16
Rhythm Generation in Spinal Cultures: Is It the Neuron or the Network?

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16.1 Rhythm Generation in Neural Networks In Vitro: Worth Studying?

16.1.1 Rhythm Generation in Intact Preparations

16.1.1.1 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 that 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 that occur during respiration, locomotion, or scratching are controlled by rhythmically active neural networks in brainstem and spinal cord (Grillner et al., 1998; Rekling and Feldman, 1998).

All these examples show that rhythmic activity in neural networks underlies many of the specific CNS functions and also suggest that the capability for rhythm generation must be a fundamental property of neural networks. In principle there are two ways a network can generate rhythms: rhythm may be produced by a well-defined circuit, usually composed of excitatory and inhibitory cells, or rhythm generation depends mainly on the cellular properties of certain neurons in the network and 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 dissociated cell cultures, they are entirely regrown from randomly seeded cells. In the case of organotypic slice cultures, networks develop out of premature

networks 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 obtained 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 intact spinal cord.

16.1.1.2 Fictive Locomotion in the Cat Spinal Cord

The neuronal system generating the stereotypic movements characteristic of 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 (1898) observed that cats and other mammals can perform locomotor movements of the legs even after a complete transection of the spinal cord. He proposed that this activity might 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 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. It was later 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 favored, 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.

16.1.1.3 The Lamprey Model

One problem in the cat experiments is the difficulty of getting direct experimental access to the CPGs in the spinal cord. Therefore, other preparations such as the spinal cord of the turtle (Mortin and Stein, 1989), or embryonic preparations such as 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 such as 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, 1998; Grillner, 2003). Here we 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+}-influx through NMDA receptor channels as a depolarizing mechanism and the subsequent activation of Ca^{2+}-dependent K^{+} currents as the hyperpolarizing mechanism. Other channels, such as voltage-dependent Ca^{2+} channels, contribute to this pacemaker mechanism. The resulting rhythm can be modulated by various agents including 5-hydroxytryptamine (5-HT).

16.1.1.4 Development of Pattern Generators: The Chick Spinal Cord

The chick spinal cord is the preparation mainly 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 made mainly by the group of Michael O'Donovan to reveal the mechanisms involved in such embryonic rhythm generation (O'Donovan, 1999). This group found that these rhythms can be mediated not only by glutamate receptors (of the $(+/-)$-α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid (AMPA) and NMDA types) but also by glycine and γ-aminobutyric acid (GABA) A receptors. These normally inhibitory receptors have excitatory effects in the embryo 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 modeled by computer simulation based on the two parameters of hyperexcitability and activity-dependent depression (Tabak et al., 2001). Because these rhythms share many properties with the bursting induced in spinal cultures by disinhibition, we discuss this model more extensively later in this chapter.

16.1.1.5 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 behavior 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). CPGs in neonatal rat spinal cord proved to share many of the properties previously described in cat and lamprey spinal cords. 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. (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, inasmuch as alternation but not rhythmic activity is suppressed by midsagittal transections or by pharmacological blockade 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, 1998). High K^+ induces alternating fictive locomotion patterns similar to those induced by NMDA and 5-HT, however, 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 that are similar to the spontaneous rhythms described in the embryonic chick (O'Donovan et al., 1998a).

16.1.2 Acute and Cultured Slices

16.1.2.1 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, although this is a widely used preparation in other areas of the CNS such as brainstem, where pattern generators for respiration have been investigated (Rekling and Feldman, 1998), are few papers report on locomotor CPG functions in acute mammalian spinal slices (example is a recent paper by Demir et al., 2002). In contrast, acute slices of spinal cord are quite often used to study sensory neurons in the dorsal horns, indicating that although this preparation is technically quite demanding, there must be other reasons for its rare use. What then is the reason for this lack of motor studies in slices?

To answer this question, we must take a deeper look into the localization of the CPGs in rat and mouse spinal cords. Although there is no doubt that in lamprey and chick CPGs are distributed along the entire spinal cord, their localization in the rat spinal cord is still debated. CPGs for 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 rhythms (Kiehn and Kjaerulff, 1998). The CPG of the upper limbs seems to be located at C5 to T1, whereas 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 consists in the minimal size of a CPG. In the rat, this was found to be at least two segments (Ballion et al., 2001). Given that a 400 μm thick slice of spinal cord of neonatal rats 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 multi-electrode arrays (MEAs) showed asynchronous activity, but no rhythms. Rhythms could be induced by disinhibition in longitudinal slices of ventral horns (Tscherter, 2002).

16.1.2.2 Slice Cultures

The first attempt to maintain intact spinal slices (explants) in culture goes back to the 1960s. 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 prepared from fetal tissue, in our lab at embryonic age 14 (E14, one week before birth), and are kept in culture for up to four 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 mimics in vivo development. This question cannot be answered in general but must be kept in mind for each phenomenon and parameter investigated. We 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 in vivo development 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 to 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 to 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 to 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 to 5 Hz (Streit, 1993). 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 light of this study it may well be that too many excitatory axons crossing 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).

16.1.3 Random Networks

16.1.3.1 Networks of Dissociated Spinal Neurons

Neural networks can form entirely in vitro from dissociated and randomly seeded neurons in culture. Although 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 P.G. Nelson's group described the receptors mediating such spontaneous activity in cultures of dissociated cells of the mouse spinal cord (Ransom et al., 1977). These authors found that GABAergic circuits develop earlier than glutamatergic circuits and that the patterns of activity observed at different in vitro ages corresponded to the ratio of GABAergic to glutamatergic transmission. Later on, some of these results were confirmed in rat cultures (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 isolated spinal cords, 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. (2000a,b) discovered that cultures of dissociated mouse spinal cord contain 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 changing culturing conditions (Latham et al., 2000b).

16.1.3.2 Emergent Properties of Random Networks

The finding that at least some of the rhythms observed in networks in intact spinal cords 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 such as 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 that are not immediately evident from the behavior of

individual neurons (Faingold, 2004). In the remaining part of this chapter we present some evidence for the second hypothesis. Furthermore, we present some insights 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 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.

16.2 Slice Cultures of Spinal Cord: Where Are the Pacemakers?

16.2.1 Collective Network Behavior Revealed by MEA Recordings

16.2.1.1 Slice Cultures on MEAs

To analyze 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 multi-site recording. They are either based on voltage-sensitive or calcium-sensitive dyes or extracellular electrodes. Dyes that 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 good spatial but limited temporal resolution. Extracellular electrodes measure potential differences between the recording electrode and the ground. Such transients are produced by the current flow that is due to changes in membrane conductance of 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 multi-electrode arrays 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. Because the speed and the memory capacity of average lab computers has increased dramatically in the last ten years, handling large amounts of data and thus 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 made

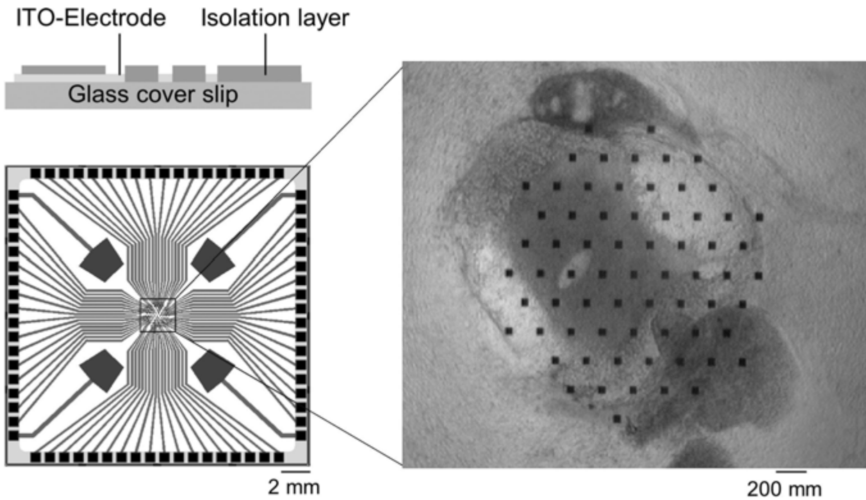

FIGURE 16.1. Spinal slice culture on MEA. Left side: cross-section and layout of the MEA chip. Note the four large ground electrodes, Right side: spinal slice culture on a hexagonal layout of black platinum electrodes after 14 days in vitro.

commercially available by Ayanda Biosystems, 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 × 21 mm), indium tin oxide (ITO) electrodes (100 nm thick, 40 × 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 an interelectrode 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.

Slices of spinal cord from embryonic rats or mice at E14 were attached to the MEAs (see Figure 16.1) with coagulated chicken plasma and kept in plastic tubes that 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 Dulbecco's MEM with glutamax, 10% fetal calf serum, and nerve growth factor. More details about the cultures are given in Tscherter et al. (2001).

16.2.1.2 Signals Recorded by MEAs

For the experiments, slice cultures with an age of 10 to 20 days in vitro (DIV) were used. The MEA with the culture was placed into a plexiglas 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 to 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 offline 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 Figure 16.2), lasting less than 4 msec, correspond to single-action potentials in neuronal somata and axons (single-unit activity). They often appear in clusters (multi-unit activity), which probably originate from closely timed action potentials of several neurons seen by one electrode. The medium transients lasted for 100 to 500 msec and probably correspond to local field potentials that are caused by synaptically induced strong depolarization

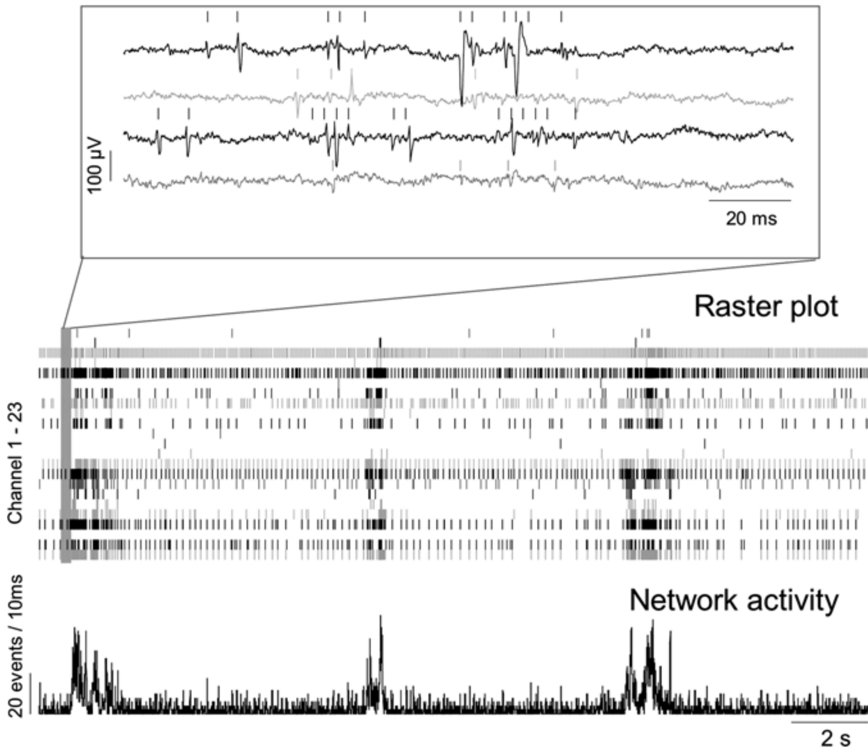

FIGURE 16.2. Spike detection: the upper graph shows original traces from four electrodes, from which the fast transients (spikes) were detected and indicated by time markers. Middle graph: the time markers of all electrodes (events) are displayed in a raster plot. Lower trace: all detected events in the network are counted in 10 msec bins and displayed as network activity plot.

in large groups of neurons. The slow signals last for several (5 to 20) seconds and are probably caused by changes in the composition or volume of the interstitial space.

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 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 Figure 16.2). For multi-unit 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 Figure 16.2). Counting the number of events in bins of 10 msec resulted in plots of the network activity versus time (Figure 16.2). The methods of spike detection and presentation are described in more detail in Tscherter et al., (2001).

16.2.1.3 Spontaneous Activity in Slice Cultures

All cultures show a high amount of spontaneous activity. As shown in Figure 16.3, 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 msec and often appear repetitively at frequencies around 4 to 5 Hz (see Figure 16.3). 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), because 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 blockade 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 motoneurons and then to skeletal muscles. In slice cultures a similar output forms when skeletal muscle is co-cultured with the spinal slices. We have previously shown that such muscle fibers are indeed innervated by spinal neurons and that the patterns of muscle contractions follow the patterns of activity in spinal cord 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 fibers can be measured together with the activity in

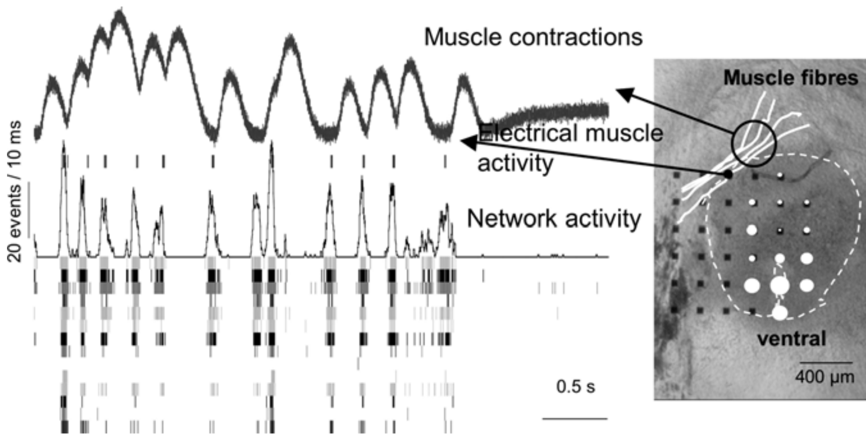

FIGURE 16.3. Spontaneous activity in spinal network and in co-cultured muscle. The raster and network activity plots (black) show synchronous network activity (population bursts). The distribution of activity in the slice is visualized by the points on the left. The size of the points is proportional to the activity at the electrodes. The population bursts in the spinal network induce contractions in co-cultured skeletal muscle fibers. The red event markers show the electrical activity and the red trace the contractions of the muscle (measured by an optical device).

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 Figure 16.3). Therefore correlated network activity seems to be necessary to activate motoneurons. In this way these in vitro networks even produce a simple form of behavior.

16.2.2 How Are Rhythms Induced?

16.2.2.1 Fast Oscillations

As mentioned before, the patterns of spontaneous activity are organized into short population bursts (waves) which often appear in short trains of four to five bursts following each other with intervals of around 200 msec. In some cultures they form persistent, highly regular oscillations of network activity at 4 to 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, as with other rhythms described in this chapter, 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 Figure 16.3).

In slice cultures of rat spinal cord, similar oscillations as described above reliably appear under disinhibition. As pointed out in more detail in the next section, disinhibition means the pharmacological removal of synaptic inhibition from the

network. Because the most prominent fast inhibitory neurotransmitters in the spinal cord are GABA and glycine, disinhibition is achieved by combining blockers of $GABA_A$ and glycine receptors, bicuculline, and strychnine. In 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 becomes longer toward the end of the episodes. A very similar pattern of "intraburst" oscillations is seen in isolated spinal cords of neonatal rats after disinhibition (Bracci et al., 1996) and in chick embryo (O'Donovan et al., 1998b). However, they are usually not found in slice cultures of embryonic mouse and in cultures of dissociated spinal neurons after disinhibition, whereas, 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 rats (Streit et al., 1992) as well as in isolated spinal cords of neonatal rats and 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 to 5 Hz (Senn et al., 1996). It remains to be shown whether the differences in the generation of oscillations between preparations can be explained by differences in synaptic depression.

16.2.2.2 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 to 20 sec 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 toward the end of the bursts oscillating as described in the preceding section. Persistent activity decreases during bursts with a rapid decay at the beginning, a subsequent plateau phase with slow decay, and usually a second rapid decay at the end. This decay is a network effect inasmuch as 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 individual neurons as described later in this chapter.

During disinhibition-induced bursting, the resting membrane potential of the neurons is hyperpolarized compared to the innate spontaneous activity 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 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 (e.g., due to a partial block of excitatory transmission by CNQX), recovery from hyperpolarization becomes evident (Darbon et al., 2003).

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

16.2.2.3 Regular Fast Bursting

Disinhibition-induced bursts appear more frequently and more regularly when NMDA is added (see Figure 16.4 and Legrand et al., 2004). This typical pattern of regular fast bursting is additionally characterized by a high level of background activity during 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 procedures lead to a depolarization of 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.

16.2.3 Where Are the Pacemakers?

16.2.3.1 Burst Sources

Some attempts have been made to localize the pattern generator networks in the transverse plane of intact spinal cords. The methods used include lesion studies, staining with dyes such as sulforhodamine, which 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

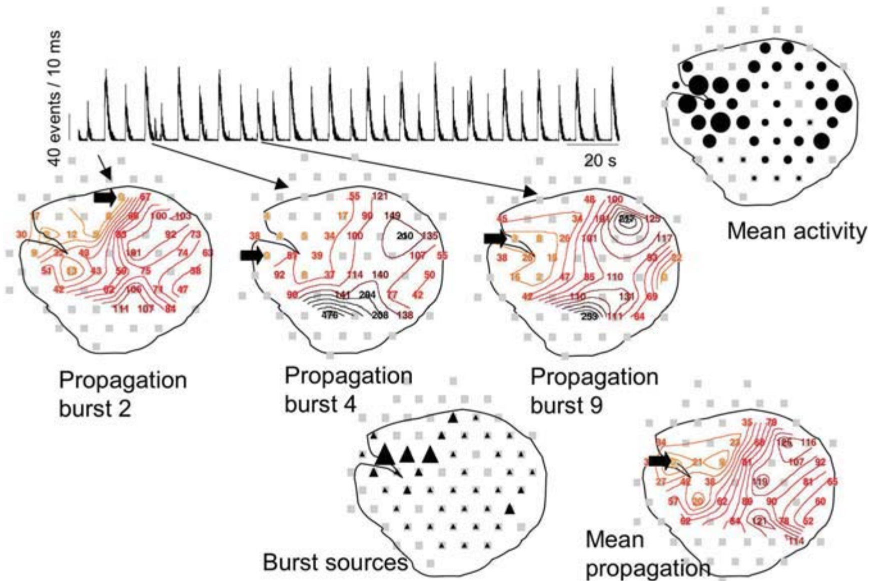

FIGURE 16.4. Burst sources and propagation: regular bursting induced by disinhibition and NMDA. The distribution of activity is shown by the black points on the right. Burst sources are determined from the raster plots at the onset of each burst. Propagation is analyzed from the distribution of the delays and the interpolated isochrones. The variability in source and propagation is shown for three bursts in the middle row. The distribution of burst sources (triangles, size is proportional to the bursts initiated at the electrode: smallest triangles show active electrodes with no sources), and the mean propagation over all bursts are shown in the lower row.

not entirely conclusive due to methodological limitations, points to a localization of the pattern 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 neurons are rhythmically modulated during fictive locomotion (Butt et al., 2002). However, these methods did not allow distinguishing between cells that drive rhythms and those that are driven by rhythms. Therefore the question of 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.

We have addressed this question using slice cultures of rat and mouse spinal cords on MEAs. Mapping the activity seen at the electrodes on the slices (see Figures 16.3 and 16.4, 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 analyzing the propagation of the wavefronts of each burst (see Figure 16.4). We found that even when rhythms are highly regular (induced by disinhibition and NMDA) there is much variability in the origin and the propagation of the wavefronts from burst to burst. Nevertheless, there are a limited number (three to eight) 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 wavefront 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 Figure 16.4 and Tscherter et al., 2001). In most 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 innate spontaneous activity as well as for all three types of induced rhythms. The findings of several burst 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.

16.2.3.2 Pacemaker Cells?

To identify intrinsic spiking cells it is necessary to block all synaptic transmission in the network. The activity that is left under such conditions can be attributed to intrinsic activity. It turns out to be sufficient to block 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 (Figure 16.5). This activity is probably entirely due to intrinsic spiking, inasmuch as 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 between 0.1 and 10 Hz. At some of the electrodes, regular tonic activity is seen, whereas in others activity fluctuates. Rarely is clear bursting seen at one electrode, and bursting is restricted to one electrode and differs also in rate and regularity from 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 neurons in the network are capable of intrinsic spiking, which, however, does not define rhythms observed in the presence of synaptic transmission. Therefore these neurons cannot be considered as true cellular pacemakers. To find out whether these cells may at least trigger bursts, we compared their spatial distribution to that of burst sources. We found a good correspondence between the distribution of intrinsic activity and burst sources (see Figure 16.5), suggesting that intrinsic spiking is indeed the source of population bursts.

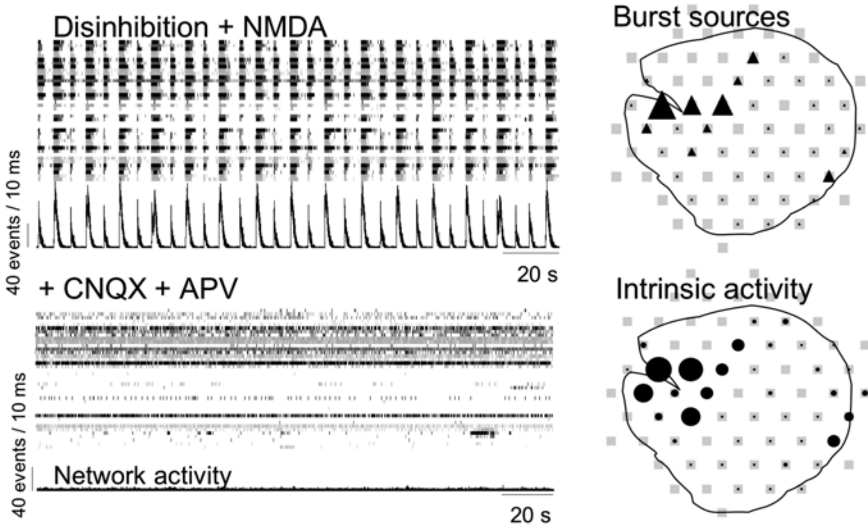

FIGURE 16.5. Burst sources and intrinsic activity: the upper row shows the distribution of burst sources during regular fast bursting (disinhibition + NMDA). The lower row shows the distribution of intrinsic activity after blockade of glutamatergic synaptic transmission by CNQX (10 μM) and APV (50 μM). Note the good correspondence between the two distributions.

16.3 Random Networks Grown In Vitro: What Can We Learn?

16.3.1 What Are Neurons Doing?

16.3.1.1 Rhythm Generation in Random Networks

The findings presented so far suggest that rhythm generation is based on repetitive recruitment of 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 cellular properties are the same. Networks can be randomized by dissociation of neurons of the dissected slices on the day of preparation. When these dissociated cells are seeded out in cultures, they form a new network within several days. After three to four weeks in cultures, these networks reach a steady state in terms of patterns of spontaneous activity they produce. It has been known for some time 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 in dissociated cultures as well that we have described before in slice cultures (compare Tscherter et al., 2001 and Streit et al., 2001).

Nevertheless, there are differences between the culture types in the protocols used to evoke 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, because activity during the bursts in mouse slice cultures does not oscillate and thus resembles the rhythms found in rat dissociated cultures. Because 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.

In addition to 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 form a "trigger network" (Yvon et al., 2005). 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 wavefront from there through the network can be smooth, leading to an even distribution of latencies (see Streit et al., 2001, Yvon et al., 2005). The recruitment of the whole network requires on average 50 to 100 msec for a network covering a rectangle of 3×1 mm. Because the conduction velocity in axons of the cultured cells is around 0.3 m/sec, it becomes clear that conduction time is only a small percentage of the total recruitment time, which depends strongly on mean synaptic density and excitability of neurons. In about half of the dissociated cultures, two or occasionally even more trigger networks are present.

16.3.1.2 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 Figure 16.6). We usually found a good correlation between network activity and postsynaptic potentials and action potentials in neurons. In the example shown in Figure 16.6, 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 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 (Figure 16.7).

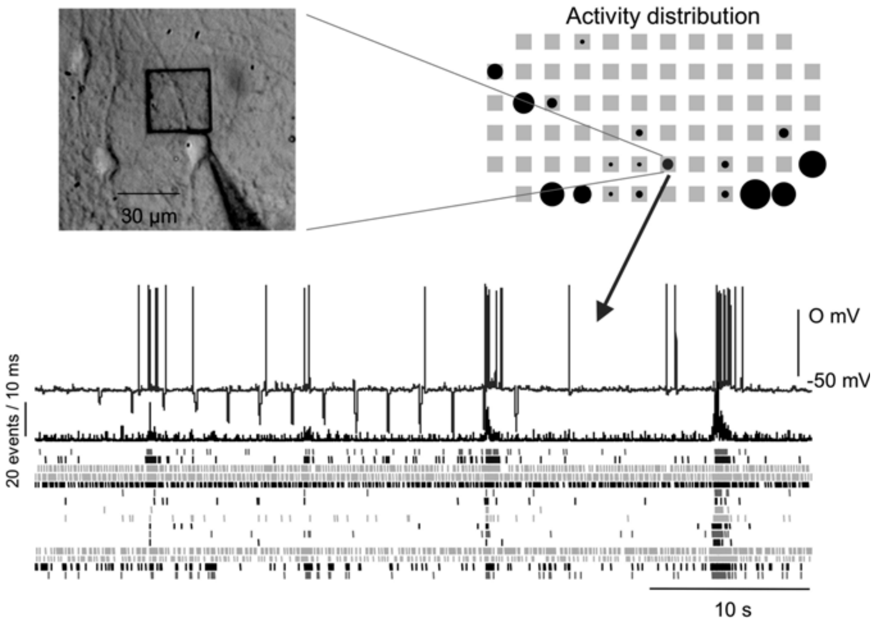

FIGURE 16.6. MEA recordings combined with whole cell recording from one cell. The picture on the left shows one MEA electrode (ITO only to keep it transparent) together with a patch-clamped neuron. Below, the raster and network activity plot of spontaneous activity are shown together with the single-cell recording (upper trace). 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.

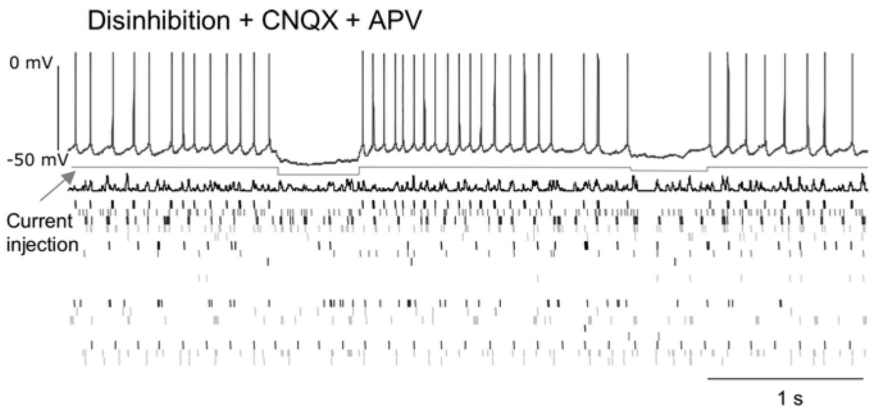

FIGURE 16.7. Intrinsic spiking persists after block of fast synaptic transmission. Raster and network activity plots are shown together with the intracellular recording from one intrinsic spiking neuron (upper trace) and the activity distribution (lower trace). All recordings were made after the blockade of fast synaptic transmission with strychnine, bicuculline, CNQX, and APV. Note that the intrinsic spiking in the neuron is stopped during hyperpolarizing pulses.

It can be switched off by a slight hyperpolarization of the membrane without leaving an oscillating membrane potential. On the other hand, 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 Figure 16.7).

We are currently investigating the conductance underlying the slow depolarization that causes intrinsic spiking. Our 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 negative than –60 mV. Both currents are present in only some of the spinal neurons, but I_{NaP} is found exclusively in intrinsic spiking cells (Darbon et al., 2004).

A critical question is whether the intrinsic spiking cells are indeed capable of driving the network, that is, recruiting 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 that are artificially induced by injection of current pulses. In the majority of 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 that were able to drive the network on their own, even with single spikes. From these findings we conclude that only a small percentage of intrinsic spiking cells are 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 are 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).

16.3.2 What Shapes Rhythms?

16.3.2.1 Spike Frequency Adaptation

Rhythms are composed of a state of high network activity and a state of low network activity (intervals). In the previous sections, 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 msec) 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 mechanisms other than synaptic inhibition are involved. We have previously mentioned that use-dependent synaptic depression is involved in shaping fast oscillations. The recovery time constant of synaptic depression is around 200 msec, 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 Figure 16.8). In individual cells, 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%), synaptic depolarization is so strong during bursts that spikes immediately die out due to an inactivation of Na^+ channels (see Figure 16.9). These cells thus respond to network bursts mainly with a depolarized plateau potential (Darbon et al., 2002a,b). The decrease in spike frequency during bursts is certainly due to decrease in synaptic input current. On the other hand, when a stable input current

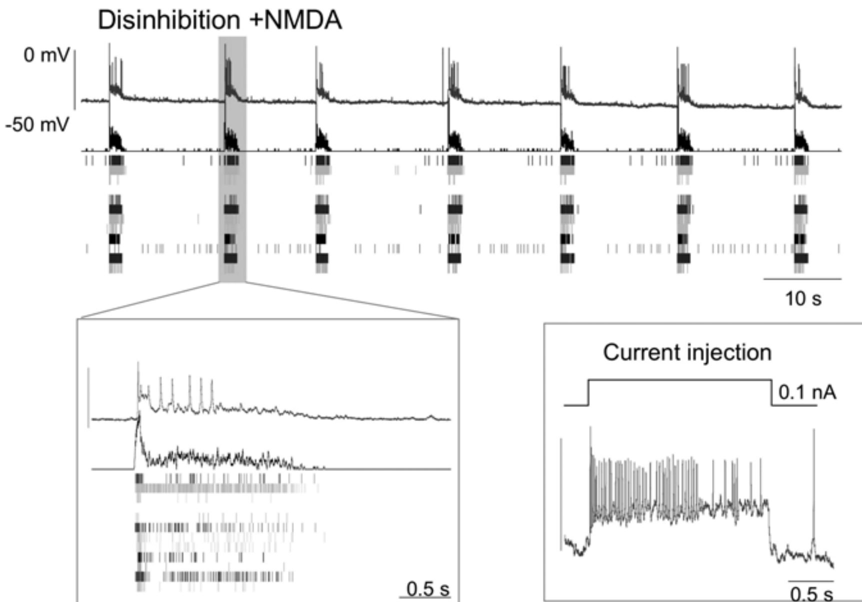

FIGURE 16.8. Spike frequency adaptation. Raster and network activity plots combined with intracellular recording from one neuron (upper trace) show the decrease in network activity and spike rate during the bursts. A similar decrease in spike rate occurs during constant pulses of depolarizing current injections of similar length and amplitude as the bursts.

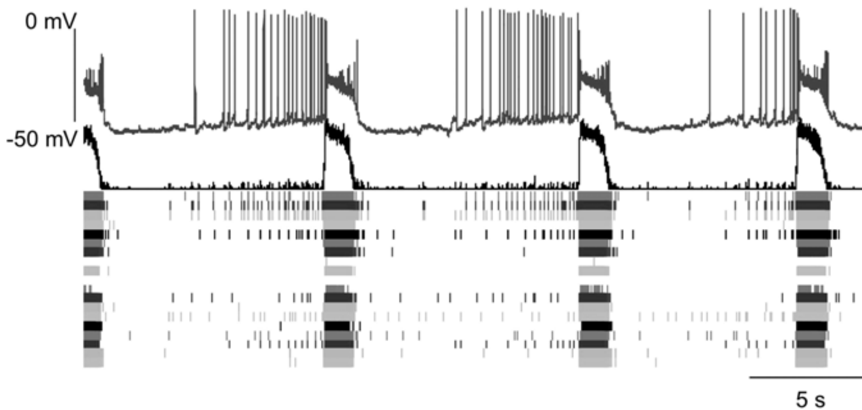

FIGURE 16.9. Intrinsic spiking is suppressed following bursts. Raster and network activity plots are shown together with the intracellular recording of an intrinsic spiking cell (upper trace) during disinhibition-induced bursting. Note the hyperpolarization combined with a suppression of spiking following the burst. In parallel, 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.

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, 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.

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 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 spikes, both characteristic of a smaller I_{Na}. Furthermore, slow inactivation of I_{Na} seems to be enhanced by riluzole, because 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 long-lasting persistent bursts.

16.3.2.2 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 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 bursts and slowly recovers during the interval (see Figure 16.9). The silent period following the burst often goes parallel with a transient hyperpolarization of the cell. As mentioned before, even those cells that show no such transient hyperpolarization following bursts are hyperpolarized during the intervals relative to the state before disinhibition (innate spontaneous activity). This suggests that bursts cause a hyperpolarization of the cells (by up to 10 mV), which 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 because we also found the hyperpolarization in intrinsically silent cells. In these cells we also observed a decrease in excitability following bursts (Darbon et al., 2002b).

16.3.3 Network Refractoriness

16.3.3.1 Pacing the Network with Electrical Stimuli

MEA electrodes cannot only be used for recording but also for stimulation at any site of the network. Single stimuli of 1 to 2 V and 0.1 msec 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 that are evoked through synaptic transmission. Most of the directly stimulated spikes occur in the first ten milliseconds following 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 cannot be evoked from all electrodes, especially not from those that, 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 stimulus can be taken as a measure of cellular excitability, because this parameter does not involve synaptic transmission. Delays after stimulus of more than ten milliseconds usually point to activity that 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, stimuli had almost no effect on the patterns or on the total amount of activity. This shows that under these conditions, 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) that is driving the network.

In contrast, when 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 stimuli fail to induce a burst (see Figure 16.10). The highest

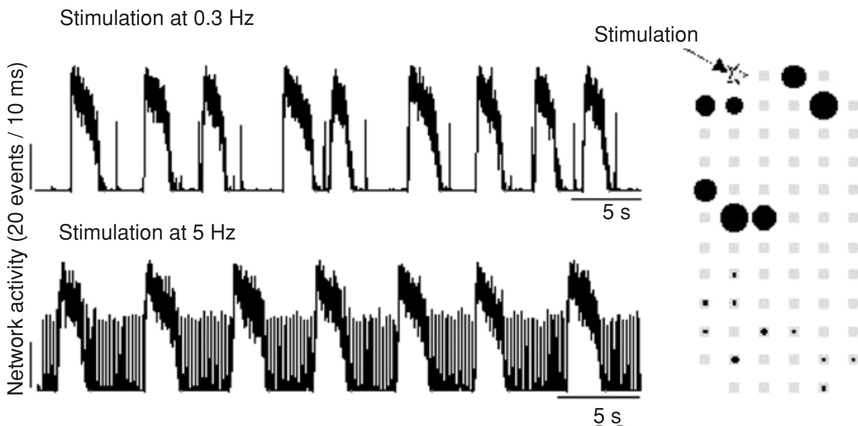

FIGURE 16.10. Network refractory period. Electrical stimuli (1 to 2 V, 0.5 msec) at one MEA electrode (star) evoke activity at several other electrodes. The size of the black points in the graph on the right side is proportional to the amount of activity, which is evoked in the first 10 msec following stimuli. The traces show the response of the network to two different frequencies of stimulation. Note that the network responds with burst 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.

frequency of stimulation to which the network reliably responds with bursts (the critical frequency), is around 10/min (range 5 to 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, because at too high frequencies of stimulation, failures occur and at too low frequencies spontaneous bursts tend to disrupt bursting from 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 because the rate of intrinsic firing is obviously the critical parameter defining burst rate.

16.3.3.2 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 intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ increases during bursts and slowly recovers during the intervals (Darbon et al., 2002a). In parallel, the rate of spontaneous postsynaptic currents is transiently increased following 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 because 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 reflect 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 bursting, burst duration is positively correlated to the preceding but not to the following interval duration (Streit, 1993; Tabak and O'Donovan, 1998). Tabak et al. (2001) have shown in a theoretical study 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 becomes higher and more reliable during depolarization (as is the case during fast regular bursting), burst initiation becomes a deterministic process, inasmuch as it is now mainly controlled by the periodic suppression and relaxation of excitability in 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 now a deterministic process.

16.4 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 pre-Bötzinger Complex of the brain stem (Rekling and Feldman, 1998). In this model, rhythm generation is based on properties of neurons that are not pacemakers themselves, but that form a pacemaker network through mutual excitatory coupling. We have identified important properties of 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 that regulates rhythms is the membrane potential. If 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 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 membrane potential: the more depolarized the neurons are, the more intrinsic spiking there is, the more reliably bursts are initiated, and the more regular bursting becomes. However, two intrinsic accommodation mechanisms, spike frequency adaptation and synaptic depression, also define the rate and regularity of rhythms. Probably the stronger one of the two mechanisms dominates 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 increase the variability and thus decrease the regularity of rhythms; on the other hand it underlies the more complex rhythms such as bursting with intraburst oscillations.

Mutual excitation of 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 acetylcholine can also support recurrent excitation during defined periods of development.

Because 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 acute in slices and in vivo.

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