Acetylcholine addition and electrical stimulation of dissociated neurons from an extended subthalamic area – A pilot study in the rat

T. Heida[1], K.G. Usunoff[2] and E. Marani[1,2]

[1] University of Twente/Faculty of Electrical Engineering, Mathematics and Computer Science, Biomedical Signals & Systems, Enschede, The Netherlands

[2] Medical University and Inst. Physiology, Bulgarian Academy of Sciences/Dept. Anatomy and Histology, Sofia, Bulgaria

Abstract—**Addition of acetylcholine to cultures of STN area's (5 steps of 10 µM with a step interval of 1000 s) show a direct and longlasting reduction of STN activity. Low frequency stimulation (20 Hz, 500 block pulses) increases STN activity, while high frequency stimulation (80 Hz, 2000 block pulses) reduces STN activity, even after the stimulation period of 25 seconds.**

Keywords— **STN, electrical stimulation, Acetylcholine.**

I. Introduction

Parkinson's disease is characterized by the progressive loss of dopamine neurons in the substantia nigra, which results in a reduction of activity in the thalamus partly due to an increased bursting activity of STN (subthalamic nucleus) cells. This abnormally increased bursting activity of STN cells was correlated with tremor in Parkinson patients [1]. The STN plays important roles in (voluntary) motor control, e.g, pathological changes in the nucleus cause hemiballism.

Manipulation of the activities of STN neurons by adding neurotransmitter agonist or antagonists strongly affects spiking behaviour [2], which indicates the importance of knowing how activities of STN neurons are regulated. It is presently firmly established that the STN projection neurons are glutamatergic, excitatory [3], and they heavily innervate the substantia nigra (SN), the internal pallidal segment (GPi), followed by the external pallidal segment (GPe) and the pedunculopontine tegmental nucleus (PPN), by widely branching axons. Some of these connections are reciprocal.

Deep brain stimulation (DBS), which is high frequency stimulation in or near the STN, results in an average reduction of: akinesia (42%); rigidity (49%), tremor (27%) and of axial symptoms. DBS produces non-selective stimulation of an unknown group of neuronal elements over an unknown volume of tissue. Therefore the actions of DBS are difficult to understand.

In slice preparations, STN neurons show rhythmic single-spike activities at resting membrane potentials. In response to depolarizing current pulses, STN neurons increase their firing frequencies linearly with the magnitude of injected current. Several studies have reported the generation of a plateau potential, a long-lasting depolarizing potential [4, 5]. A plateau potential can induce long-lasting high-frequency discharge in the absence of synaptic inputs. STN neurons can generate a plateau potential only when the cells are hyperpolarized in advance. By way of this voltage dependent generation of a plateau potential, STN neurons can transform short-lasting synaptic excitatory inputs into long-lasting bursts and change their spontaneous activities from single-spike to a burst firing pattern [6]. In addition, the voltage-dependency of a plateau potential may play important roles in the generation of oscillatory bursting activity of the STN neurons, characterized by bursts of long duration and repeating at low frequency.However, the mechanism of this voltage dependency in the generation of a plateau potential remains unknown. Opening of K+ channels by metabolic pathways is one possibility; high-frequency inhibitory input from, for example, the Globus Pallidus or PPN stimulation of the STN, is another.

According to Otsuka [6], L-type Ca++ channels play an important role in the generation of a plateau potential due to the slow inactivation kinetics. The burst frequency gradually decreases after which the neuron returns to its normal firing behaviour. It is assumed that dopamine depletion, as occurring in Parkinson's disease, results in hyperpolarization of STN neurons, and thus bursting activities are more likely to be induced than in the normal situation.

Is increased bursting activity due to network activity, within the STN itself or due to influence of the GP or other structures that project to the STN? In order to answer this question, STN cell cultures may provide a useful instrument. Therefore, dissociated STN area cells of the rat are cultured on a micro-electrode array (MEA). The influence of acetylcholine (PPN input) and high frequency stimulation (as in deep brain stimulation) on STN dissociated neuron activity is investigated experimentally.

II. Methods

A. Cell culturing

STN cells (rats) were dissociated using chemical (trypsine/EDTA) and mechanical dissociation techniques, and

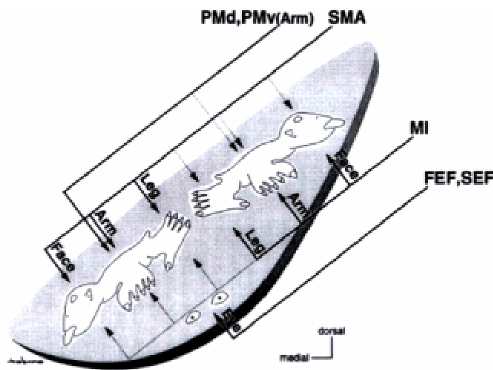

Fig. 1 STN (3*7*12 mm ~ 250 mm3) with presumed somatotopic organization (Source: A. Nambu et al., Neurosci. Res. 43, 2002).

cultured on a micro-electrode array (MEA) consisting of 64 electrodes. The surface of the array was coated with Poly-EthylenImine (PEI, 30 ngram/ml) to support attachment and growth of the neurons. During recording periods the electrode array was placed in an incubator while the temperature was kept at 37°C.

B. Measurement set up

A MC1060BC pre-amplifier and FA60s filter amplifier (both MultiChannelSystems) was used to prepare the signals for AD-conversion. Amplification is 1000 times in a range from 100 Hz to 6000 Hz. A 6024E data-acquisition card (National Instruments, Austin, TX) was used to record all 60 channels at 16 kHz. Custom-made Labview (National instruments, Austin, TX) programs are used to control the data acquisition (DAQ). These programs 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 ºC, using a TC01 (MultiChannel Systems) temperature controller. Recording starts after a minimum of 20 minutes, to prevent any transient effects. Noise levels were typically 3 to 5 μV_{RMS}, somewhat depending on the MEA and electrode. We use commercially available MEA's from MultiChannel 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.

C. Addition of Acetylcholine

Acetylcholine was applied in 5 steps of 10 µM with a step interval of 1000 s using a small pipet positioned through the cover placed over the electrode array for sterility.

D. Electrical stimulation

Stimulation through these electrodes occurred at 20 Hz and 80 Hz. Stimulation artefacts are removed from the recorded data. Electrodes with spontaneous activity of at least 1 Hz prior to stimulation were used and translated to a minimum total number of spikes within the period prior to stimulation. One electrode out of 60 was chosen for stimulation. Stimulation settings: 20 Hz, 500 block pulses, start at 300 s (end 325 s); 80 Hz, 2000 block pulses, start at 300 s (end 325 s).

III. Results

A. Addition of Acetylcholine

Under normal culturing conditions single spike activity with an average frequency of 5.5 Hz was recorded. Bursts, i.e. sequences of at least four spikes with an inter-spike interval less than or equal to 20 ms, were also recorded but no synchrony was found.

Acetylcholine was applied in 5 steps of 10 µM with a step interval of 1000 s. After application neuronal activity was significantly decreased for about 100 s, after which spiking activity was restored. The total measurement time was 2.25 hr (including preceding normal registration). Up to 1000 s after the last acetylcholine application a total reduction of 25% of the spike activity was measured ($p=0.01$). The occurrence of bursts did not significantly change during and after the application of acetylcholine. In conclusion, two spike phenomena in STN cultures could be discerned: an acute diminishing effect of acetylcholine and an overall reduction or late acetylcholine effect.

B. Electrical stimulation

Stimulation of the cultures occurred via one of the 60 electrodes for 25 seconds and were carried out at 20 Hz and 80 Hz and repeated in the experiments. Experiments lasted nearly 1.5 hour and the summation bin was 5 seconds. At low frequency stimulation the total normalized firing rates during the stimulation period increased, while at high frequency stimulation the total normalized firing rate decreased during and after the stimulation period.

VI. Discussion

The connection that is mimicked by addition of acetylchline is part of the PPN-STN connection. This part of the connetion is cholinergic, but other cell groups are also present (glutamaterg, GABA-ergic and dopaminergic).

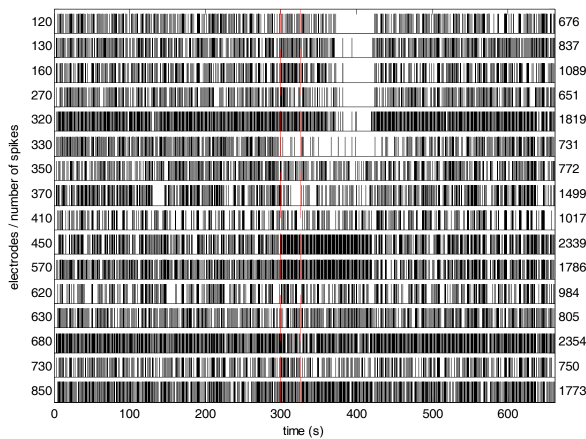

Fig. 2 Activity of STN cells recorded from several electrodes. The moments of stimulation are indicated by red lines; Spikes are indicated by a single line.

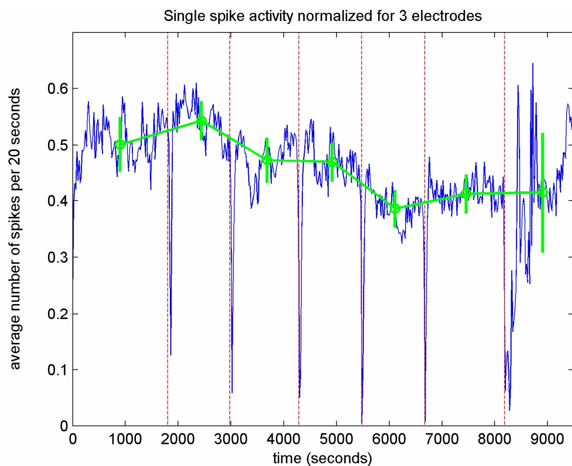

Fig. 3 Activity of STN cells recorded from several electrodes. The moments of application of ACh are indicated by red lines; after the fifth step (a total of 50 µMol) ACh was washed out.

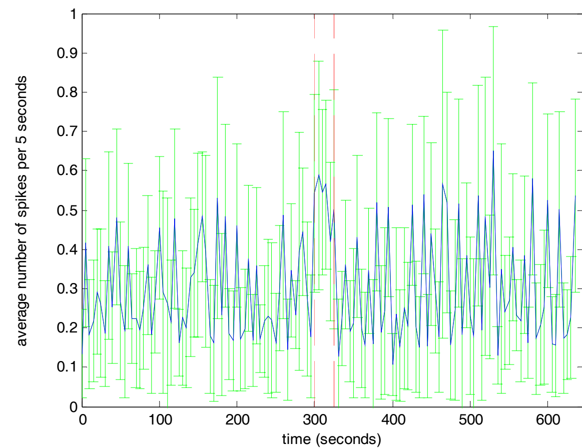

Fig. 4 Normalized mean firing rate of selected electrodes; electrode 330 and 410 were deselected on the basis of their activity during the stimulation period with a stimulation frequency of 20 Hz, and a total of 500 pulses (25 seconds).

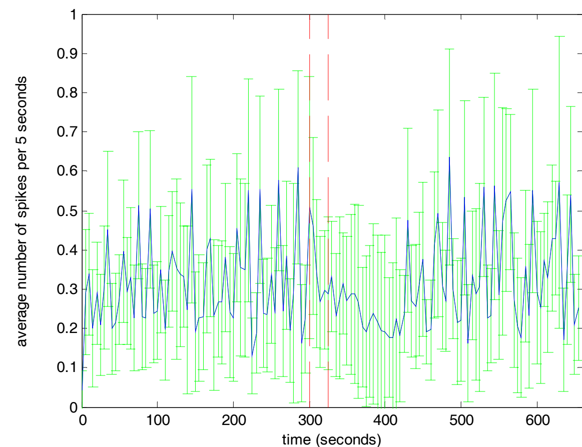

Fig. 5 Normalized mean firing rate of selected electrodes. Stimulation period is indicated by red lines. Electrode 28 is the stimulation site with a stimulation frequency of 80 Hz, and a total of 2000 pulses (25 seconds).

Destruction of the PPN ends up with hyperactivity of the STN [7]. PPN lesioning was shown to induce akinesia in primates [8, 9]. It is now well established that the cholinergic agonists brought into the rat STN contributes to an higher excitation of the STN neurons [3]. However, muscarine agonists in slices diminished the amplitude of both EPSP's and IPSP's in the STN [10, 11].

The reduction of IPSP's is which leads to a final excitation of STN neurons [11, 12]. Adverse results are found in literature as to the effect of acetylcholine on the subthalamic neurons. This could well be due to the still existing connections. Taking away one connection by lesion, adding neurotransmitters or their agonists, therefore, does not show the pure effect of connections, neurotransmitters or receptors. Too many parameters are involved to understand the effect of these experiments. Culturing subthalamic neurons at least restricts the amount of parameters, but adds others! and it is rather unexpected that addition of acetylcholine to such cultures shows a short term and a long term effect. One should notice that addition of 10µM acetylcholine to rat cortex neurons increases their activity (unpublished results). If hyperactivity of STN is induced by reducing the PPN neurotransmitters, among them acetylcholine, and motohypoactivity is the consequence, than this MEA culturing experiment explains by the long term effect how such an hyperactivity can result from this type of neurotransmitter, neglecting all the other effects of other PPN neurotransmitters. The results show no effect on bursting activity, and

therefore the long term effect of acetylcholine on subthalamic cultured cells may be related to the synchrony or pacemaker effect, stressing the role of the PPN.

DBS is carried out in humans under monopolar cathodic stimulation with 120-180 Hz frequency, 1-5 V amplitude and 60-200 ms pulse duration. Although the stimulation conditions in the reported experiments at high frequencies differ from those of human DBS, still some conclusions can be drawn. Compared to low frequency stimulation an overall increase in the spike activity in culture during stimulation is noticed, after which the activity returns to normal. While at high frequency stimulation (80 Hz in our cultures) a decreases in spike activity in cultures appears not only during stimulation but also a certain period afterwards (25 till 100 seconds).

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Author:	T. Heida
Institute:	University of Twente, Biomedical Signals & Systems
Street:	Drienerlolaan 5
City:	Enschede
Country:	The Netherlands
Email:	t.heida@utwente.nl