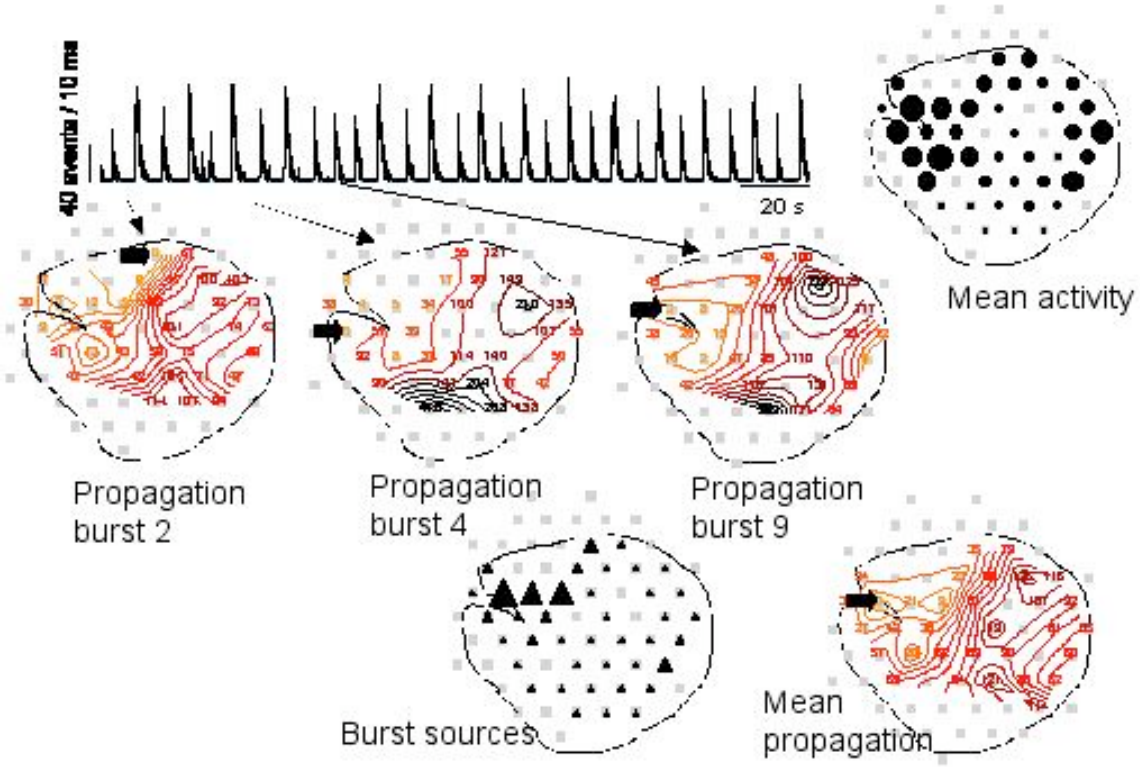


Figure 4. Burst sources and propagation. Regular bursting induced by disinhibition and NMDA. The distribution of the activity is shown by the black points on the right. Burst sources are determined from the raster plots at the onset of each burst (inset). Propagation is analyzed from the distribution of the delays and the interpolated isochrones. The variability in source and propagation is shown for three bursts in the middle row. The distribution of the burst sources (red triangles, size is proportional to the bursts initiated at the electrode. Small points show active electrodes with no sources) and the mean propagation over all bursts are shown in the lower row.

We have addressed this question using slice cultures of rat and mouse spinal cord on MEAs. Mapping the activity seen at the electrodes on the slices (see Figs. 3,4 and Tscherter *et al*. 2001) reliably shows that activity is highest in the ventral parts of the slices around the central fissure. This

finding strongly suggests that the rhythm generating networks in spinal slice cultures in fact belong to the central pattern generators. We then looked for the origin of rhythmic activity by analysing the propagation of the wave-fronts of each burst (see Fig. 4). We found that even when the rhythms are highly regular (induced by disinhibition and NMDA) there is much variability in the origin and the propagation of the wave-fronts from burst to burst. Nevertheless, there is a limited number (3 –8) of sites from which bursts originate (burst sources). These burst sources are usually grouped around the central fissure, although single "ectopic" sites are sometimes seen in the dorsal part of the slices, at the sites of the entrance of the dorsal roots. In spite of the variability from burst to burst, there is also a tendency in the propagation of the wave-front in the slice: Bursts start from a source at one side of the central fissure, propagate to the opposite ventral horn and finally to the dorsal parts of the slice (see Fig. 4 and Tscherter *et al*. 2001). In most of the slices, burst sources are found on both sides of the slice. Although bursts always reach both ventral horns of the slice, they sometimes fail to propagate to the dorsal parts. This general pathway of propagation is the same for the innate spontaneous activity as well as for all three types of induced rhythms. The findings of several bursts sources and the variability of propagation rule out the hypothesis of a single pacemaker as the source of rhythm generation. However, they are compatible with the existence of a network of pacemaker cells distributed around the central fissure.

2.3.2 Pacemaker cells?

To identify intrinsic spiking cells it is necessary to block all synaptic transmission in the network. The activity which is left under such conditions can be attributed to intrinsic activity. It turns out to be sufficient to block the glutamatergic receptors by a combination of CNQX and APV to suppress bursting in the network. Under such conditions asynchronous activity remains at about 30 % of the electrodes (Fig. 5). This activity is probably entirely due to intrinsic spiking, since it is not further changed when synaptic release is totally blocked by a bath solution containing 0 Ca^{2+} and 3 Mg^{2+}. Looking at individual electrodes the rate of such intrinsic activity varies in the between 0.1 and 10 Hz. At some of the electrodes regular tonic activity is seen, whereas in others the activity fluctuates. Rarely clear bursting is seen at one electrode, and this bursting is restricted to one electrode and differs also in rate and regularity from the bursting seen in the presence of synaptic transmission. The interpretation of these data is compromised by the fact that more than one cell could contribute to the activity recorded by one electrode. Nevertheless we can conclude that up to 30 % of the neurons in the network are capable of intrinisic spiking that, however, they do not define the rhythms observed in the presence of synaptic transmission. Therefore they cannot be considered as true cellular pacemakers. To find out whether these cells may at least trigger the bursts, we compared their spatial distribution to that of the burst sources. We found a good correspondence between the distribution of intrinsic activity and burst sources (see Fig. 5), suggesting that the intrinsic spiking is indeed the source of the population bursts.

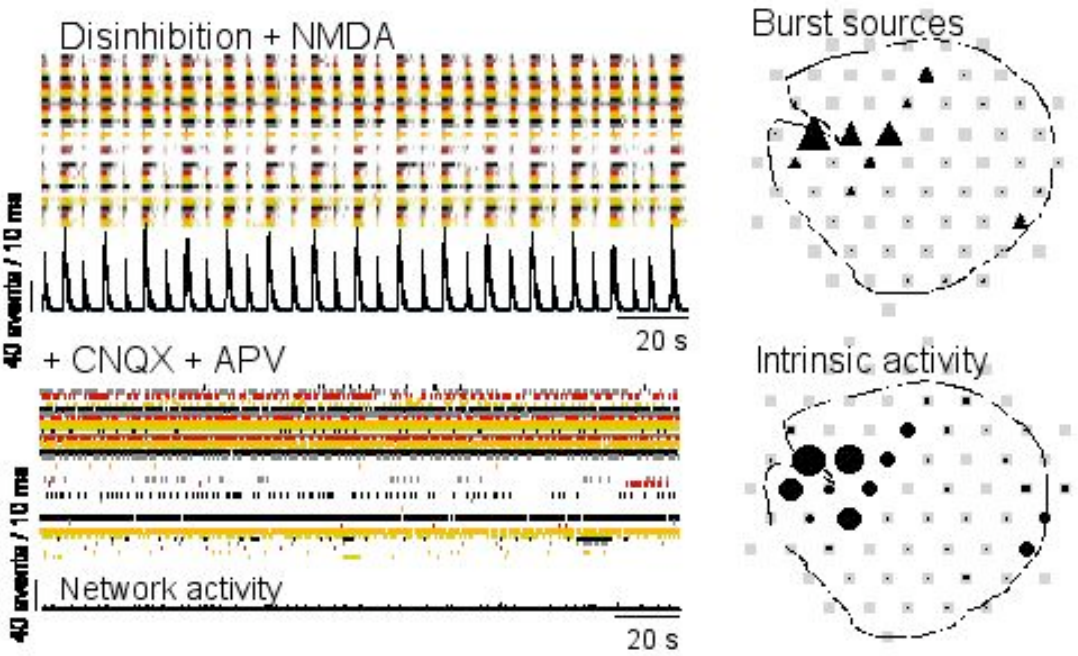


Figure 5: Burst sources and intrinsic activity: The upper row shows the distribution of burst sources during regular fast bursting (disinhibition + NMDA). The lower row shows the distribution of intrinsic activity after block of glutamatergic synaptic transmission by CNQX (10μM) and APV (50 μM). Note the good correspondence between the two distributions.

3. RANDOM NETWORKS GROWN *IN VITRO*: WHAT CAN WE LEARN?

3.1. What are the neurons doing?

3.1.1 Rhythm generation in random networks

Our findings presented so far suggest that rhythm generation is based on repetitive recruitment of the neurons in the network by intrinsic spiking cells through recurrent excitation. Such a mechanism does not depend on a specific network architecture. Therefore, the same rhythms as seen in organotypic slices should also appear in randomized networks , provided the cellular properties are the same. Networks can be randomized by dissociation of the neurons of the dissected slices on the day of preparation. When these dissociated cells are seeded out in culture, they form a new network within several days. After 3 – 4 weeks in culture, these networks reach a steady state in terms of the patterns of spontaneous activity they produce. It has been known for sometime that such networks are capable of rhythm generation. We therefore used them to test the hypothesis that random networks reproduce population bursting. Indeed, we found all three patterns of rhythmic activity that we have described before in slice cultures and in dissociated cultures as well (compare Tscherter *et al.*, 2001 and Streit *et al.*, 2001). Nevertheless there are differences between the culture types in the protocols used to evoke the rhythms and in the ease with which they appear. A major difference is that the fast oscillations during the bursts, which are reliably found during disinhibition in rat slice cultures, are rarely seen in dissociated cultures, where activity persists during the bursts (as shown before). The reason for this discrepancy is still unknown. It is, however, not related to structural differences between slice and dissociated cultures, since the activity during the bursts in mouse slice cultures does not oscillate and thus resembles the rhythms found in rat dissociated cultures. Since the fast oscillations are probably based on synaptic depression, the observed differences between the culture systems may reflect differences in the frequency response of the synapses. This remains to be shown experimentally.

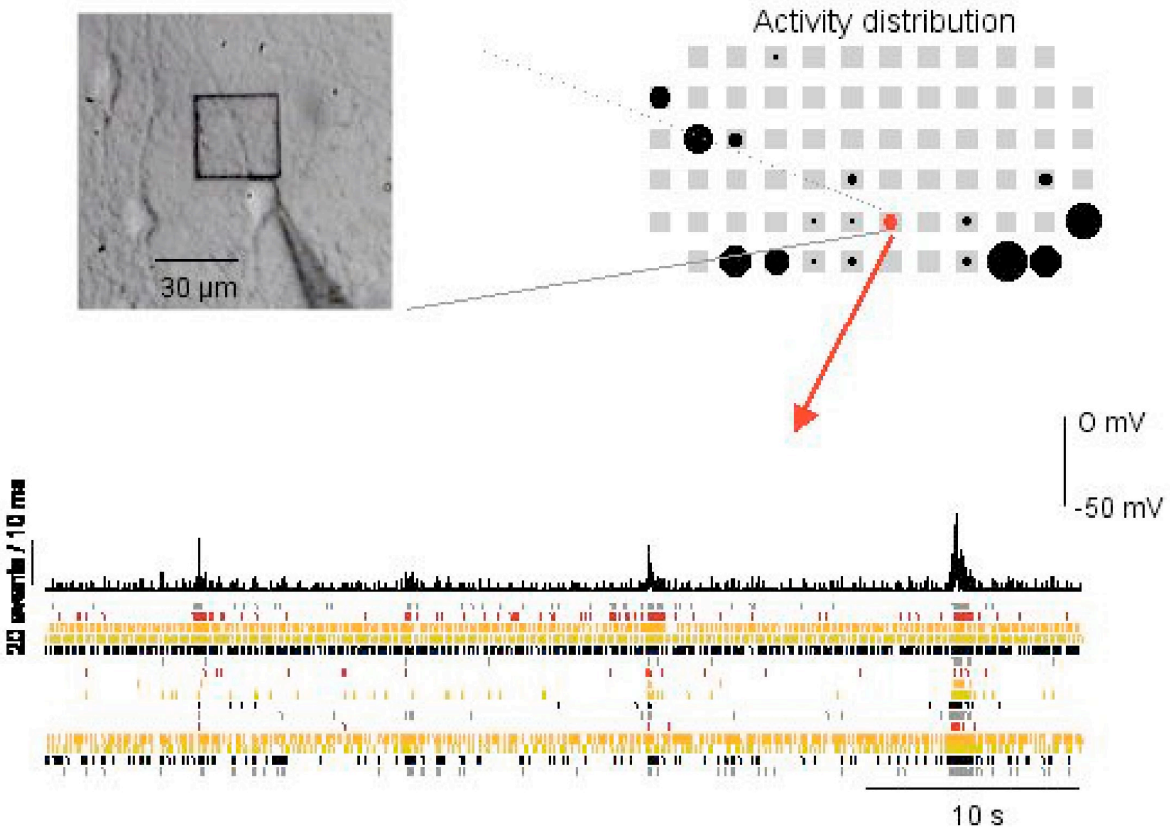
Besides this difference, rhythms in dissociated and slice cultures are similar, suggesting a common mechanism of rhythm generation. As in slice cultures, highly regular fast rhythms appear with disinhibition and NMDA in dissociated cultures (Legrand et al., 2004). The bursts also originate from several sources and propagate in the network along variable paths, which, however, follow a general pattern from each source. Several sources can even share the same general pattern of network recruitment, suggesting that the intrinsic spiking neurons are strongly interconnected and thus from a “trigger network”. The cells belonging to such trigger networks have short delays for recruitment.

When they are distributed over the whole network, this leads to an uneven spatial distribution of delays. When they are concentrated at one site, the propagation of the wave-front from there through the network can be smooth, leading to an even distribution of the latencies (see Streit *et al*. 2001). The recruitment of the whole network requires on average 50 – 100 ms for a network covering a rectangle of 3 x 1 mm. Since the conduction velocity in the axons of the cultured cells is around 0.3 m/s, it becomes clear that the conduction time is only a small percentage of the total recruitment time, which depends strongly on the mean synaptic density and the excitability of the neurons. In about half of the dissociated cultures, two or occasionally even more trigger networks are present.

3.1.2 Intrinsic spiking neurons

Cultures of dissociated cells have the advantage over slice cultures that the networks form a monolayer and therefore individual cells are more directly accessible for intracellular single cell recordings. We therefore used this preparation to combine network activity recordings by MEA with single cell recordings by the whole cell patch clamp method (see Fig. 6). We usually found a good correlation between network activity and postsynaptic potentials and action potentials in the neurons.

Figure 6. MEA recordings combined with whole cell recording from one cell. The picture on the left shows one MEA electrode (ITO only to keep it transparent) together with a patch clamped neuron. Below the raster and network activity plot of spontaneous activity are shown together with the single cell recording (in red). Note the correlation of the firing of the cell with the ensemble activity of the network. The distribution of the activity and the location of the recorded neuron in the network are shown in the upper right graph.

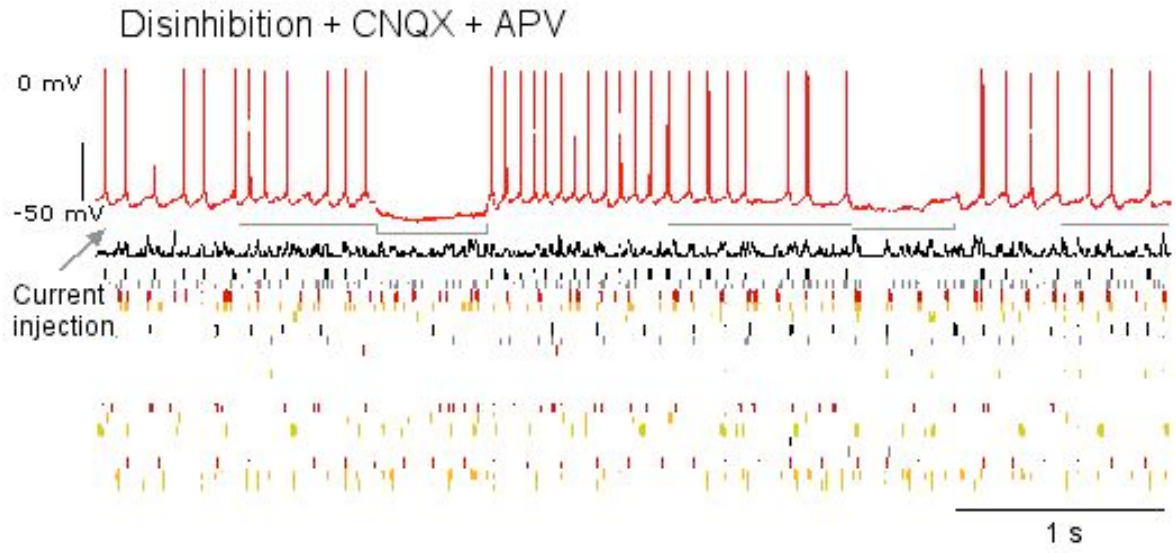


In the example shown in Fig. 6 the recorded neuron responds to increased network activity with trains of spikes. This example illustrates the typical case in which the neuron is driven by the network. However, in some of the neurons episodes of repetitive spiking appear that are unrelated to network activity. Such spiking is based on a slowly depolarizing membrane potential and persists when glutamatergic synaptic transmission is blocked by CNQX and APV (Fig. 7). It can be switched off by a slight hyperpolarization of the membrane without leaving an oscillating membrane potential. On the

other hand the spiking frequency can be increased by a depolarization of the membrane. Thus even small fluctuations of the membrane potential cause immediate changes in spike rate. This explains the irregular tonic rates of intrinsic activity recorded by the MEA electrodes (see Fig. 7).

We are currently investigating the conductance underlying the slow depolarization that causes the intrinsic spiking. Our yet unpublished findings suggest that the major component is a TTX-sensitive persistent Na^+ -current (I_{NaP}) which is activated at a membrane potential between –60 and –20 mV. In addition there is a co-operative effect of the hyperpolarization-activated cation current (I_h), which is activated at potentials more negativ than –60 mV. Both currents are present in only some of the spinal neurons, but I_{NaP} is found exclusively in intrinsic spiking cells.

Figure 7. Intrinsic spiking persists after block of fast synaptic transmission. Raster and network activity plots are shown together with the intracellular recording from one intrinsic spiking neuron (red trace) and the activity distribution (upper right). All recordings were made after the block of fast synaptic transmission with strychnine, bicuculline, CNQX and APV. Note that the intrinsic spiking in the neuron is stopped during hyperpolarizing pulses.



A critical question is whether the intrinsic spiking cells are indeed capable of driving the network that is to recruit enough cells to start a population burst. This is clearly not the case for most of these neurons. Even when they fire intrinsically, the network does not follow. This can be easily demonstrated by spikes which are artificially induced by injection of current pulses. In the majority of the experiments in which this has been done, the network did not follow these spikes, not even repetitive spiking at high frequency. However, we occasionally found cells which were able to drive the network on their own, even with single spikes. From these findings we conclude that only a small percentage of the intrinsic spiking cells is able to recruit the network. These cells are functionally connected to one or several highly excitable trigger networks, thus explaining the reliable repetitive network recruitment during fast regular bursting. In addition, one has to consider that fast regular bursting occurs when the network is depolarized (see above). Under such conditions both the number of intrinsic spiking cells as well as the excitability of the trigger networks is increased. The receptors underlying the recruitment of the trigger networks are mainly AMPA / kainate, and, less importantly, NMDA receptors (Legrand *et al*. 2004). This seems to be different from networks of dissociated neurons of mouse spinal cord, in which regular oscillatory activity can be maintained entirely by NMDA receptors (Keefer *et al*., 2001).

3.2. What shapes the rhythms?

3.2.1. Spike frequency adaptation

Rhythms are composed of a state of high network activity and a state of low network activity (intervals). In the previous subchapters, we have seen that intrinsic spiking drives the network through recurrent excitation from the low state to the high state. What brings it back then to the low state? In the original half-cycle model of pattern generation in the spinal cord, this is achieved by mutual synaptic inhibition. In innate spontaneous activity, population bursts are indeed much shorter (around 100 ms) than during disinhibition, suggesting that synaptic inhibition is indeed involved in burst termination. Nevertheless, we have shown that the most reliable and regular rhythms appear under disinhibition, indicating that other mechanisms than synaptic inhibition are involved. We have previously mentioned that use-dependent synaptic depression is involved in shaping the fast oscillations, The recovery time constant of synaptic depression is around 200 ms, which agrees well with the frequency range of fast oscillations of around 5 Hz; however, it is too fast to shape the slower patterns. Looking at the persistent network activity during the slow and regular fast bursts induced by disinhibition in dissociated cultures, activity decreases in three phases during the burst: A first rapid decrease is followed by a plateau with only a slight decrease and finally a second rapid fall terminates the burst (see Fig. 8). In individual cells the network activity causes a depolarization with a concomitant increase in spike rate. Spike frequency is initially high and decreases during the burst. Often spiking even ceases during the burst. In some cells (about 30%) the synaptic depolarization is so strong during the bursts that the spikes immediately die out due to an inactivation of the Na^+ channels (see Fig. 9). These cells thus respond to the network bursts mainly with a depolarized plateau potential (Darbon *et al.*, 2002a,b). The decrease in spike frequency during the bursts is on the one hand certainly due to the decrease in synaptic input current. On the other hand, when a stable input current of the same length is injected into the cell, the frequency of the evoked spikes also decreases and can cease after seconds during the pulse, much as during the bursts. Furthermore the plateau potentials can also be induced by injection of large currents. From these observations we conclude that such slow spike frequency adaptation is the primary mechanism leading to termination of these bursts. The early rapid decrease in network activity may additionally be caused by synaptic depression.

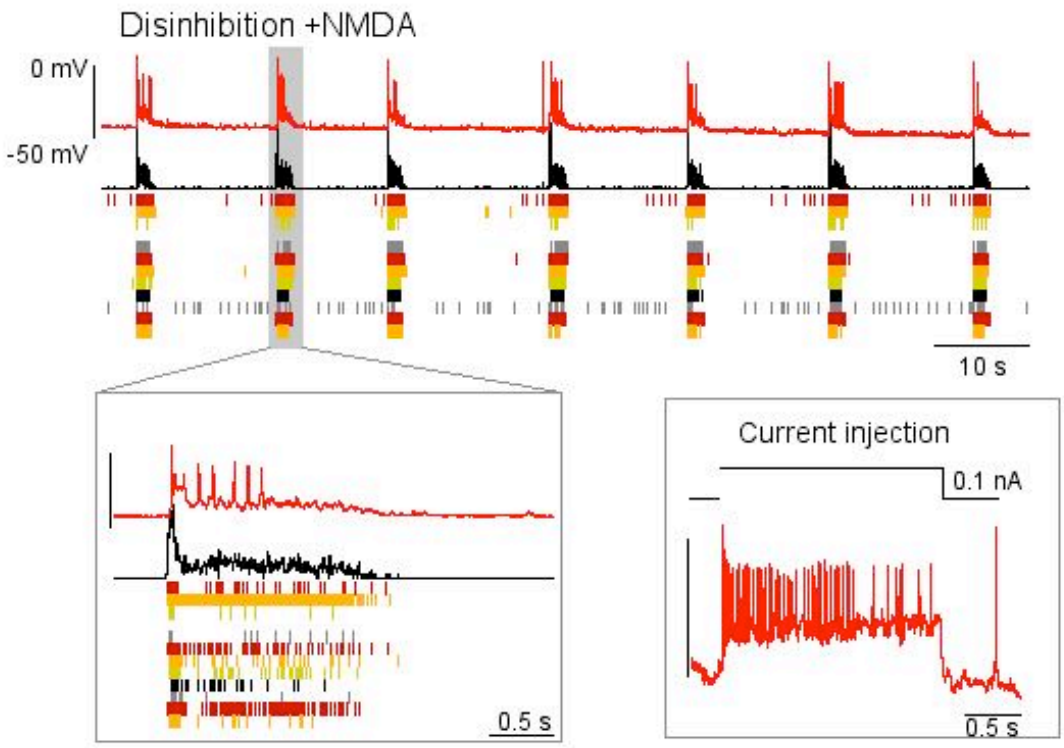


Figure 8. Spike frequency adaptation. Raster and network activity plots combined with intracellular recording from one neuron (red trace) show the decrease in network activity and spike rate during the bursts. A similar decrease in spike rate occurs during constant pulses of depolarizing current injections of similar length and amplitude as the bursts.

Spike frequency adaptation can be based on accumulation of Ca^{2+}-dependent K^+ currents or on a slow inactivation of Na^+ currents (I_{Na}) (Ellerkmann *et al.*, 2001). We have previously shown that bursts cannot be terminated by an artificial increase in the intracellular Ca^{2+} concentration (Darbon et al., 2002a). In addition, apamin and charybdotoxin, blockers of the Ca^{2+}-dependent K^+ currents, have only minor effects on bursting. Therefore we suggest that the slow spike frequency adaptation is due to a progressive inactivation of I_{Na}. In line with this hypothesis is the finding that spike frequency adaptation is accompanied by a decrease in spike amplitude and a slowing of the spikes, both characteristic of a smaller I_{Na}. Furthermore, slow inactivation of I_{Na} seems to be enhanced by riluzole since it enhances spike frequency adaptation (unpublished experiments). Thus we conclude that slow inactivation of I_{Na} is the major mechanism involved in the termination of the long-lasting persistent bursts.

3.2.2. Auto-regulation of intrinsic spiking

When the level of intrinsic activity at individual MEA electrodes is compared to the asynchronous activity in the intervals of slow bursting at the same electrodes (the first measured in the absence of synaptic transmission, the second during disinhibition), there is usually less asynchronous activity than intrinsic activity. This suggests that intrinsic spiking is suppressed following the bursts. Indeed, at some electrodes, the background activity is very low following the burst and increases with time during the interval. In intrinsic spiking cells, spontaneous spiking is suppressed following the bursts and slowly recovers during the interval (see Fig. 9). The silent period following the burst often goes parallel with a transient hyperpolarization of the cell. As mentioned before, even those cells which show no such transient hyperpolarization following the bursts are hyperpolarized during the intervals relative to the state before disinhibition (innate spontaneousactivity).

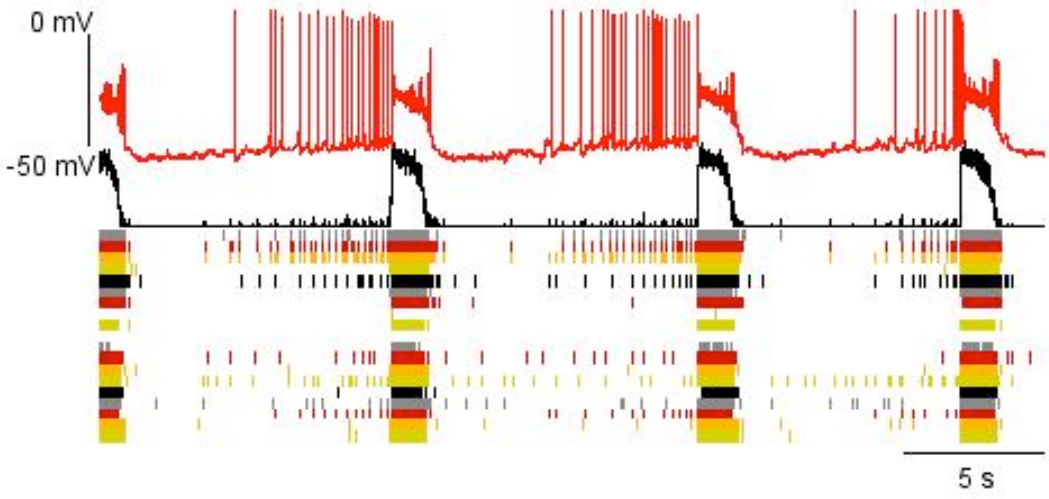


Figure 9. Intrinsic spiking is suppressed following bursts. Raster and network activity plots are shown together with the intracellular recording of an intrinsic spiking cell (red trace) during disinhibition-induced bursting. Note the hyperpolarization combined with a suppression of spiking following the burst. In parallel, the asynchronous background activity in the intervals is low following the burst and recovers during the interval. During the bursts spiking ceases in the recorded neuron due to the strong depolarization.

This suggests that bursts cause a hyperpolarization of the cells (by up to 10 mV) that slowly recovers during the intervals. Only when the intervals are long enough (due to a low burst rate), does the transient nature of the hyperpolarization become evident; otherwise it appears as a persistent hyperpolarization. In such experiments the transient nature of the hyperpolarization is revealed when the intervals are prolonged by low concentrations of CNQX (Darbon et al. 2002b). The suppression of intrinsic spiking and the hyperpolarization can be induced in a neuron in the absence of synaptic transmission, when bursts are mimicked by repetitive long-lasting pulses of current injection (see Darbon *et al.* 2002b). During trains of such pulses intrinsic spiking disappears and slowly recovers after the train. This shows that the hyperpolarization is not synaptically mediated but is an intrinsic property of the neuron itself. It can be regarded as a negative feedback mechanism that stabilizes cellular excitability, because intensely spiking neurons hyperpolarize and thus in turn decrease their

spike rate. The mechanism involved in this auto-regulation is an up-regulation of the electrogenic Na/K pump by a Na^+ load of the cell, as occurs during frequent spiking (Darbon *et al.*,2003). This mechanism is not only present in intrinsic spiking neurons since we also found the hyperpolarization in intrinsically silent cells. In these cells we also observed a decrease in excitability following the bursts (Darbon *et al.* 2002b).

3.3. Network refractoriness

3.3.1. Pacing the network with electrical stimuli

MEA electrodes can not only be used for recording but also for stimulation at any site of the network. Single stimuli of 1-2 V and 0.1 ms duration applied at one electrode usually cause activity at several other electrodes. This activity includes spikes, which are due to direct electrical stimulation of neurons and those which are evoked through synaptic transmission. Most of the directly stimulated spikes occur in the first ten milliseconds following the stimulus. They appear at several electrodes which are usually located close to the stimulating electrode, but which can be distributed over the whole network for strong stimuli (Darbon *et al.*, 2002b). Such a wide distribution is probably due to the activation of several axons that cross the stimulation electrode. In line with this hypothesis, such activity can not be evoked from all electrodes, especially not from those which, when used for recording, show no activity. Electrodes are manually switched from recording to stimulation. The number of spikes in the first ten milliseconds following the stimulus can be taken as a measure for cellular excitability, since this parameter does not involve synaptic transmission. Delays after the stimulus of more than ten milliseconds usually point to activity which is mediated through excitatory synaptic transmission. In consequence, such activity is suppressed by CNQX and APV. Its intensity depends on the amount of spontaneous activity. During innate spontaneous activity and during fast regular bursting, when intrinsic activity is high, the stimuli had almost no effect on the patterns or on the total amount of activity. This shows that under these conditions the intrinsic spiking is so intense that the additional spiking induced by external stimulation does not have much influence on the total activity in the network. Spontaneous activity is thus dominated by the various sources of refractoriness in the network (including synaptic inhibition) and not by the amount of activity (intrinsic plus stimulated), which is driving the network. In contrast, when the neurons are hyperpolarized during slow population bursting, intrinsic activity is reduced. Under such conditions external stimulation can evoke bursts and pace the network if its frequency is properly adjusted. External stimuli do not evoke bursts when they are applied immediately after a burst, showing that bursts are followed by a network refractory period. Only after this refractory period can stimuli evoke bursts. Therefore, when stimulated at too high frequencies, every second or more stimulus fails to induce a burst (see Fig. 10). The highest frequency of stimulation to which the network reliably responds with bursts (the critical frequency), is around 10 / min (range 5 – 20 / min, see Darbon *et al.*, 2002b). In dissociated cultures, the frequency of spontaneous bursting (after disinhibition) is often close to the critical frequency, showing that it is determined by the refractory period and not by the amount of intrinsic spiking. In these cultures it is difficult to pace the rhythm, since at too high frequencies of stimulation, failures occur and at too low frequencies spontaneous bursts tend to disrupt the bursting from the stimulation. In slice cultures of the rat, however, bursting is usually slower than in dissociated cultures and the spontaneous rates are lower than the critical frequency. In these cultures pacing the rhythm is easily possible since the rate of intrinsic firing is obviously the critical parameter defining the burst rate.

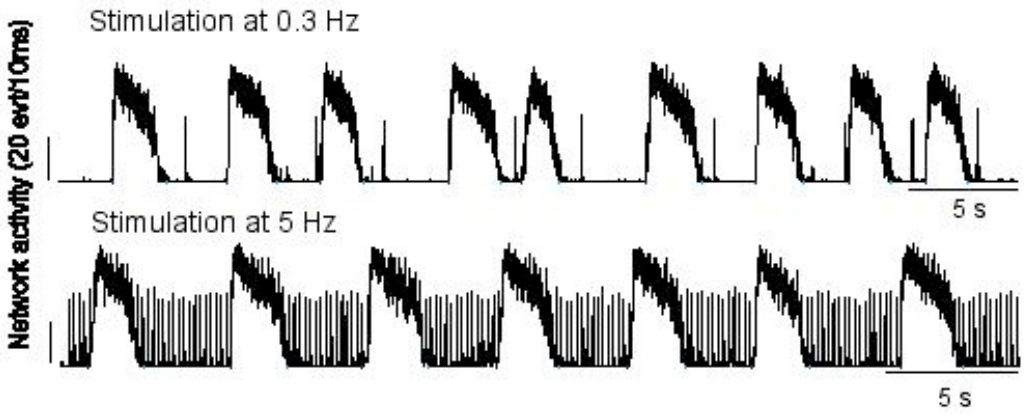


Figure 10. Network refractory period. Electrical stimuli (1-2 V, 0.5 ms) at one MEA electrode (yellow star) evokes activity at several other electrodes. The size of the black points in the upper graph is proportional to the amount of activity, which is evoked in the first 10 ms following the stimuli. The traces show the response of the network to two different frequencies of stimulation. Note that the network responds with busts rate, which is defined by the network refractory period and not by the frequency of stimulation. Stimuli, which are falling into the refractory period, fail to trigger a burst.

3.3.2. Network refractory period

Several factors may contribute to network refractoriness. We have seen before that the slow inactivation of I_{Na} and the up-regulation of the Na/K pump both contribute to a decrease in cellular excitability. In addition, a slowly recovering component of synaptic depression has been proposed to be involved in network refractoriness (Tabak *et al*., 2000). In spinal cultures we know that the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ increases during the bursts and slowly recovers during the intervals (Darbon *et al*., 2002a). In parallel, the rate of spontaneous postsynaptic currents is transiently increased following the bursts (Darbon *et al*., 2002b). This observation can be interpreted as an increased rate of spontaneous release of transmitter due to elevated $[Ca^{2+}]_i$. We have previously proposed that these experiments argue against a slow component of synaptic depression being present during the intervals. Nevertheless they do not exclude this possibility since evoked and spontaneous synaptic release need not necessarily behave the same way.

It is certain that the network refractory period is based on recovery processes. Therefore, it is relative in terms of network activation. This means that the network can be activated at different levels of network excitability by a sufficiently strong stimulus (be it intrinsic or stimulus–evoked spiking). This has been shown previously in slice cultures, in which bursting was paced at different frequencies by electrical stimulation. In these experiments several parameters, which indicate network excitability (burst duration, time to peak, cellular excitability) correlate with the burst rate in the sense that the shorter the interval is, the lower is the network excitability (Darbon *et al*., 2002b). It was in fact recognized even earlier that during spontaneous population busting, the burst duration is positively correlated to the preceding but not to the following interval duration (Streit, 1993, Tabak and O'Donovan, 1998). Tabak has shown in a theoretical study (Tabak *et al*., 2001) that such a correlation points to a stochastic process for burst initiation and a deterministic process for burst termination. We have shown here that burst initiation is based on network recruitment by intrinsic spiking. This is a stochastic process if the number of intrinsic spiking neurons and their spike frequency is mainly dependent on stochastic fluctuations of the membrane potential in individual neurons, as is the case during slow population bursting. When the number of intrinsic spiking neurons and their spike frequency gets higher and more reliable during depolarization (as is the case during fast regular bursting), burst initiation becomes a deterministic process, since it is now mainly controlled by the periodic suppression and relaxation of excitability in the neurons. (Giugliano *et al*., 2004). Thus, we found a strong correlation between burst duration and the preceding interval in disinhibition-induced slow population bursting. This correlation was lost when in the same culture slow bursting was switched to fast regular bursting by NMDA (Legrand *et al*., 2004), showing that burst initiation is a deterministic process now.

4. SUMMARY: IS IT THE NEURON OR THE NETWORK?

The mechanism involved in rhythm generation as proposed here is similar to the group pacemaker model, which has been proposed to explain the generation of respiratory rhythms in the preBötzinger Complex of the brainstem (Rekling and Feldman 1998). In this model, rhythm generation is based on the properties of neurons which are not pacemakers themselves, but which form a pacemaker network through their mutual excitatory coupling. We have identified the important properties of the neurons as an intrinsic spiking mechanism based on I_{Nap} and I_h (present in about 30% of the neurons), spike frequency adaptation based on a slow inactivation of I_{Na}, the regulation of the activity of the Na/K pump, and use-dependent synaptic depression. An important parameter which regulates the rhythms is the membrane potential. If the neurons are in general hyperpolarized (as during high activity of the Na/K pump induced by disinhibition), population bursting is slow with long bursts and low levels of intrinsic spiking in the intervals. If the neurons are in general depolarized (as with NMDA or high $[K^+]_e$), population bursting is fast with short bursts and high levels of intrinsic spiking in the intervals. Also the regularity of the rhythms depends on the membrane potential: the more depolarized the neurons are, the more intrinsic spiking there is, the more reliably bursts are initiated, the more regular bursting becomes. However, the two intrinsic accommodation mechanisms, spike frequency adaptation and synaptic depression, also define the rate and regularity of the rhythms. Probably the stronger one of the two mechanisms dominates the rhythms, the more regular they are. Synaptic depression, due to its shorter relaxation time constant, produces faster rhythms than spike frequency adaptation. The interaction of both mechanisms may on the one hand increase the variability and thus decrease the regularity of the rhythms, on the other hand it underlies the more complex rhythms like as bursting with intra-burst oscillations.

Mutual excitation of the neurons can be mediated by any receptors that have an excitatory function. In our cultures (as in the isolated spinal cord of the neonatal rat) AMPA/kainate and NMDA receptors seemed to be the major components. However, NMDA receptors alone, GABA/glycine or acetycholine can also support recurrent excitation during defined periods of development.

Since the proposed mechanisms are based on fundamental properties of neurons they should apply to networks at various levels of organization as found not only in cultures but also in slices and *in vivo* preparations.

References

Atsuta, Y., E., Garcia-Rill, E., and Skinner, R. D. (1990). Characteristics of electrically induced locomotion in rat in vitro brain stem-spinal cord preparation. *J. Neurophysiol.* 64: 727-735.

Ballerini, L., Galante, M., Grandolfo, M., and Nistri, A. (1999). Generation of rhythmic patterns of activity by ventral interneurones in rat organotypic spinal slice culture. *J. Physiol.* 517: 459-475.

Ballion, B., Morin, D., and Viala, D. (2001). Forelimb locomotor generators and quadrupedal locomotion in the neonatal rat. *Eur. J. Neurosci.* 14: 1727-1738.

Barbeau, H., McCrea, D. A., O'Donovan, M. J., Rossignol, S., Grill, W. M., and Lemay, M. A. (1999). Tapping into spinal circuits to restore motor function. *Brain Res. Brain Res. Rev.* 30: 27-51.

Beato, M., and Nistri, A. (1999). Interaction between disinhibited bursting and fictive locomotor patterns in the rat isolated spinal cord. *J. Neurophysiol.* 82: 2029-2038.

Bekoff, A., and Trainer, W. (1979). The development of interlimb co-ordination during swimming in postnatal rats. *J. Exp. Biol.* 83: 1-11.

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

Bracci, E., Ballerini, L., and Nistri, A. (1996). Spontaneous rhythmic bursts induced by pharmacological block of inhibition in lumbar motoneurons of the neonatal rat spinal cord. *J. Neurophysiol.* 75: 640-647.

Bracci, E., Beato, M., and Nistri, A. (1998). Extracellular K+ induces locomotor-like patterns in the rat spinal cord in vitro: Comparison with NMDA or 5-HT induced activity. *J. Neurophysiol.* 79: 2643-2652.

Brown, T. G. (1911). The intrinsic factors in the act of progression in the mammal. *Proc. R. Soc. Lond. (Biol.)* 84: 308-319.

Butt, S. J., Harris-Warrick, R. M., and Kiehn, O. (2002). Firing properties of identified interneuron populations in the mammalian hindlimb central pattern generator. *J. Neurosci.* 22: 9961-9971.

Cazalets, J. R., Sqalli-Houssaini, Y., and Clarac, F. (1992). Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.* 455: 187-204.

Cazalets, J. R., Borde, M., and Clarac, F. (1995). Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. *J. Neurosci.* 15: 4943-4951.

Cowley, K. C., and Schmidt, B. J. (1995). Effects of inhibitory amino acid antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. *J. Neurophysiol.* 74: 1109-1117.

Cowley, K. C., and Schmidt, B. J. (1997). Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.* 77: 247-259.

Crain, S. M., and Peterson, E. R. (1967). Onset and development of functional interneuronal connections in explants of rat spinal cord-ganglia during maturation in culture. *Brain Res.* 6: 750-762.

Dale, N. (1995). Experimentally derived model for the locomotor pattern generator in the xenopus embryo. *J. Physiol.* 489: 489-510.

Darbon, P. Pignier, C., Niggli, E., and Streit, J. (2002a). Involvement of calcium in rhythmic activity induced by disinhibition in cultured spinal cord networks. *J. Neurophysiol.* 88: 1461-1468.

Darbon, P., Scicluna, L., Tscherter, A., and Streit, J. (2002b). Mechanisms controlling bursting activity induced by disinhibition in spinal cord networks. *Eur. J. Neurosci.* 15: 671-683.

Darbon, P., Tscherter, A., Yvon, C., and Streit, J. (2003). Role of the electrogenic Na/K pump in disinhibition-induced bursting in cultured spinal networks. *J. Neurophysiol.* 90: 3119-3129.

Demir, R., Gao, B. X., Jackson, M. B., and Ziskind-Conhaim, L. (2002). Interactions between multiple rhythm generators produce complex patterns of oscillation in the developing rat spinal cord. *J. Neurophysiol.* 87: 1094-1105.

Dietz, V., Wirz, M., Colombo, G., and Curt, A. (1998). Locomotor capacity and recovery of spinal cord function in paraplegic patients: A clinical and electrophysiological evaluation. *Electroencephalogr. Clin. Neurophysiol.* 109: 140-153.

Ellerkmann, R. K., Riazanski, V., Elger, C. E., Urban, B. W., and Beck, H. (2001). Slow recovery from inactivation regulates the availability of voltage-dependent Na(+) channels in hippocampal granule cells, hilar neurons and basket cells. *J. Physiol.* 532: 385-397.

Faingold, C. L. (2004). Emergent properties of CNS neuronal networks as targets for pharmacology: Application to anticonvulsant drug action. *Prog. Neurobiol.* 72: 55-85.

Fetcho, J. R., and O'Malley, D. M. (1995). Visualization of active neural circuitry in the spinal cord of intact zebrafish. *J. Neurophysiol.* 73: 399-406.

Galante, M., Nistri, A., and Ballerini, L. (2000). Opposite changes in synaptic activity of organotypic rat spinal cord cultures after chronic block of AMPA/kainate or glycine and GABAA receptors. *J. Physiol.* 3: 639-651.

Giugliano, M., Darbon, P., Arsiero, M., Luescher, H. R., and Streit, J. (2004). Single-neuron discharge properties and network activity in dissociated cultures of neocortex. *J. Neurophysiol.* (march 24, 2004) 10-1152/jn.00067.2004

Grillner, S., and Zangger, P. (1979). On the central generation of locomotion in the low spinal cat. *Exp. Brain Res.* 34: 241-261.

Grillner, S., Wallen, P., Brodin, L., and Lansner, A. (1991). Neuronal network generating locomotor behavior in lamprey: Circuitry, transmitters, membrane properties, and simulation. *Annu. Rev. Neurosci.* 14: 169-199.

Grillner, S., Parker, D., and el Manira, A. (1998). Vertebrate locomotion--a lamprey perspective. *Ann. N. Y. Acad. Sci.* 860: 1-18.

Grillner, S. (2003). The motor infrastructure: From ion channels to neuronal networks. *Nat. Rev. Neurosci.* 4: 573-586.

Gross, G. W., Williams, A. N., and Lucas, J. H. (1982). Recording of spontaneous activity with photoetched microelectrode surfaces from mouse spinal neurons in culture. *J. Neurosci. Meth.* 5: 13-22.

Heuschkel, M. (2001), Fabrication of multi-electrode array devices for electrophysiological monitoring of in-vitro cell/tissue cultures. *Thesis* EPFL, Lausanne.

Iizuka, M., Nishimaru, H., and Kudo, N. (1998). Development of the spatial pattern of 5-HT-induced locomotor rhythm in the lumbar spinal cord of rat fetuses in vitro. *Neurosci. Res.* 31: 107-111.

Jankowska, E., Jukes, M. G., Lund, S., and Lundberg, A. (1967). The effect of DOPA on the spinal cord. 6. Half-centre organization of interneurones transmitting effects from the flexor reflex afferents. *Acta Physiol. Scand.* 70: 389-402.

Keefer, E. W., Gramowski, A., and Gross, G. W. (2001). NMDA receptor-dependent periodic oscillations in cultured spinal cord networks. *J. Neurophysiol.* 86: 3030-3042.

Kiehn, O., and Kjaerulff, O. (1998). Distribution of central pattern generators for rhythmic motor outputs in the spinal cord of limbed vertebrates. *Ann. N. Y. Acad. Sci.* 860: 110-129.

Kiehn, O., and Butt, S. J. (2003). Physiological, anatomical and genetic identification of CPG neurons in the developing mammalian spinal cord. *Prog. Neurobiol.* 70: 347-361.

Kiehn, O., and Kullander, K. (2004). Central pattern generators deciphered by molecular genetics. *Neuron* 41: 317-321.

Kjaerulff, O., Barajon, I., and Kiehn, O. (1994). Sulphorhodamine-labelled cells in the neonatal rat spinal cord following chemically induced locomotor activity in vitro. *J. Physiol.* 478: 265-273.

Kjaerulff, O., and Kiehn, O. (1996). Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: A lesion study. *J. Neurosci.* 16: 5777-5794.

Kremer, E., and Lev-Tov, A. (1997). Localization of the spinal network associated with generation of hindlimb locomotion in the neonatal rat and organization of its transverse coupling system. *J. Neurophysiol.* 77: 1155-1170.

Kudo, N., and Yamada, T. (1987). N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci. Lett.* 75: 43-48.

Kudo, N., and Nishimaru, H. (1998). Reorganization of locomotor activity during development in the prenatal rat. *Ann. N. Y. Acad. Sci.* 860: 306-317.

Kullander, K., Butt, S. J., Lebret, J. M., Lundfald, L., Restrepo, C. E., Rydstrom, A., Klein, R., and Kiehn, O. (2003). Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. *Science* 299: 1889-1892.

Kwiecien, R., and Hammond, C. (1998). Differential management of Ca2+ oscillations by anterior pituitary cells: A comparative overview. *Neuroendocrinology* 68: 135-151.

Latham, P. E., Richmond, B. J., Nelson, P. G., and Nirenberg, S. (2000a). Intrinsic dynamics in neuronal networks. I. Theory. *J. Neurophysiol.* 83: 808-827.

Latham, P. E., Richmond, B. J., Nirenberg, S., and Nelson, P. G. (2000b). Intrinsic dynamics in neuronal networks. II. Experiment. *J. Neurophysiol.* 83: 828-835.

Legrand, J. C., Darbon, P., and Streit, J. (2004). Contributions of NMDA receptors to network recruitment and rhythm generation in spinal cord cultures. *Eur. J. Neurosci.* 19: 521-532.

Magnuson, D. S., and Trinder, T. C. (1997). Locomotor rhythm evoked by ventrolateral funiculus stimulation in the neonatal rat spinal cord in vitro. *J. Neurophysiol.* 77: 200-206.

Marchetti, C., Beato, M., and Nistri, A. (2001). Alternating rhythmic activity induced by dorsal root stimulation in the neonatal rat spinal cord in vitro. *J. Physiol.* 530: 105-112.

McPherson, D. R., McClellan, A. D., and O'Donovan, M. J. (1997). Optical imaging of neuronal activity in tissue labeled by retrograde transport of Calcium Green Dextran. *Brain Res. Brain Res. Protoc.* 1: 157-164.

Milner, L. D., and Landmesser, L. T. (1999). Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J. Neurosci.* 19: 3007-3022.

Mortin, L. I., and Stein, P. S. G. (1989). Spinal cord segments containing key elements of the central patterns generators for three forms of scratch reflex in the turtle. *J. Neurosci.* 9: 2285-2296.

Muller, W., and Swandulla, D. (1995). Synaptic feedback excitation has hypothalamic neural networks generate quasirhythmic burst activity. *J. Neurophysiol.* 73: 855-861.

Nakayama, K., Nishimaru, H., Iizuka, M., Ozaki, S., and Kudo, N. (1999). Rostrocaudal progression in the development of periodic spontaneous activity in fetal rat spinal motor circuits in vitro. *J. Neurophysiol.* 81: 2592-2595.

Nishimaru, H., Iizuka, M., Ozaki, S., and Kudo, N. (1996). Spontaneous motoneuronal activity mediated by glycine and GABA in the spinal cord of rat fetuses in vitro. *J. Physiol.* 497: 131-143.

O'Brien, R. J., and Fischbach, G. D. (1986). Excitatory synaptic transmission between interneurons and motoneurons in chick spinal cord cell cultures. *J. Neurosci.* 6: 3284-3289.

O'Donovan, M. J., and Rinzel, J. (1997). Synaptic depression: A dynamic regulator of synaptic communication with varied functional roles. *Trends Neurosci.* 20: 431-433.

O'Donovan, M. J., Chub, N., and Wenner, P. (1998a). Mechanisms of spontaneous activity in developing spinal networks. *J. Neurobiol.* 37: 131-145.

O'Donovan, M. J., Wenner, P., Chub, N., Tabak, J., and Rinzel, J. (1998b). Mechanisms of spontaneous activity in the developing spinal cord and their relevance to locomotion. *Ann. N. Y. Acad. Sci.* 860: 130-141.

O'Donovan, M. J. (1999). The origin of spontaneous activity in developing networks of the vertebrate nervous system. *Curr. Opin. Neurobiol.* 9: 94-104.

Ozaki, S., Yamada, T., Iizuka, M., Nishimaru, H., and Kudo, N. (1996). Development of locomotor activity induced by NMDA receptor activation in the lumbar spinal cord of the rat fetus studied in vitro. *Brain Res. Dev. Brain Res.* 97: 118-125.

Pinco, M., and Levtov, A. (1993). Modulation of monosynaptic excitation in the neonatal rat spinal cord. *J. Neurophysiol.* 70: 1151-1158.

Ransom, B. R., Christian, C. N., Bullock, P. N., and Nelson, P. G. (1977). Mouse spinal cord in cell culture. II. Synaptic activity and circuit behavior. *J. Neurophysiol.* 40: 1151-1161.

Rekling, J. C., and Feldman, J. L. (1998). PreBotzinger complex and pacemaker neurons: Hypothesized site and kernel for respiratory rhythm generation. *Annu. Rev. Physiol.* 60: 385-405.

Ren, J., and Greer, J. J. (2003). Ontogeny of rhythmic motor patterns generated in the embryonic rat spinal cord. *J. Neurophysiol.* 89: 1187-1195.

Robinson, H. P., Torimitsu, K., Jimbo, Y., Kuroda, Y., and Kawana, A. (1993). Periodic bursting of cultured cortical neurons in low magnesium: cellular and network mechanisms. *Jpn J. Physiol.* 43: S125-130.

Rossignol, S., Giroux, N., Chau, C., Marcoux, J., Brustein, E., and Reader, T. A. (2001). Pharmacological aids to locomotor training after spinal injury in the cat. *J. Physiol.* 533: 65-74.

Schmidt, B. J., and Jordan, L. M. (2000). The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.* 53: 689-710.

Senn, W., Wyler, K., Streit, J., Larkum, M., Lüscher, H.-R., Mey, H., Müller, L., Stainhauser, D., Vogt, K., and Wannier, T. (1996). Dynamics of a random neural network with synaptic depression. *Neural Networks* 9: 575-588.

Sherrington, C. (1898). Decerebrate rigidity and reflex coordination of movements. *J. Physiol.* 22: 319-332.

Shik, M. L., and Orlovsky, G. N. (1976). Neurophysiology of locomotor automatism. *Physiol. Rev.* 56: 465-501.

Spenger, C., Braschler, U. F., Streit, J., and Lüscher, H.-R. (1991). An organotypic spinal cord - dorsal root ganglion - skeletal muscle coculture of embryonic rat.I. The morphological correlates of the spinal refelex arc. *Eur.. J. Neurosci.* 3: 1037-1053.

Steriade, M. (2001). Impact of network activities on neuronal properties in corticothalamic systems. *J. Neurophysiol.* 86: 1-39.

Streit, J., Spenger, C., and Lüscher, H.-R. (1991). An organotypic spinal cord-dorsal root ganglia-skeletal muscle coculture of embryonic rat. II. Functional evidence for the formation of spinal reflex arcs in vitro. *Eur. J. Neurosci.* 3: 1054-1068.

Streit, J., Lüscher, C., and Lüscher, H.-R. (1992). Depression of postsynaptic potentials by high frequency stimulation in embryonic motoneurons grown in spinal cord slice cultures. *J. Neurophysiol.* 68: 1793-1803.

Streit, J. (1996). Mechanisms of pattern generation in cocultures of embryonic spinal cord and skeletal muscle. *Int. J. Dev. Neurosci.* 14: 137-148.

Tabak, J., and O'Donovan, M. J. (1998). Statistical analysis and intersegmental delays reveal possible roles of network depression in the generation of spontaneous activity in the chick embryo spinal cord. *Ann. N. Y. Acad. Sci.* 860: 428-431.

Tabak, J., Senn, W., O'Donovan, M. J., and Rinzel, J. (2000). Modeling of spontaneous activity in developing spinal cord using activity-dependent depression in an excitatory network. *J. Neurosci.* 20: 3041-3056.

Tabak, J., Rinzel, J., and O'Donovan, M. J. (2001). The role of activity-dependent network depression in the expression and self-regulation of spontaneous activity in the developing spinal cord. *J. Neurosci.* 21: 8966-8978.

Tresch, M. C., and Kiehn, O. (2000). Motor coordination without action potentials in the mammalian spinal cord. *Nat. Neurosci.* 3: 593-599.

Tscherter, A., Heuschkel, M. O., Renaud, P., and Streit, J. (2001). Spatiotemporal characterization of rhythmic activity in rat spinal cord slice cultures. *Eur. J. Neurosci.* 14: 179-190.

Tscherter, A. (2002) Rhythmic activity in cultured spinal cord networks: A multielectrode array study. Thesis, University of Bern, Bern.

Vertes, R. P., and Kocsis, B. (1997). Brainstem-diencephalo-septohippocampal systems controlling the theta rhythm of the hippocampus. *Neuroscience* 81: 893-926.

Vinay, L., Brocard, F., Pflieger, J. F., Simeoni-Alias, J., and Clarac, F. (2000). Perinatal development of lumbar motoneurons and their inputs in the rat. *Brain Res. Bull.* 53: 635-647.

Westerga, J., and Gramsbergen, A. (1990). The development of locomotion in the rat. *Brain Res. Dev. Brain Res.* 57: 163-174.