The number of spikes that are generated per electrode per burst is relatively small, when compared to the number of spikes in the AWSR. Therefore, more averaging in the time domain is required. This can be done by increasing the width of the Gaussian inter polation function, or by including several aligned bursts. It was chosen to include multiple bursts because a wider Gaussian would obscure the very details one is looking for.

4
STN Activity Recorded in Vitro: Dissociated Cell Cultures

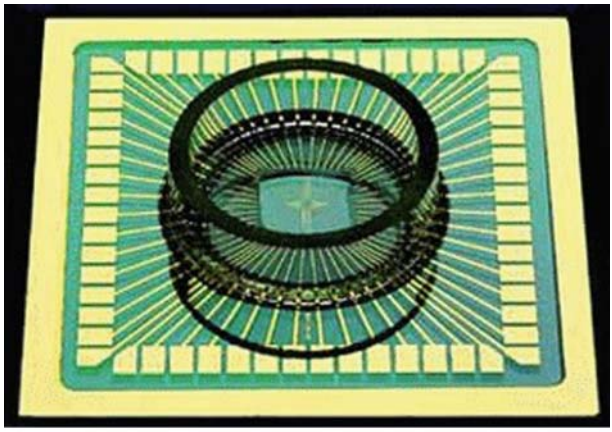
In PD, because of the loss of the dopaminergic nigrostriatal connection, the basal ganglia neurons, including the STN, fire by low-frequency oscillatory bursts and fire in synchrony. Under normal conditions, the basal ganglia neurons fire irregularly. The reciprocal pallido–subthalamic connection can be mimicked in vitro and is capable of burst firing. This pattern can be influenced by co-cultures of striatum and cortex (Plenz and Kitai 1999). The observed bursting activity was unaccompanied by synchronous activity in STN slice preparations after the addition of glutamate, dopamine, GABA, or muscarine receptor agonists and/or antagonists. Therefore, synchrony of STN activity in the parkinsonian state is dependent on its extrinsic connections (Wilson et al. 2004).

Dissociation of central nervous system areas of P1 (day 1 postnatal) rat pups makes it possible to culture these neurons in a chemically defined medium (Heida 2003). By placing these cultures on MEAs, their spontaneous electrical activity can be recorded. By using polyethylenimine as substrate, a seemingly monolayered network can be created in culture on the MEA (see Rutten et al. 2001 for an overview).

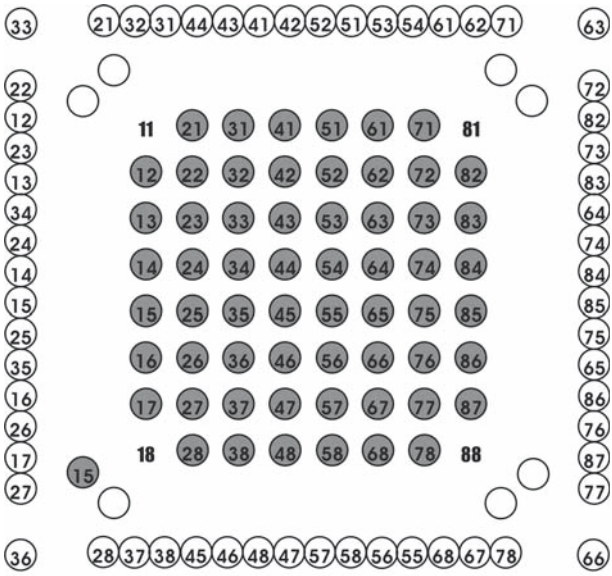
4.1
Experimental Set-up

In short, microelectrode arrays could be constructed because of the progress in the field of microtechnology and were applied in the biological sciences for cardiac cells (Thomas et al. 1972); later it became a tool in neurotechnology for recording and stimulation purposes (Gross et al. 1979). Until now, the MEA that has been used for stimulation and recording experiments with neuronal cells and tissue at the Twente University consists of a glass substrate (5×5 cm) on top of which an array of electrodes is created using lithographic and etching methods (Multi Channel Systems). Figure 16 shows the layout of the MEA. Around the centre, a glass ring provides a culture chamber for neurons suspended in a specially prepared culture medium (Romijn et al. 1984).

If dissociated subthalamic neurons are placed on a MEA they are devoid of their connections and after approximately 7 days, when a network has been created, their activity can be recorded.



A



B

Fig. 16 MEA for culturing neurons (Multi Channel Systems); **A** the MEA; **B** the inner electrode structure in detail and electrode numbering. Electrode diameter is 10 μm; inter-electrode distance is 100 μm

From Part I, Sects. 2.2 and 5.2, it is clear that a cholinergic innervation of the STN area is present. From the presence of N-type Ca^{2+} channels, as described in Sect. 3.3 (this volume), we may expect that acetylcholine can change the activity of STN neurons. Therefore, adding acetylcholine is a mode to test the activity of the STN neurons in terms of increased or decreased firing rate, changes in the firing pattern, or inducing or changing burst activity behaviour in culture.

4.1.1
Cell Culture

Subthalamic areas were dissected from rat pups (postnatal day 1, P_1), mechanically dissociated and trypsin-treated and cultured in a serum-free medium (R12, Romijn et al. 1984), with NGF added, for at least 10 days in vitro (DIV) at a concentration of nearly 10^4 subthalamic cells/ml. An extended description of the technique can be found in van Welsum et al. (1989) and van Dorp et al. (1990).

4.1.2
Measurement Set-up

A MC1060BC preamplifier and FA60s filter amplifier (both Multi Channel Systems) was used to prepare the signals for AD conversion. Amplification is 1,000 times in a range from 100 Hz to 6,000 Hz. A 6024E data-acquisition card (National Instruments, Austin, TX, USA) was used to record all 60 channels at 16 kHz (Figure 17 shows the measurement set-up). Custom-made Labview (National Instruments) programmes are used to control the data acquisition (DAQ). These programmes also apply a threshold detection scheme with the objective of data reduction. Actual detection of action potentials is performed in an offline fashion. During the experiments, the temperature was controlled at 36.0°C using a TC01 (Multi Channel System) temperature controller. Recording starts after a minimum of 20 min to prevent any transient effects. Noise levels were typically 3–5 μV_{RMS}, somewhat depending on the MEA and electrode. We use commercially available MEAs from Multi Channel Systems with 60 titanium nitride electrodes in a square grid. The inter-electrode distance is 100 μm, and the diameter of the electrodes is 10 μm.

4.2
Spontaneous Activity

Spontaneous activity was observed using several MEAs for a total of several hours. Figure 18 shows the result of one of the measurements in terms of the average spike rate as a function of time over a period of 5 min. Electrodes that showed a minimum firing activity of at least 1 Hz were selected for further analysis, which in this case resulted in the selection of 22 electrodes. The average firing rate in Fig. 18

Fig. 17 Measurement set-up in which the MEA is connected to the computer while culturing conditions are controlled

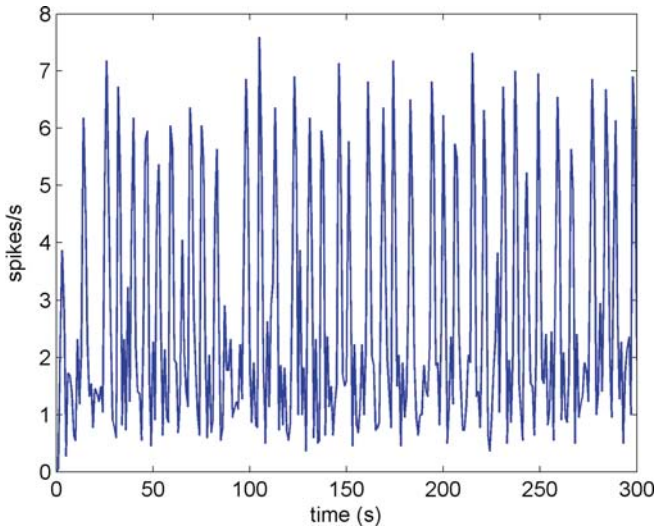


Fig. 18 Spontaneous activity in an in vitro STN network represented by the average number of spikes as a function of time. An average spiking frequency of 2.7 Hz was detected selecting those electrodes with at least 1 Hz as a baseline activity

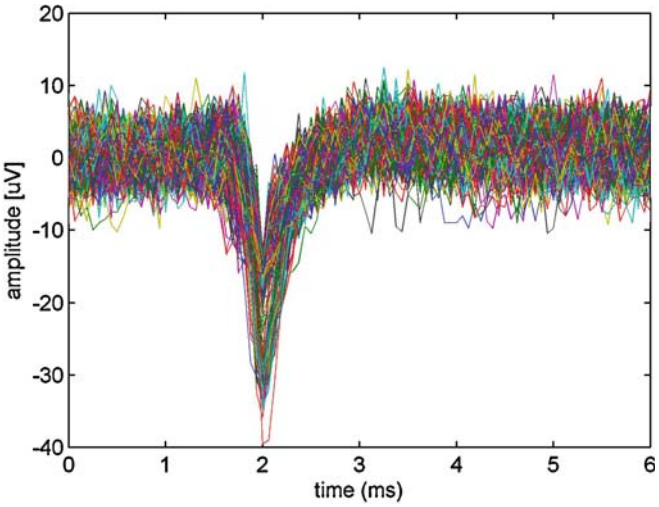


Fig. 19 Typical waveforms of recorded STN spontaneous spiking activity; this graph consists of 500 randomly selected wave forms from one of the electrodes

is 2.7 Hz. However, the average firing was found to vary among MEAs, possibly resulting from different network architectures. A number of wave forms from the measurement are shown in Fig. 19.

4.3 Addition of Acetylcholine

To test the effect of acetylcholine on the activity of STN cells, acetylcholine was added to a concentration of 10 μM in 50 μl of culture medium to a 2-ml culture bath, each 1,000 ms. The total recording lasted 1.5 h; the recorded activity before addition of acetylcholine was considered the spontaneous STN activity in culture. Whether bursts occur in the network of STN cells was tested. A burst was defined as consisting of at least four action potentials with an inter-spike interval of 20 ms or less, i.e. a minimum intra-burst spike rate of 50 Hz.

Addition of acetylcholine induced a direct substantial reduction of spontaneous activity in the cultures, as shown in Figs. 20 and 21. At the application, for a time period of nearly 50–100 s no activity was noticed, after which network activity returned. Measuring the total spike activity over the entire period, a 25% reduction of activity occurred as compared to the spontaneous control activity before acetylcholine addition (3.1 and 4.2 Hz, respectively). In a control experiment, it was shown that the addition of 50 μl R12 (normal culture medium) without acetylcholine did not disturb the activity at all.

Bursting activity was not convincingly detected in the networks. Only one of the MEAs showed bursting activity in accordance with the burst definition, with

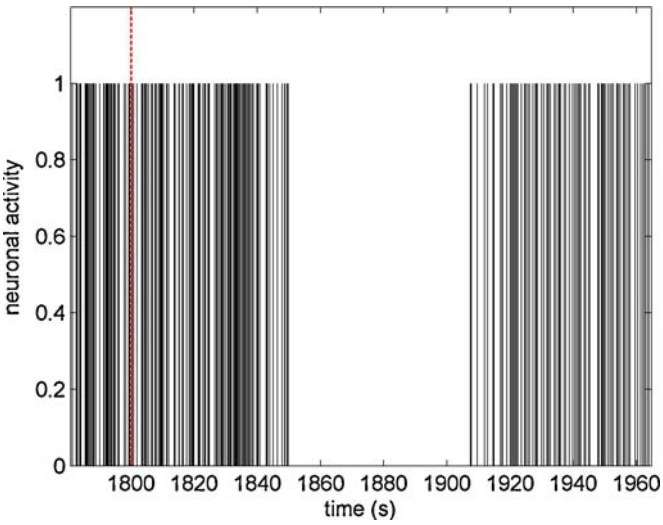


Fig. 20 Activity recorded at electrode 14 during and after the addition of acetylcholine (step 1, addition of 10 μMol ACh). The data clearly show a total reduction of activity after the moment ACh was added

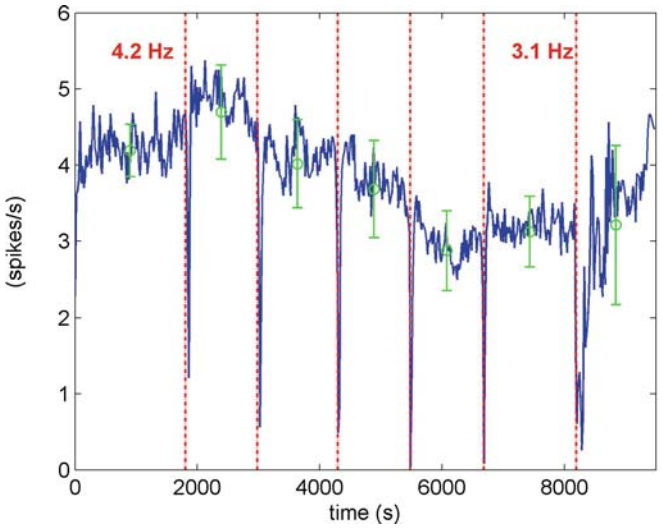


Fig. 21 Average spike activity over 8,000 s with five steps of ACh addition and a washing step after 8,100 ss, indicated by the broken lines. After the removal of ACh, network activity returned to the normal level of spontaneous activity

an average burst length of 4.2 spikes. This bursting activity did not change significantly before and during a period of 1,000 s after the addition of acetylcholine.

From these results, we can conclude that the addition of 50 μM acetylcholine to cultured STN area neurons shows two effects:

1. A direct strong reducing effect on the spike activity of the STN neurons after each addition step that lasts about 50–100 seconds.
2. A long-term effect reducing the activity with, in this case 25% as compared to the spontaneous activity in culture.

The connection that is mimicked by addition of acetylcholine is part of the PPN–STN connection (see Part I, Sect. 5.2.5). This part of the PPN–STN connection is cholinergic, but other cell groups are also involved (glutamatergic, and possibly GABA-ergic). Destruction of the PPN ends up with hyperactivity of the STN (Breit et al. 2005). PPN lesioning was shown to induce akinesia (in fact only motor hypoactivity) in primates (Matsumura and Kojima 2001; Matsumura 2001). It is well established that the cholinergic agonists brought into the rat STN contribute to an excitation of the STN neurons (Feger et al. 1979). However, it was also shown that muscarine agonists in slices diminished the amplitude of both EPSPs and IPSPs in the STN (Flores et al. 1996; Shen and Johnson 2000). The reduction of IPSPs is higher, which leads to a final excitation of STN neurons (Rosales et al. 1994; Shen and Johnson 2000).

Contradictory results are found in the literature as to the effect of acetylcholine on the subthalamic neurons (see above). This could well be caused by the still existing connections. Taking away one connection by lesioning, adding neurotransmitters or their agonists, therefore, does not show the pure effect of connections, neurotransmitters or receptors. Too many parameters are involved to fully understand the effect of these experiments. Culturing subthalamic neurons at least restricts the amount of parameters, but adds others!

It is rather unexpected that addition of acetylcholine to such cultures shows a short-term and a long-term effect. If hyperactivity of STN is induced by reducing the PPN neurotransmitters, among them acetylcholine, and motor hypoactivity is the consequence, then this MEA culturing experiment explains by the long-term effect how such hyperactivity can result from this type of neurotransmitter, neglecting all the other effects of other PPN neurotransmitters. One should remember that addition experiments of Plenz and Kitai (1999) do not show any effects on synchrony. Our results did not show any effect on bursting activity, which may suggest that the long-term effect of acetylcholine on subthalamic cultured cells is related to this synchrony or pacemaker effect, stressing the role of the PPN.

4.4 Electrical Stimulation

Electrical stimulation through one of the electrodes of the multi-electrode array was applied at 20 Hz and 80 Hz with the following stimulation and measurement settings:

- 20 Hz, 500 charge-balanced block pulses (+10/−10 μA, 400 μs/phase), start at 300 s (end 325 s).
- 80 Hz, 2,000 charge-balanced block pulses (+10/−10 μA, 400 μs/phase), start at 300 s (end 325 s).

Stimulation artefacts were removed from the recorded data. Electrodes with average spontaneous activity of at least 1 Hz prior to stimulation were selected.

The recorded data show that at low-frequency (20 Hz) as well as at high-frequency stimulation (80 Hz) the average firing rate increased right after the onset of stimulation, Figures 22 and 23, respectively. During the stimulation period, the firing rate rapidly decreased in both situations; however, at high frequency (80 Hz) this decrease was more rapid. At the time that stimulation is switched off, network activity is quickly diminished before regaining its spontaneous activity. This silencing effect coincides with the effects observed by Benazzouz et al. (2000) and Garcia et al. (2003), as described in Sect. 3.5 (this volume).

All observed effects were found to be reproducible. In Fig. 23B, stimulation at 80 Hz is applied for 120 s, which shows that during stimulation network activity stabilizes and the deviation from the average spike rate is decreased, suggesting a more regulated network activity.

The dissociated STN cells form a network in vitro, while in vivo the STN does not show recurrent connections. No definite conclusions on the stimulation experiments in relation to the use of high-frequency stimulation in DBS can be

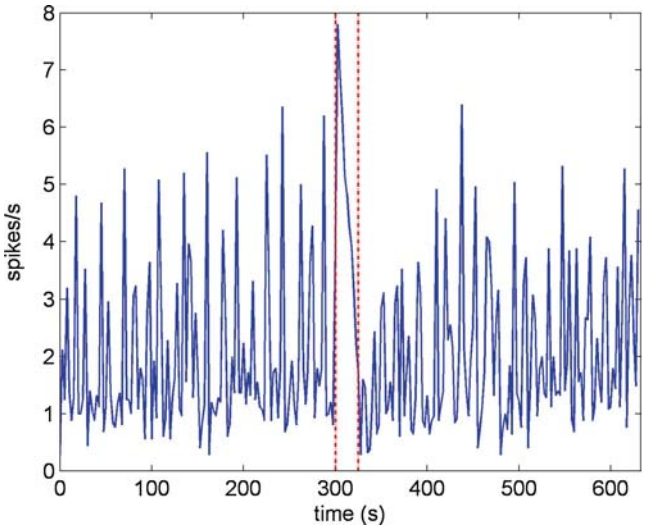


Fig. 22 Average firing rate of selected electrodes. The stimulation period is indicated by broken lines; stimulation frequency is 20 Hz, and a total of 500 pulses are applied (i.e. 25 s). Electrode 28 is the stimulation site

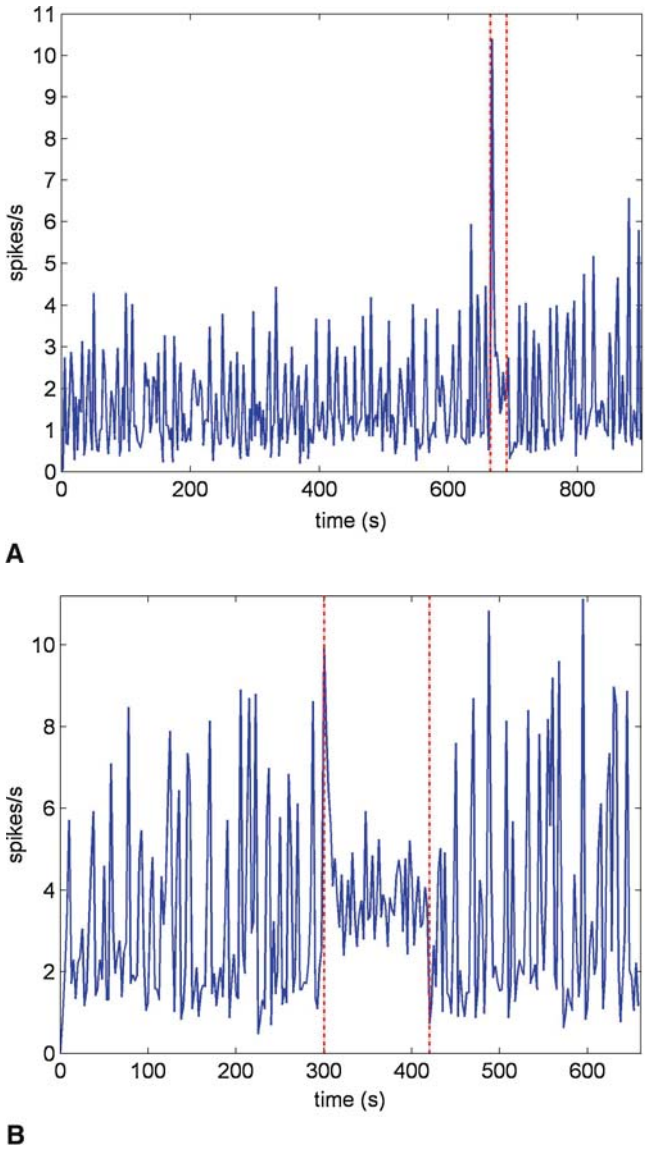


Fig. 23 A,B The effect of electrical stimulation at 80 Hz on the average firing rate of STN network activity; the stimulation period is indicated by broken lines. **A** A total of 2000 pulses is applied (i.e. 25 s); **B** an extended period of stimulation (120 s) indicates that network activity is regulated by stimulation. In both situations, electrode 28 is the stimulation site

drawn. Further research including the use of different types of neurotransmitters and stimulation protocols is required. Modelling studies as described in Sect. 5 may also shed some light on the network dynamics and the influence of stimulation.