VILNIUS UNIVERSITY

ROBERTAS GUZULAITIS

The organisation principles of spinal neural network: temporal integration of somatosensory input and distribution of network activity

Summary of doctoral dissertation

Biomedical sciences, biophysics (02 B)

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The study was carried out at Vilnius University during the period 2009 – 2013.

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VILNIAUS UNIVERSITETAS

ROBERTAS GUZULAITIS

Nugaros smegenų neuronų tinklo veikimo principai: somatosensorinės informacijos integracija ir aktyvumo išplitimas

Daktaro disertacijos santrauka

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Abbreviations

AP – action potential;

CPG – central pattern generator;

CSP – cutaneous silent period;

DLF – dorsolateral funiculus;

ENG – electroneurogram;

HE – hip extensor;

HF – hip flexor;

V_m – membrane potential;

VPP – ventral posterior pocket;

V_{th} – threshold of action potential.

1. Introduction

A century ago it was found that spinalized animals are able to generate coordinated movements (Sherrington 1906, Sherrington 1910, Brown 1911, Brown 1914). Now it is accepted that movements such as locomotion and reflexes are organised by neural network of spinal cord. Spinal cord injuries in human often lead to chronic paralysis. The loss of mobility is dramatic for injured and costly for society. Understanding the principles of organisation of spinal neural network might lead to new strategies of treatment and rehabilitation.

Spinal neural network receive and integrate somatosensory input. After processing of somatosensory information the neural network has to make a decision whether the sensory input is meaningful. If the stimulus is strong enough – neural network starts a motor program. But the stimulus is of intermediate intensities can be meaningful too. In this case the decision can made depending on stimulus repeatability. If stimuli of intermediate intensities appear at certain frequencies – network reacts.

The classical example of processing of temporal events is temporal summation of sensory input in the neuron (Kandel, Schwartz et al. 2000). Neuron depending on its characteristic time constant is able to integrate synaptic input at millisecond time range. However, the spinal neural network integrates somatosensory input in sub-second time range (Sherrington 1906, Crowe and Linnartz 1985, Currie and Stein 1988). This means that the information about somatosensory input remains in spinal cord for more than 1 s. The mechanism of this temporal integration in spinal cord in sub-second time range is still poorly understood.

As it was mentioned before spinal neural network is able to generate locomotion and reflexes. Neural network is distributed in entire spinal cord in the fishes (Grillner 1974, Cohen and Wallen 1980). However in limbed animals the key elements of neural network controlling hind and front limb

movements are located in lumbar and cervical enlargements respectively (Mortin and Stein 1989, Cazalets, Borde et al. 1995, Ballion, Morin et al. 2001). These enlargements are connected with thoracic segments of spinal cord. It has been recently shown that synaptic connections in thoracic spinal segments is important for interlimb coordination and information transmission from higher brain centers (Cowley, Zaporozhets et al. 2008, Cowley, Zaporozhets et al. 2010). Moreover, the plasticity in these spinal segments could restore voluntary movements after spinal lesions (van den Brand, Heutschi et al. 2012). These studies indicate that neural network in thoracic segments contributes to limb movement generation. However, the particular function of these spinal segments is not known.

The dissertation is focussed on uncovering the mechanism of temporal integration in spinal neural network and evaluating the spatial distribution of neural network controlling hind limb movements.

Aim and objectives

The aim of the study is to evaluate the spatial distribution of neural network activity and to determine the mechanism causing temporal integration of somatosensory input.

Objectives:

- To evaluate how excitability of motoneurons contributes to temporal integration;
- To assess the activity of pre-motor neurons during temporal integration;
- To evaluate the spatial distribution of neural network generating pocket scratch reflex.

Actuality and scientific novelty

The intrinsic properties of motoneurons and its contribution to temporal integration were tested. It was found that temporal integration of

somatosensory input can occur without noticeable change in excitability of motoneurons. However, the activity of pre-motor neurons increases during temporal integration.

Inhibition of motoneurons during cutaneous silent period was showed for the first time directly by intracellular recordings of motoneurons.

It was found that neurons in thoracic segments receive information about activity of hind limbs. Some of these neurons send their axons back towards lumbar enlargement. This suggests wide distribution of neural network controlling movements of hind limbs.

Defensive statements

- Motoneurons are inhibited during somatosensory silent period;
- Temporal integration of somatosensory input might occur without changes of excitability of motoneurons;
- Activity of pre-motor neurons increases during temporal integration of somatosensory input;
- Thoracic segments of spinal cord are involved in pocket scratch generation.

2. Methods

2.1. Ethical approval

Experiments were performed in University of Copenhagen, neuronal signalling laboratory and Vilnius university, department of neurobiology and biophysics.

The surgical procedures complied with Danish legislation and were approved by the controlling body under The Ministry of Justice. Experiments in Vilnius university were performed with permission from State food and veterinary service (no. 0240).

2.2. The integrated carapace-spinal cord preparation

Red-eared turtles (*Chrysemys scripta elegans*) with 10-15 cm carapace length were obtained from Nasco, Fort Atkinson, WI, USA. Surgical procedures were described before (Alaburda and Hounsgaard 2003). Briefly, turtles (n=68) were placed on crushed ice 2 hr before surgery (Melby and Altman 1974, Lennard and Stein 1977) to induce drowsiness and reduce stress and pain by hypothermia. In this way the head and neck could be protracted using minimal force. Brain functions were terminated immediately upon decapitation by crushing the head. The blood was substituted by perfusion through the heart with a Ringer solution containing (mM): 120 NaCl; 5 KCl; 15 $NaHCO_3$; 2 $MgCl_2$; 3 $CaCl_2$; and 20 glucose, saturated with 98% O_2 and 2% CO_2 to obtain pH 7.6.

Transverse cuts were made at rostral (D3-D4 segments) and caudal (D9-S2 segments) parts of spinal cord. The hip flexor (HF) (innervating *puboischiofemoralis internus, pars anteroventralis* muscle) nerve was exposed and cut. In some experiments cutaneous VPP (ventral posterior pocket) nerve was exposed and cut too (Figure 2.2.1) (Currie and Stein 1988).

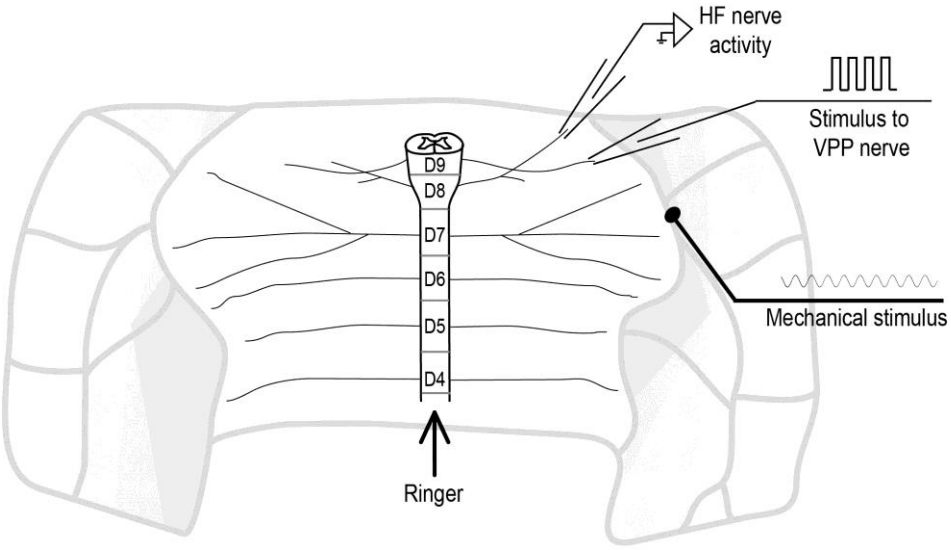

Figure 2.2.1 Carapace-spinal cord preparation from adult turtle. Scratch reflex could be induced by mechanical stimulation of carapace or electrical stimulation of VPP nerve.

The cut-end of the carapace was glued to a Plexiglas platform and mounted in a holder to visualize transverse cut surface of spinal cord. The caudal (Figure 2.2.2 A) or rostral (Figure 2.2.2 B) cut-end of the carapace was glued to a Plexiglas platform and placed upwards for motoneuron or interneuron recordings respectively. Intracellular electrodes were inserted by visual guidance. A small Plexiglas slab was glued to the opposite carapace edge and a plastic tube was installed for perfusion of the spinal canal.

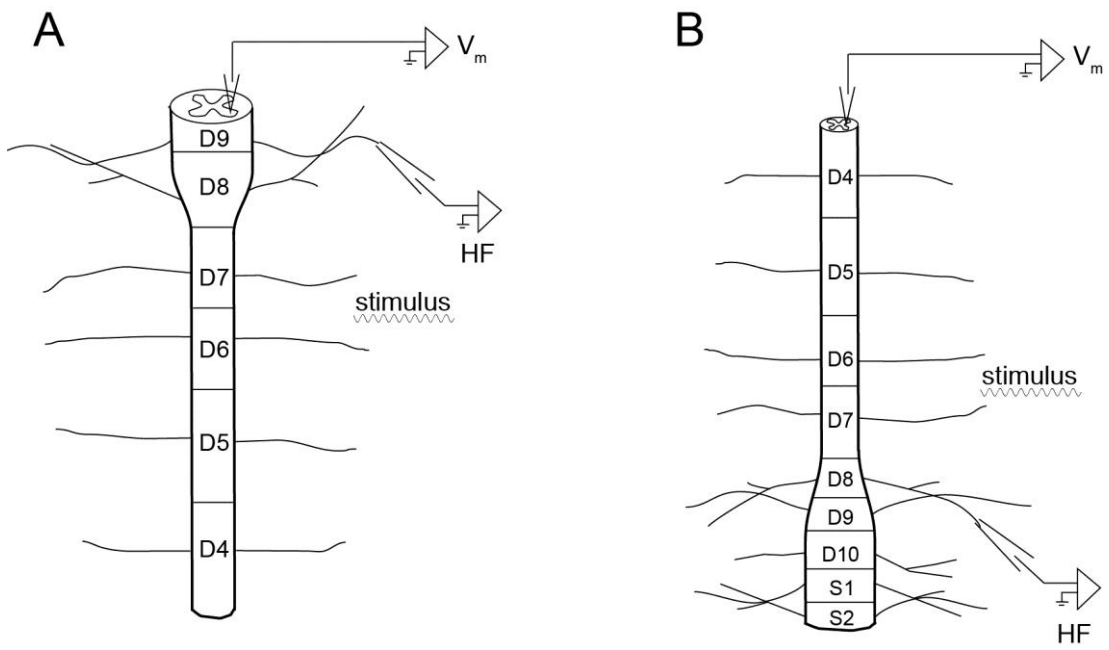

Figure 2.2.2 Carapace-spinal cord preparations for motoneuron (A) and interneuron (B) recordings. Mechanical stimulus was applied on carapace or skin of the turtle. Recordings of HF motor nerve were performed to monitor scratch reflex.

2.3. Stimulation

Mechanical stimulation for induction of the fictive scratch reflex (rhythmic activity in ipsilateral HF nerve) was performed by pinching the pocket skin with a pair of tweezers or with fire-polished glass rod mounted to the membrane of a loudspeaker controlled with a function generator (Rosenberg 1986, Alaburda and Hounsgaard 2003). M9.5 dermatome innervated by D7 spinal segment afferents (Mortin and Stein 1990) was stimulated to induce pocket scratch (Mortin, Keifer et al. 1985). M8.5 and M6.5 dermatomes inervated by D6 and D4 spinal segment afferents respectively (Mortin and Stein 1990) were stimulated to induce rostral scratch (Mortin, Keifer et al. 1985).

Electrical stimuli for induction of fictive scratch reflex were applied to cut ipsilateral cutaneous nerve (VPP nerve) at D8-D9 spinal segments (Currie and Stein 1988, Currie and Lee 1996) with suction electrode.

Graded activation of scratch network activity was achieved by varying intensity and frequency of stimulus (Currie and Stein 1988, Currie and Stein 1990). With the stimulus intensities used in this study scratching was induced at frequencies ≥0.2 Hz. To avoid scratch reflex induction the frequency of stimulation was reduced to <0.05 Hz.

2.4. Recordings

Intracellular recordings in current-clamp mode were performed with a Multiclamp 700B amplifier (Molecular Devices). Sharp glass electrodes from thin-walled borosilicate glass were filled with a mixture of 0.9 M CH_3CO_2K and 0.1 M KCl. In staining experiments 2 % of biocytin was added to the pipette solution. Resistance of electrodes was 40-60 MΩ. Intracellular recordings in bridge mode were obtained from motoneurons in ventral horn of D9-D10 segments. Recordings were accepted if motoneurons had a stable membrane potential more negative than -50 mV. Intracellular recordings in bridge mode were obtained from interneurons in ventral horn and intermedium zone of D4 segment. Most of interneurons were spontaneously active and to prevent interneurons from spiking sustained negative current was injected.

Data were sampled at 10 or 20 kHz with analog-to-digital converter (Digidata 1440 or Digidata 1322A, Molecular Devices), displayed by means of Axoscope and Clampex software (Molecular Devices), and stored on a hard disk for later analysis.

The electroneurogram (ENG) of the HF nerve was recorded with a differential amplifier Iso-DAM8 (World Precision Instruments, Sarasota, FL, USA) using a suction electrode. The bandwidth was 100 Hz – 1 kHz, amplification $x10^4$.

2.5. Staining of neurons

Micrographs of neurons and their axons were produced using a confocal microscope, Zeiss LSM 700 with diode lasers, on a Zeiss Axiolmager M2

using 10x/0.3 EC Plan-Neofluar and 40x/0.6 EC Plan-Neofluar objectives (Zeiss). The flourophore Cy3 was excited at 555 nm and detected in the range 560-1000 nm. Images were handled with ZEN 2009 software (Zeiss) in the LSM and 8-bit TIFF format.

Neurons were filled with biocytin by passing positive current steps (100-500 pA, 2 Hz, 250 ms) for 5-20 min. After filling neurons with biocytin D4-D5 spinal segments were removed from preparation. The tissue was perfused and left in phosphate buffered saline (PBS) with 4 % paraformaldehyde for 24-48 hrs at 4ºC. Spinal cord was then rinsed with and stored in PBS. The tissue was dissected longitudinally at midline and glued on an agar block. The tissue was sliced into longitudinal 100 µm slices using Leica VT1000S vibrotome. The slices were incubated overnight at 4ºC with Cyanine-3-conjugated to streptavidin (1:500, Jackson Immunoresearch labs, Inc) in PBS with 0.3 % Triton X-100 and 5 % donkey serum. After washing in in PBS-T, the slices were mounted on cover slit with a drop of ProLong® Gold antifade reagent (Invitrogen Molecular Probes, USA) and cured overnight at room temperature before microscopy.

2.6. Data analysis

Latency and duration of CSP

CSPs were analysed from sweeps of ENG recordings (from 200 ms before until 300 ms after a single cutaneous nerve stimulus) during motor nerve activity (during ENG burst). ENG sweeps were superimposed for qualitative demonstration of CSP. For quantitative evaluation of CSP at least 20 ENG sweeps were rectified and averaged. The baseline was defined as average of 100 ms of rectified ENG before the stimulus (from 50 to 150 ms before stimulus). The CSP (the onset and the end) was identified as the most prominent downward deflection of the rectified ENG signal below 80 % of baseline (Kofler 2003, Rodi and Springer 2011). The latency of CSP was

defined as the time from the stimulus to the onset of CSP and the duration of CSP as the time from the onset to the end of CSP.

Intensity of CSP

The intensity of CSP was evaluated by suppression index (SI) i.e. the ratio of rectified ENG during CSP and rectified ENG before stimulus (baseline) (Rodi and Springer 2011).

The rebound of ENG was defined as the average of the rectified ENG activity 50 ms after the end of CSP. The strength of rebound after CSP was evaluated as rebound index (RI) i.e. the ratio of the rectified ENG during the rebound and the rectified ENG before stimulus (baseline) (Kumru, Opisso et al. 2009).

Conductance of neurons

To measure the resting and the evoked synaptic conductance in motoneurons during CSP the response to nerve stimulation was recorded at least at 3 different levels of DC current injected through the intracellular recording electrode. The conductance was calculated from current – voltage (I-V) characteristic for every 0.1 ms.

In other experiments conductance of neurons was evaluated from V_m deflections induced by hyperpolarizing current pulses. 250 ms current pulses were applied at 2 Hz frequency and/or 100 ms current pulses were applied at 5 Hz frequency.

V_m of neurons

The membrane potential (V_m) in different states (rest, before scratch reflex etc.) was quantified as the average of the instantaneous membrane potential over 0.5 s.

Excitability of neurons

The excitability of motoneurons was monitored by the response to 250 ms depolarizing current pulses that evoked a single action potential (AP) in the resting state prior to stimulation at 2 Hz frequency (Russo and Hounsgaard 1994, Delgado-Lezama, Perrier et al. 1997, Alaburda and Hounsgaard 2003).

A change in the number of evoked APs was taken as an indication of change in excitability.

Threshold for AP generation

Threshold for AP generation (V_{th}) was defined as the membrane potential at which depolarization increased ≥10 V/s during onset of an AP evoked by a depolarizing current pulse. Before electrical stimulation V_{th} was determined as the average for 10 successive APs. During the stimulus train prior to fictive scratching V_{th} was taken as the average value for last 3 APs evoked by current pulses.

Fluctuations of V_m

The magnitude of the synaptically induced fluctuations of V_m was estimated as standard deviation (SD) of V_m (Pare, Shink et al. 1998, Destexhe and Pare 1999) for 0.5 s at appropriate time (before the first stimulus, 1 s after the stimulus, etc.). Notch (50 Hz) and high pass (2 Hz) filters were used.

Reduction of neural network

Pocket scratch was evoked by stimulating receptive fields innervated by afferents of D6-D7 spinal segments. Scratch reflex was evoked every 4-5 min and repeated at least 5 times in every state (control, D3, D4 and D5 segment removed). After 5 or more repetitions of scratch reflex one mid-thoracic segments of spinal cord was removed.

Scratch reflex activity was evaluated by calculating integral of rectified HF nerve ENG activity, number and frequency of HF bursts during scratch episode. The onset and offset of HF burst were defined when rectified and averaged ENG crossed 150% of baseline ENG activity. Baseline ENG activity was calculated before stimulation.

Phase analysis of activity of neurons

Double-referent phase analysis (Berkowitz and Stein 1994, Stein and Daniels-McQueen 2002) was performed on D4 neurons that fired rhythmically. The phase of activity was calculated in respect of HF nerve activity. HF bursts were normalized. The onset of HF burst was defined as 0 and 1 and end of HF burst

was defined as 0.5 and -0.5. The phase analysis of neurons was performed if neuron fires at least 3 AP during depolarization during scratch cycle and there were at least 3 successive scratch cycles.

Statistical analysis

Data were analysed statistically using a Student's paired t-test (Origin software; Microcal Software, Northampton, MA). Significance of differences was accepted at $p<0.05$. The level of significance is indicated as n.s. ($p>0.05$); * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$). Data are presented as means ± SD (n = a number of neurons).

3. Results

3.1. Protracted initiation of scratch network activity

In the integrated carapace-spinal cord preparation mechanical stimulation (pinch of the hind limb pocket skin with a pair of tweezers) evokes fictive scratch activity as recorded in the ipsilateral motor (HF) nerve (Figure 3.1.1 A) (Keifer and Stein 1983, Alaburda and Hounsgaard 2003). Fine adjustment of mechanical stimulation leads to protracted initiation of scratch episodes (Figure 3.1.1 B). First application of a brief mechanical stimulus with a fire-polished rod does not evoke scratching but reapplication of the same stimulus after 3 s induces a scratch episode as revealed by the rhythmic motor nerve activity. This shows that the spinal neural network in isolated preparation is able to store the information about sensory input for more than 1 s as in the spinal cord in vivo (Sherrington 1906, Crowe and Linnartz 1985, Currie and Stein 1988, Currie and Stein 1990).

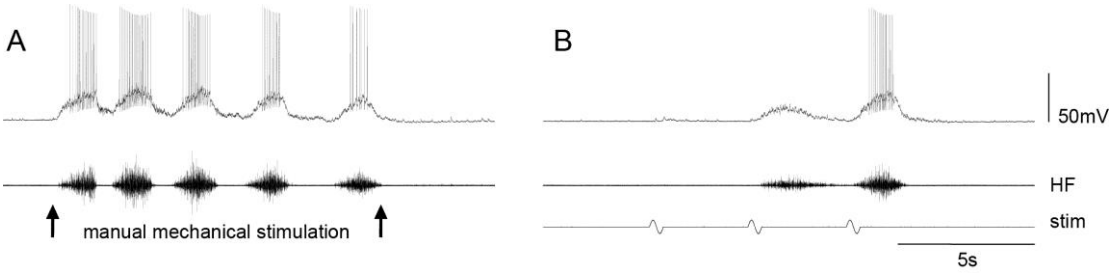

Figure 3.1.1 Protracted scratch initiation by repeated sub-threshold mechanical stimulation. Mechanical stimulation of the pocket skin evokes an episode of scratch network activity (A). Fine adjustment of stimulus intensity and duration leads to protracted activation of a scratch episode (B) – activity in the motor nerve starts only after the second brief stimulus. From top: V_m of motoneuron; ENG activity from HF nerve; stimulus.

To control the applied stimuli we adopted electrical stimulation of appropriate cutaneous nerves to evoke fictive scratch (Currie and Stein 1988). The induction of scratch activity during a train of electrical stimuli depends on stimulus intensity (Figure 3.1.2 A) and stimulus frequency (Figure 3.1.2 B).

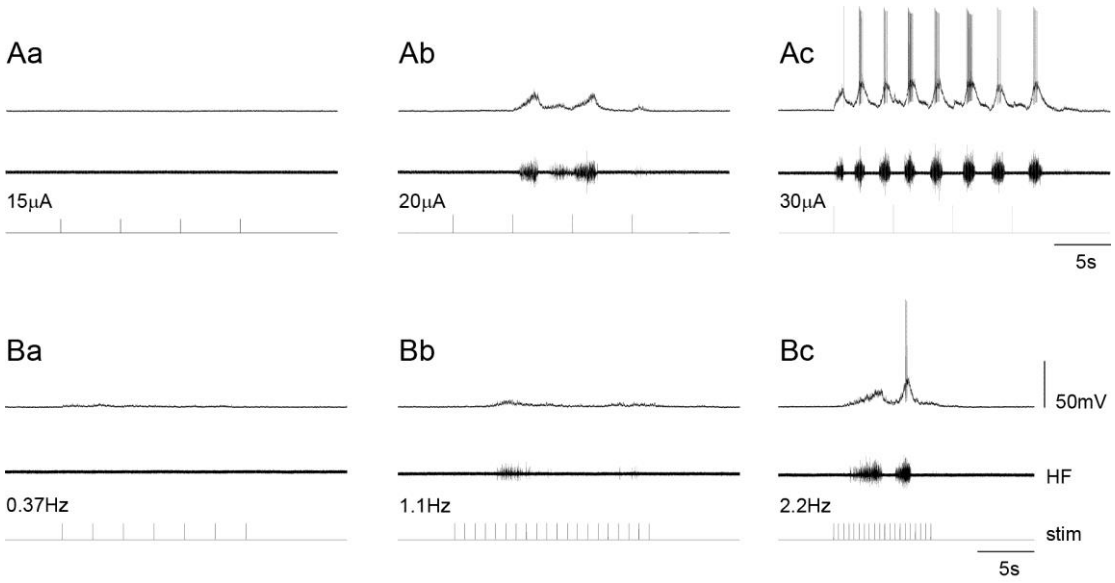

Figure 3.1.2 Protracted scratch initiation by repeated sub-threshold electrical nerve stimulation. Induction of scratch network activity by electrical stimulation of cutaneous nerve depends on stimulus intensity (A) and frequency (B). A weak stimulus does not induce activity in HF nerve ENG (Aa). At appropriate intensity even a single stimulus initiates scratching (Ac). At intermediate intensities stimulation induces protracted initiation of scratch episodes (Ab). From top: V_m of motoneuron; ENG activity from HF nerve; stimulus.

The low intensity stimuli of 15 μA (Figure 3.1.2 Aa) did not evoke spike activity in the HF motor nerve (middle sweep) during a train of 4 stimuli (lower sweep) applied every 5 seconds. At a higher intensity of 20 μA (Figure 3.1.2 Ab) no activity was evoked by the first stimulus but the subsequent three stimuli of the train evoked synaptic activity in the motoneuron (upper sweep) and spike activity in the motor nerve (middle sweep). Finally, when the stimulus intensity was increased to 30 μA (Figure 3.1.2 Ac) the first stimulus evoked a full scratch episode.

Induction of scratch network activity also depended on stimulus frequency (Figure 3.1.2 B). A train of stimuli at the low intensity of 0.17 μA evoked no motor nerve activity when applied at a frequency 0.37 Hz (Figure 3.1.2 Ba). When the stimulus frequency was increased to 1.1 Hz an episode of associated

increases in postsynaptic activity in the motoneuron and spike activity in the motor nerve was apparent (Figure 3.1.2 Bb). Finally, at a frequency of 2.2 Hz two associated rhythmic bouts of synaptic activity in the motoneuron and spike activity in the motor nerve were evoked (Figure 3.1.2 Bc).

In most preparations (15 out of 22) it was possible to adjust stimulus intensity so that a scratch episode was initiated in a protracted way by the second or subsequent stimulus during a train at a stimulus frequency <1 Hz in the absence of evoked motor nerve activity following the first stimulus as in Figure 3.1.2 Ab. These results conform closely to the findings obtained in the spinal turtle in vivo (Crowe and Linnartz 1985, Currie and Stein 1988).

3.2. Cutaneous silent period – CSP

The electric stimulus evoking scratch also induced a transient reduction in motor nerve and motoneuron activities when applied in the on-phase of the scratch cycle. This reduction is illustrated by the sweep in Figure 3.2.1 A. The quantification of ENG reduction after cutaneous stimuli for 30 successive sweeps is shown in Figure 3.2.1 B.

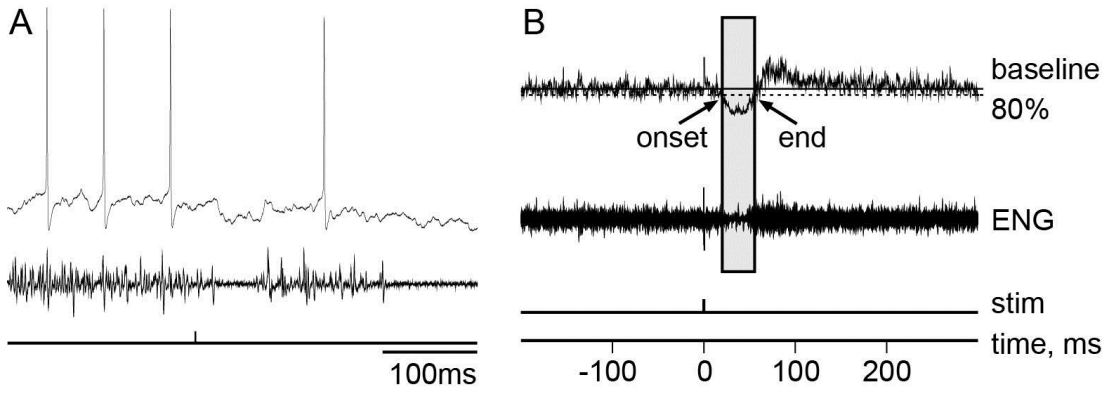

Figure 3.2.1 Suppression of motor activity after cutaneous nerve stimulation during scratch reflex. Suppression of ENG activity and motoneuron firing after a single electrical stimulus in (A). Averaged suppression of ENG activity in (B). Suppression of HF activity is shown as grey column in (B) and black arrows mark the onset and the end of it. A from top: V_m of motoneuron; ENG

of HF; applied stimuli. B from top: rectified and averaged ENG; superimposed sweeps of ENG; stimulus.

The suppression of ENG activity was found in 9 out of 10 preparations. The latency of suppression was 22.2 ± 6.8 ms, it ended after 47.1 ± 11.1 ms with a duration of 24.9 ± 10.7 ms (n=9). The suppression of ENG activity was substantial and significant: SI was 0.52 ± 0.14 (n=9).

It was noticed that activity of ENG after reduction increased dramatically and overshoot the baseline level (Figure 3.2.1 B). The suppression was followed by a rebound in nerve activity in 8 out of 9 preparations. The increase of ENG activity was significant, RI was 1.3 ± 0.21 (n=8).

It is known that a single stimulus to a cutaneous nerve can induce a suppression of on-going motor activity in several agonist and antagonist muscle groups in humans (Uncini, Kujirai et al. 1991, Floeter 2003). This suppression of motor activity is called cutaneous silent period or CPS. The time parameters of motor activity observed here is very similar to time parameters of CSP in humans. For this reason the motor activity suppression in this study is called CSP as well.

Mechanism generating CSP

CSP could be evoked by direct inhibition of motoneurons or reduction of excitation to motoneurons during motor activity. To investigate the mechanism underlying the CSP the responses in motoneurons when electric stimulus was applied in phase with activity of motoneurons was analysed (Figure 3.2.2). In parallel with the reduced ENG activity during the CSP (middle and lower sweeps in Figure 3.2.2) the spike activity ceased during the depolarizing wave in the motoneurons recorded from (top sweep in Figure 3.2.2).

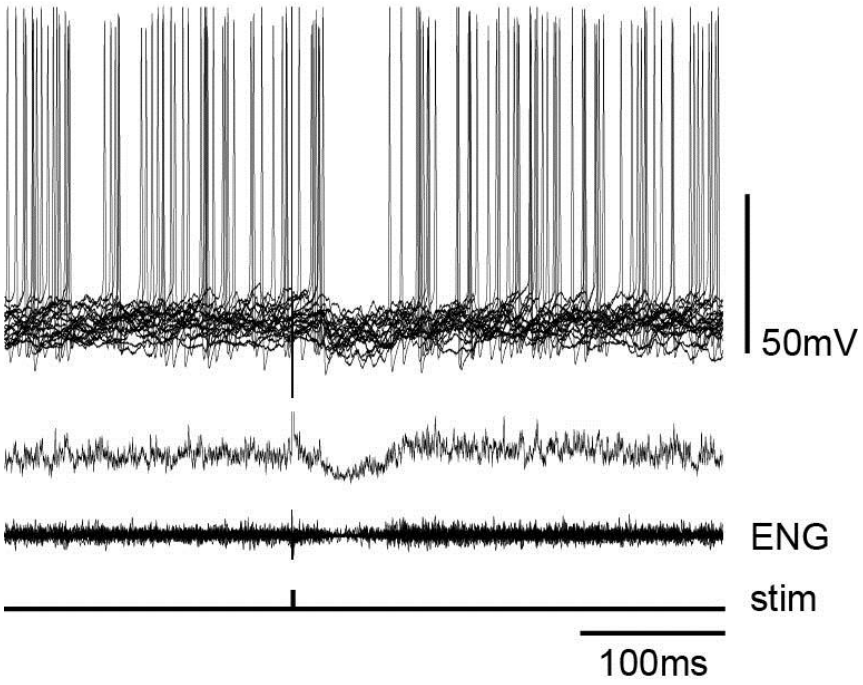

Figure 3.2.2 Suppression of on-going motor activity after cutaneous stimulus. Electrical stimulus applied to sensory stimulus evokes suppression of motor nerve activity and reduces AP activity in moteneurons. From the top: V_m of motoneuron (sweeps superimposed); rectified and averaged ENG; ENG activity (sweeps superimposed); stimulus.

However, during scratch-like activity turtle motoneurons receive intense synaptic input (Alaburda, Russo et al. 2005). In this high conductance state the spike activity is driven by synaptic fluctuation in membrane potential produced by concurrent synaptic inhibition and excitation (Berg, Alaburda et al. 2007, Berg, Ditlevsen et al. 2008). This precludes a clear distinction between a stimulus induced increase in synaptic inhibition and a decrease in synaptic excitation during the CSP. To resolve these complications advantage of the gradual activation of the scratch network activity was taken and postsynaptic responses in motoneurons at a low stimulus frequency (<0.05 Hz) and intensity that failed to induce scratching was analysed (Figure 3.2.3).

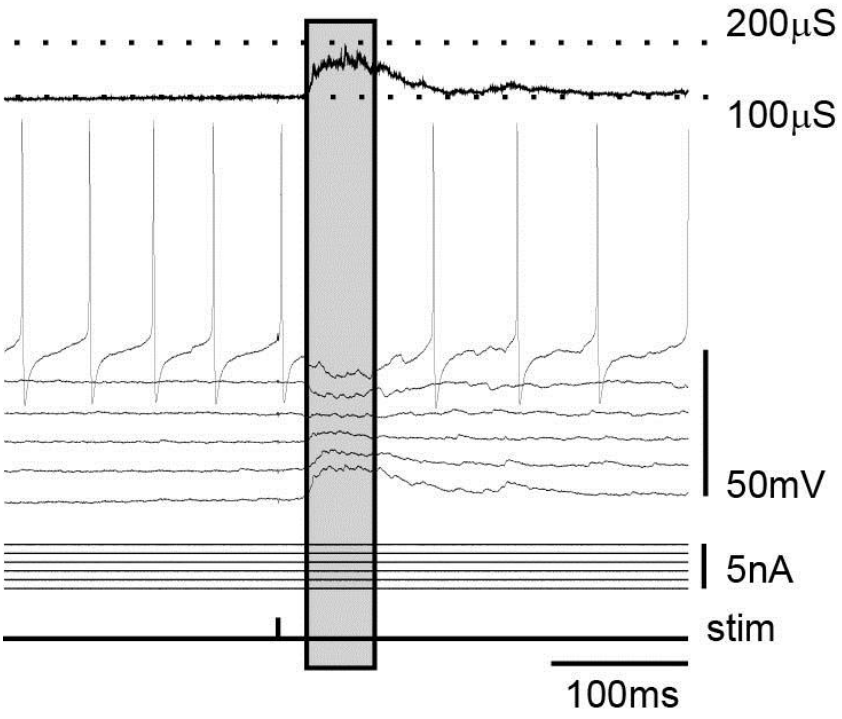

Figure 3.2.3 Direct inhibition of motoneurons during CSP. Cutaneous nerve stimulus evokes inhibition in motoneurons at rest. From the top: conductance of motoneuron; V_m of motoneuron at different holding currents; current injected to motoneuron; stimulus. The synaptic potentials during CSP reverse at around -70 mV.

It was observed that each electrical stimulus induced compound polysynaptic postsynaptic potentials with a delay similar to the delay for the CSP in motor nerve during scratching. The synaptic response evoked by the cutaneous nerve stimulus was hyperpolarising near the threshold for action potential and inhibited spike activity evoked by steady depolarizing current in all 9 cells tested (Figure 3.2.3). These results show that the cutaneous nerves stimuli that induce CSPs during motor activity activate polysynaptic spinal network that converge on motoneurons to reduce their excitability by net postsynaptic inhibition, with a reversal potential below the threshold for action potentials.

The magnitude of the postsynaptic response to cutaneous nerve stimulation was evaluated by calculating the changes in motoneuron membrane conductance during CSPs (top sweep in Figure 3.2.3). The conductance at rest

(before stimuli) of motoneurons was compared with conductance 20-50 ms after stimuli (values were taken from measurements of CSP in motor nerve). The conductance at rest was 80.8 ± 34.3 nS, during the time when CSP occurs – 124.6 ± 60.4 nS (n=15). Thus, the conductance of motoneurons significantly increased by 55 ± 38 % during the CSP.

3.4. Temporal integration of somatosensory input

V_m and excitability of motoneurons prior to scratch onset

The information about sensory input is stored in neural network for more than 1 s during gradual initiation of scratch reflex. It has been suggested that motoneurons could be dedicated to changing functional needs by the metabotropic modulation of their intrinsic response properties (Delgado-Lezama and Hounsgaard 1999). Synaptically released glutamate up-regulates the excitability of turtle motoneurons in response to brief trains of stimuli in slices (Delgado-Lezama, Perrier et al. 1997, Delgado-Lezama, Perrier et al. 1999) and during scratch episodes in the carapace-spinal cord preparation (Alaburda and Hounsgaard 2003). Here it was tested if modification of intrinsic properties of motoneurons is necessary during protracted activation of scratch.

First it was examined if the sub-threshold stimulus caused a substantial change in V_m of motoneurons prior to fictive scratching. V_m in motoneurons at rest (-64.8 ± 6.7 mV) and just before the stimulus that evoked a motor response (-64.5 ± 6.5 mV) (grey bars in Figure 3.4.1) differed by less than 1 mV (n=20).

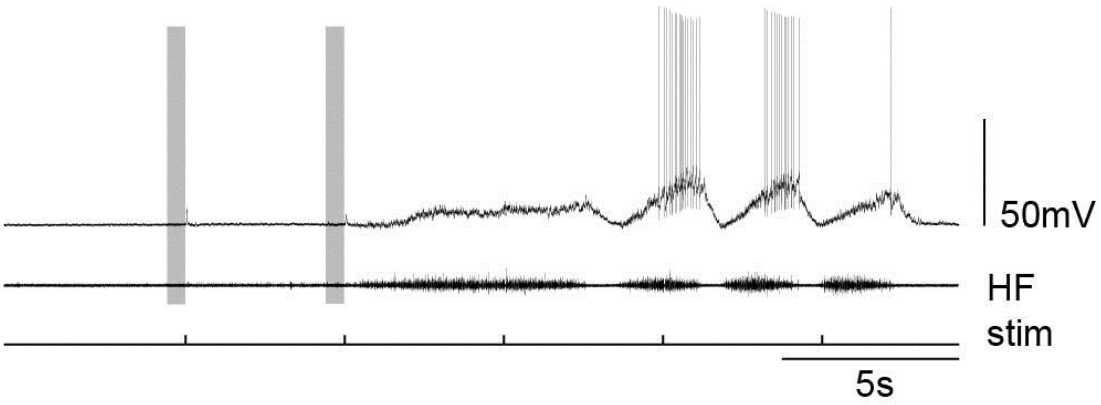

Figure 3.4.1 V_m of motoneurons does not wind up during protracted scratch initiation. V_m of motoneurons is at resting level just before the stimulus that induces motor nerve activity (grey bars). From top: V_m of motoneuron; ENG activity from HF nerve; stimulus.

The excitability of neurons can be changed by synaptic modulation even without changes in V_m (Delgado-Lezama, Perrier et al. 1997, Russo, Nagy et al. 1997). Therefore, the response of motoneurons to a supra-threshold depolarizing current pulse at rest and during protracted initiation of scratch network activity was compared. The excitability after cutaneous nerve stimuli increased in 7 out of 12 motoneurons, decreased in one and did not change in the remaining 4 cells. The changes of excitability just after the stimulus could be due to increased synaptic input from the pre-motor network. However, the response to the depolarizing test pulse was unchanged in all 12 neurons when applied just prior to the stimulus that preceded the motor activity (grey bars in Figure 3.4.2).

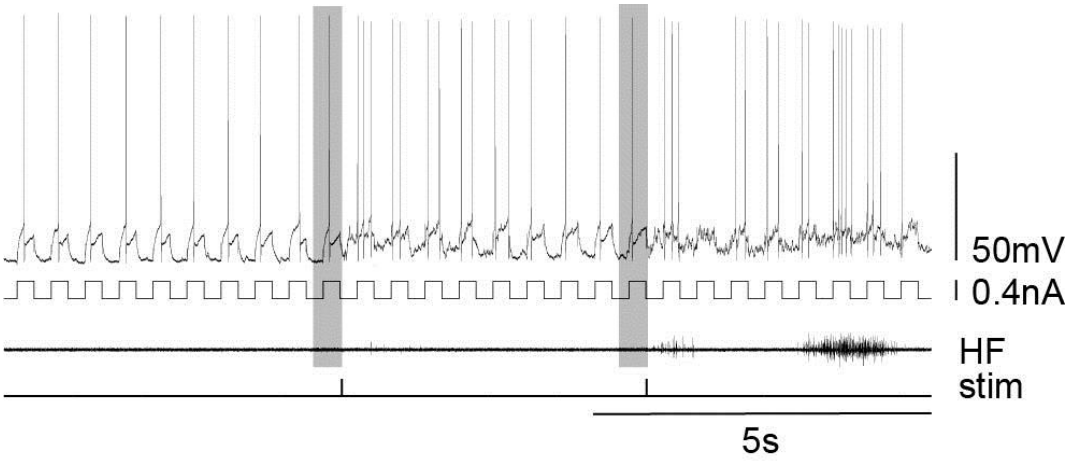

Figure 3.4.2 Excitability of motoneurons does not wind up during protracted scratch initiation. The intrinsic excitability of motoneurons is at the resting level just before the stimulus that induces motor nerve activity (grey bars). Positive current pulses with appropriate intensity to evoke a single action potential at rest were applied to motoneurons during protracted scratch initiation. From top: V_m of motoneuron; injected current pulses; ENG activity from HF nerve; stimulus.

The excitability of mammalian motoneurons increases during fictive locomotion (Krawitz, Fedirchuk et al. 2001) and scratching (Power, McCrea et al. 2010) due to hyperpolarization of V_{th}. The changes of V_{th} during protracted scratch initiation were evaluated. However, any differences between V_{th} at rest (-41.6 ± 5.2 mV) and prior to onset of fictive scratch (-41.8 ± 5.5 mV) were not observed ($p>0.05$) (n=12). These findings revealed no detectable sustained changes in V_m and excitability in motoneurons during protracted initiation of scratch network activity.

Pre-motor network activity during protracted scratch initiation

V_m of motoneurons fluctuates during protracted scratch initiation (Figure 3.4.3 A). The fluctuations of V_m evoked by synaptic activity in motoneurons prior to scratch onset signals the recruitment of the pre-motor network. In the example shown in Figure 3.4.3 A the first electrical stimulus induced a long lasting (>1

s) sub-threshold barrage of synaptic fluctuations in V_m in a motoneuron (upper trace) without activity in the HF nerve (middle trace).

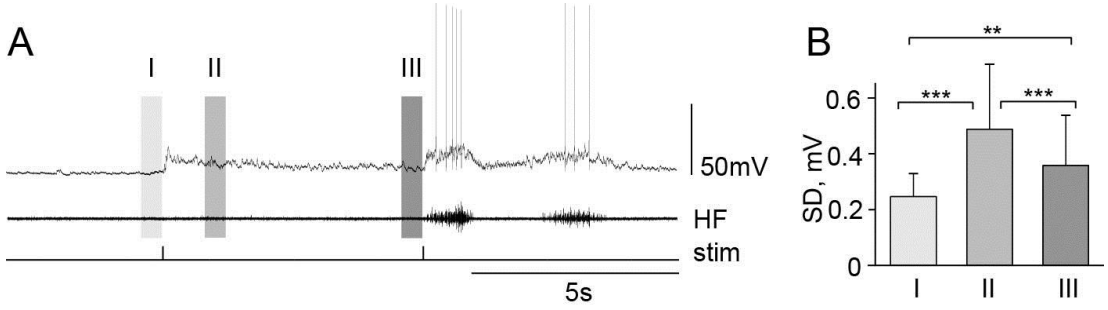

Figure 3.4.3 Increased pre-motor network activity during protracted scratch initiation. Long lasting (>1 s) V_m fluctuations in motoneurons after single electrical stimulus signals increased activity in the pre-motor network (A). V_m fluctuations are higher 1s after the first stimulus (II) and before motor activity (III) than at rest (I) (grey bars in A). V_m fluctuations evaluated as SD of V_m. A from top: V_m of motoneuron; ENG activity from HF nerve; stimulus. Significance of differences at $p<0.01$ (**) and at $p<0.001$ (***) levels, n=20.

The intensity of the prolonged synaptic activity recorded in motoneurons was evaluated as SD of V_m (Pare, Shink et al. 1998, Destexhe and Pare 1999). SD of V_m in motoneurons at rest prior to the first stimulus (I), 1 s after first stimulus (II) and just before the stimulus that evoked activity in HF nerve (III) were compared (grey bars in Figure 3.4.3 A). SD of V_m at rest was 0.25 ± 0.08 mV, increased to 0.49 ± 0.23 mV 1 s after the first stimulus and remained elevated at 0.36 ± 0.18 mV in the 0.5 s time period just prior to the onset of scratch motor activity (n=20) (Figure 3.4.3 B). The SD of V_m 1 s after the first stimulus and just before the scratch episode was significantly higher than at rest ($p<0.01$). Moreover, the SD of V_m 1 s after the first stimulus was significantly higher than immediately before the onset of scratch network activity i.e. the activity of the pre-motor network during protracted activation of fictive scratch gradually decreased over time. In addition, any scratch-like rhythmicity in fluctuations of V_m during protracted scratch initiation was not observed. Thus, the pattern of activity of the pre-motor network during

protracted scratch initiation is tonic and gradually decreases over time from an early peak after each stimulus.

With proper adjustment of stimulus intensity the onset of fictive scratching could be delayed to occur after the third or subsequent stimuli during the train (Figure 3.4.4 A1 and A2).

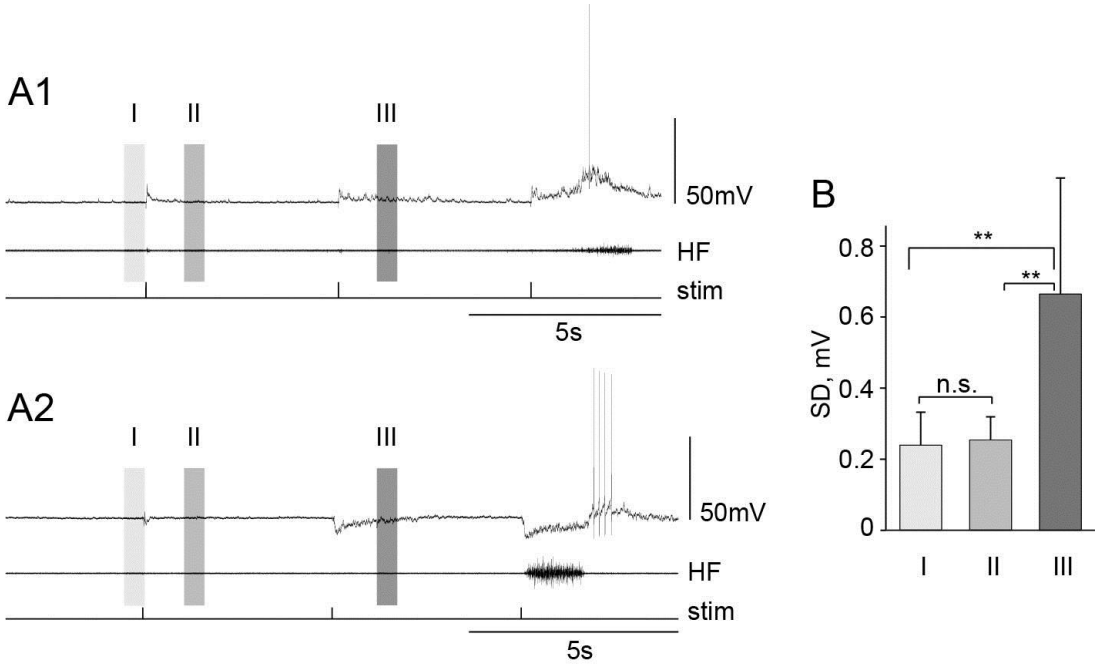

Figure 3.4.4 Wind up of pre-motor network activity evoked by repeated sub-threshold stimuli. Wind up of net excitatory (A1) and net inhibitory (A2) synaptic activity in motoneurons in response to repeated sub-threshold stimuli. V_m fluctuation evaluated as SD of V_m at rest (I), 1s after first stimulus (II) and 1s after second stimulus (III) (grey bars in A1 and A2). V_m fluctuations increases (winds up) with repeated sub-threshold stimuli (B). Recordings in A1 and A2 from different motoneurons. A1 and A2 from top: V_m of motoneuron; ENG activity from HF nerve; stimulus. Significance of differences at $p>0.05$ (n.s.) and at $p<0.01$ (**) levels, n=8.

Under these conditions the pre-motor network activity as reflected in the synaptic input to motoneurons increases (winds up) with repeated stimuli. Note that not only excitatory (Figure 3.4.4 A1) input winds up prior to the onset of

scratch network activity. In 3 out of 8 motoneurons there was a net increase of inhibition during synaptic wind up as illustrated in Figure 3.4.4 A2.

SD of V_m in motoneurons at rest (I), 1 s after the first stimulus (II) and 1 s after the second stimulus (III) were compared (grey bars in Figure 3.4.4 A1 and A2). SD was 0.24 ± 0.09 mV at rest, 0.25 ± 0.06 mV after the first stimulus and 0.66 ± 0.33 mV after the second stimulus (n=8) (Figure 3.4.4 B). The pre-motor network activity was significantly higher after the second than after the first stimulus or at rest.

3.5. The role of mid-thoracic spinal segments in pocket scratch generation

Mid-thoracic spinal segments participate in pocket scratch generation

Stimulus applied on receptive fields of carapace around hind limb evokes three forms of scratch reflex: rostral, pocket and caudal (Mortin, Keifer et al. 1985). Afferents inervating receptive fields of pocket scratch enter spinal cord at lumbar enlargement (D7-D8 spinal segments) (Mortin and Stein 1990). Motoneurons locted in lumbar enlargement (D8-S2 spinal segments) (Ruigrok and Crowe 1984) are activated to perform movements aiming to remove an irritant from body surface. It is tempting to hypothesize that neural network generating pocket scratch is located within lumbar enlargement (D7-S2 spinal segments). The involvement mid-thoracic spinal segments (D3-D5) in pocket scratch generation was tested.

Mechanical stimulation of hind limb pocket region evokes scratch reflex monitored in HF nerve activity (Figure 3.5.1. B).

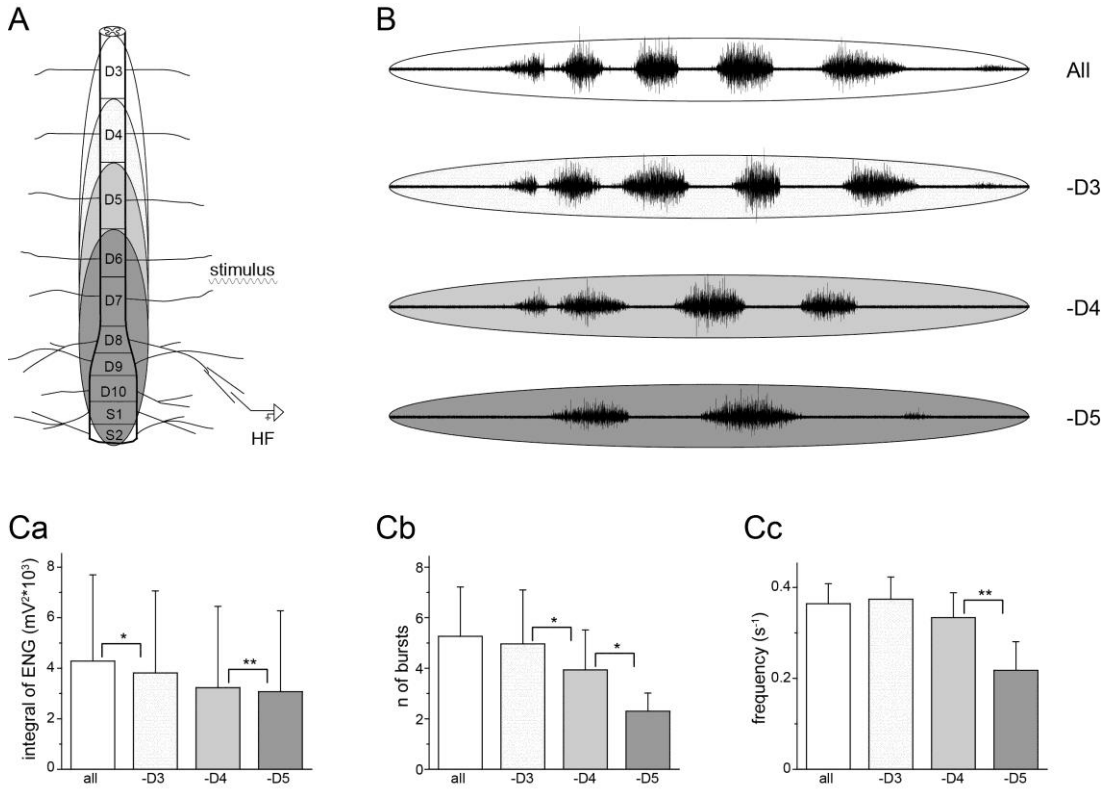

Figure 3.5.1 Mid-thoracic segments of spinal cord contribute to pocket scratch generation. Mechanical stimulus applied on receptive field of D7 spinal segment evokes pocket scratch response (top B) in integrated carapace-spinal cord preparation (A). Removal of mid-thoracic spinal segments (D3-D5) changes HF activity evoked with the same mechanical stimulation (B). After removal of mid-thoracic spinal segments mechanical stimulus induces pocket scratch with weaker HF activity (Ca), fewer HF bursts (Cb) and lower frequency of HF bursts (Cc). Significance of differences at $p<0.05$ (*) and at $p<0.01$ (**) levels, n=6.

Scratch reflex (HF nerve activity) was changed after removing mid-thoracic segments of spinal cord (Figure 3.5.1 B). It was noticed that scratch reflex tends to be "weaker" after removing these segments. The same stimulus evokes scratch episodes with fewer bursts and bursts tend to be prolonged.

The experiment was repeated in 3 turtles inducing scratch reflex in left and right sides in alternation and removing mid-thoracic spinal segments gradually (see methods) (n=6). Integral of rectified HF ENG activity (Figure 3.5.1 Ca) number of HF bursts per scratch episode (Figure 3.5.1 Cb) and frequency of

HF nerve bursts during scratch episode were evaluated (Figure 3.5.1 Cc). All parameters decrease after removing each of mid-thoracic segments (Figure 3.5.1 C).

Similar experiments were repeated after removing D4 and then D5 spinal segments (n=1) or D3-D5 spinal segments at once (n=2). In all experiments the removal of mid-thoracic spinal segments change scratch reflex in the same manner – decreases HF intensity, reduces number of HF bursts and prolongs HF bursts.

Activation of neurons of D4 spinal segments during pocket scratch

In order to understand how mid-thoracic neural network is involved in pocket scratch generation neurons from D4 spinal segment during pocket scratch were recorded. In total 105 neurons from ventral horn and intermedium zone of D4 spinal segments during pocket scratch were recorded. The responses of D4 neurons to pocket scratch were evaluated qualitatively.

Some of interneurons (10 out of 105) in D4 segment did not respond to ipsilateral pocket scratch (Figure 3.5.2 A). Other interneurons (95 out of 105) of D4 segment responded to ispilateral pocket scratch. These neurons were divided in to two groups. One group of neurons (44 out of 105) received tonic input (Figure 3.5.2 B) during pocket scratch. Another group of active neurons (51 out of 105) was activated rhythmically during pocket scratch (Figure 3.5.2 C). The rhythmicity was related to motor output – HF nerve activity. This suggests that these inputs originate from network generating motor output. The summary of responses of D4 neurons during pocket scratch is presented in Figure 3.5.2 D.

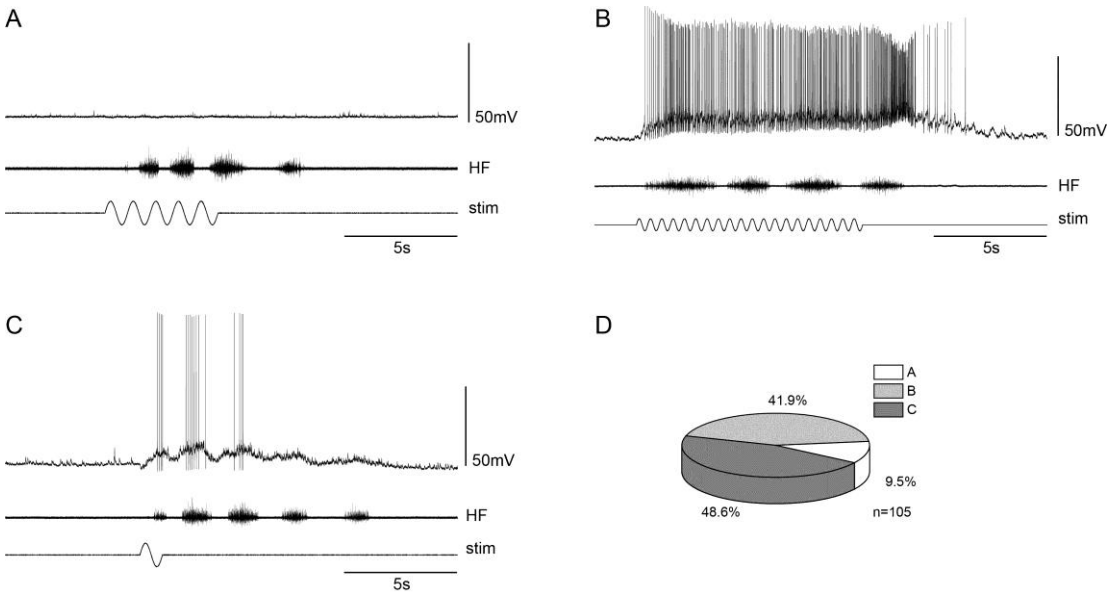

Figure 3.5.2 Responses of D4 neurons to pocket scratch. Some neurons of D4 segments do not receive any input during pocket scratch (A). Most of neurons of D4 spinal segments are activated during pocket scratch (B and C). The summary of responses of D4 neurons to pocket scratch is presented in D. A, B and C from top: V_m of neuron; ENG activity from HF nerve; stimulus.

To summarize – about 90 % of neurons of D4 spinal segment are activated (receive postsynaptic potentials and generate APs) during pocket scratch generation. Rhythmically activated neurons confirm that input to these neurons come from neural network generating motor output and located in lumbar enlargement.

Phase activity of D4 neurons during pocket scratch

Mechanical stimulation of ipsilateral receptive fields of pocket scratch induces rhythmic activation of some (51 out of 105) D4 neurons (Figure 3.5.3 A). The phase of 34 out of 51 D4 neurons which fulfilled criteria of phase analysis (see methods) was analysed. Each phasic D4 neuron is active at its own characteristic phase relation to the HF nerve activity during ipsilateral scratching. The activity of the population of D4 neurons cover the entire HF period (Figure 3.5.3 B).

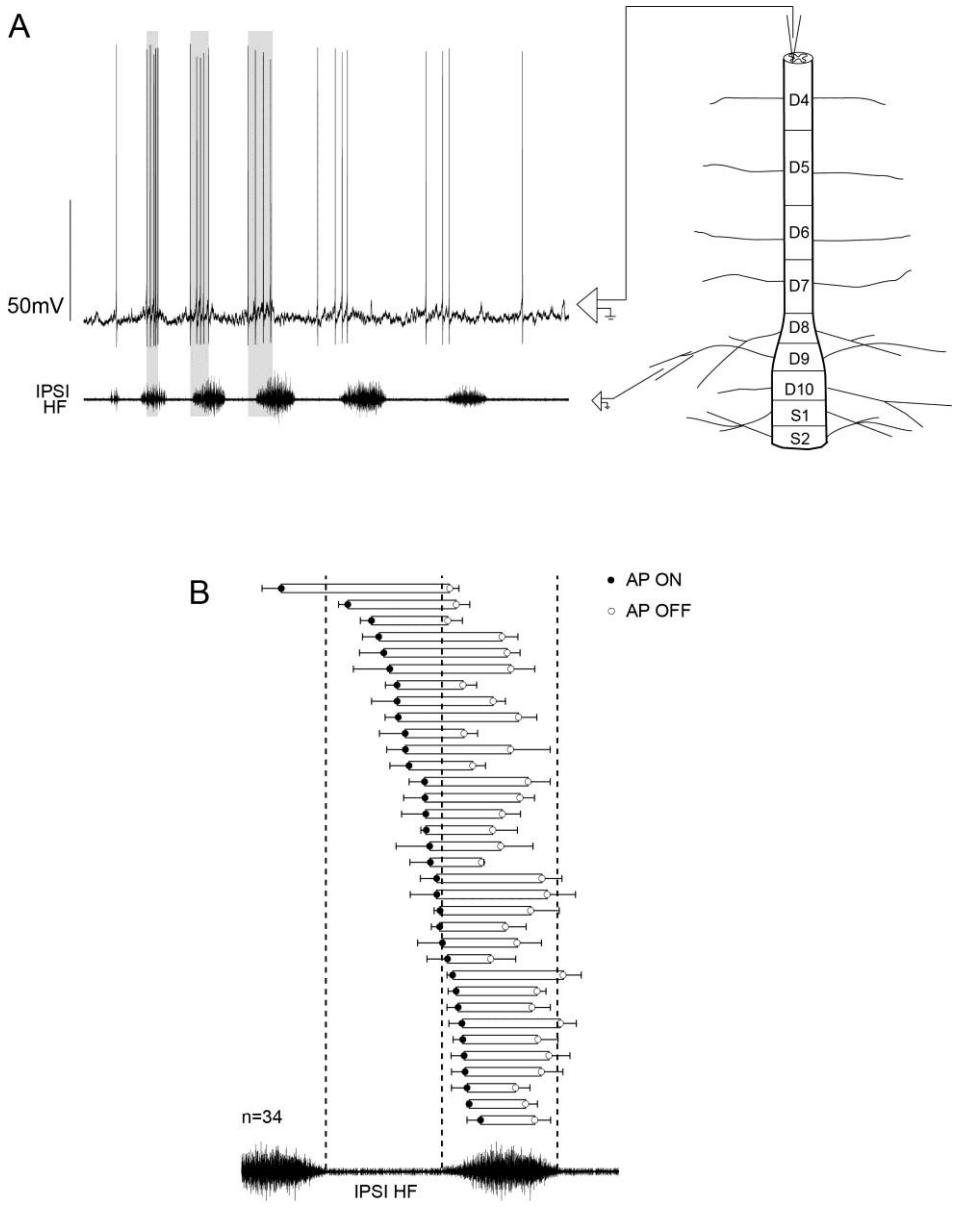

Figure 3.5.3 Phase activity of D4 neurons during ipsilateral scratch reflex. Intracellular recording of D4 neuron showed that this neuron is activated rhythmically during pocket scratch (A). D4 neuron has characteristic phase relation to the HF nerve bursts. Analysis of all (n=34) neurons showed that D4 neurons are activated in entire HF period (B).

Axons of neurons of D4 spinal segment

D4 neuron activated rhythmically during pocket scratch (Figure 3.5.4 A) was filled with biocytin. The piece of spinal cord (D4-D5 spinal segments) was removed from preparation and fixed (see methods). The tissue was sliced longitudinally. In one of the ends of the slices it is possible to detect cell body

(Figure 3.5.4 B and Ca) or pieces of dendrites. Distantly from cell body (more than 1 mm) it was possible to find pieces of descending axon (Figure 3.5.4 Cb and Cc). In total 14 D4 neurons in 5 integrated carapace-spinal cord preparations were stained. Pieces of at least 8 descending axons were found.

This demonstrated that D4 neurons not only receive input from scratch network in lumbar enlargement but also send their axons back toward scratch network in lumbar enlargement.

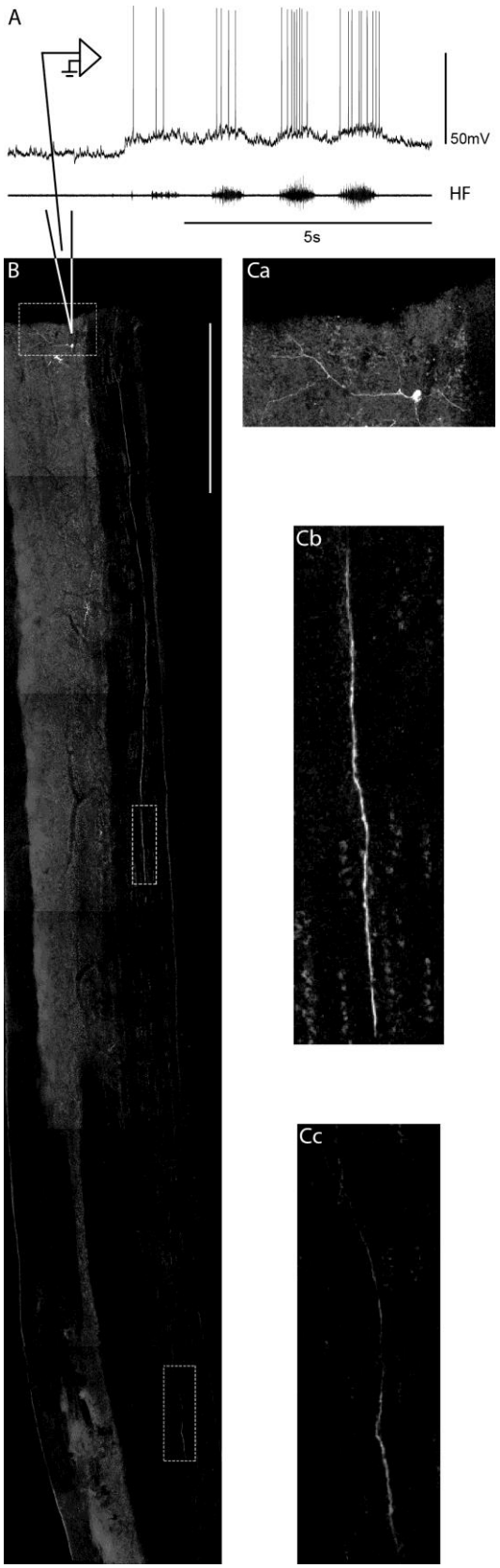

Figure 3.5.4 D4 neurons project towards lumbar enlargement. After intracellular recording of rhythmically activated D4 neuron (A) the cell was filled with biocytin and stained (B). The cell body at the end of slice showed the region of recording (Ca) and the axon (Cb and Cc) descending toward the lumbar enlargements was found. Scale bar in B 1 mm.

4. Discussion

4.1. Cutaneous silent period

Electrical stimulation of cutaneous nerve induces suppression of on-going motor activity in turtle carapace-spinal cord preparation. The transient suppression of motor activity in spinal cord after a cutaneous stimulus is termed the cutaneuos silent period (CSP) (Caccia, McComas et al. 1973, Uncini, Kujirai et al. 1991). It is not known if CSP is due to suppression of the pre-motor network or direct inhibition of motoneurons.

In this study the synaptic mechanisms underlying a CSP-like response in motoneurons were investigated in the isolated carapace-spinal cord preparation of the adult turtle. Electrical stimulation of cutaneous VPP nerve evokes synaptic activity in motoneurons that inhibits ongoing motor activity. The conductance during the postsynaptic response is more than 50 % higher than at rest and the synaptic response suppressed sustained firing evoked by depolarizing current injection. In conclusion direct postsynaptic inhibition of motoneurons contributes to the CSP during scratch network activity. This conclusion does not exclude that reduced excitatory drive also play a role during CSPs.

Experimental paradigm used in this study differs in two ways from the standard procedure for CPS tests in humans. First, voluntary muscle contraction in humans was substituted with rhythmic scratch network activity in turtle preparation. For this reason it is not possible to control and vary the level of motor neuron firing. To avoid errors due to non-sustained motor activity at least 20 ENG sweeps were averaged (see methods). Nevertheless, the evoked CSP was readily observed in 9 out of 10 preparations.

Secondly, the CSP in humans is less expressed and has shorter duration in proximal than in distal limb muscles (Inghilleri, Cruccu et al. 1997, Leis,

Stokic et al. 2000, Serrao, Parisi et al. 2001). Nevertheless, robust CSPs in the proximal hip-related motor nerve were obtained.

The shorter latency of the CSP in turtle experiments than reported for humans (Shefner and Logigian 1993, Serrao, Parisi et al. 2001, Kofler 2003, Rodi and Springer 2011) can be explained by differences in experimental set up. Cut nerves were recorded and stimulated close to spinal cord while in human experiments the stimulation and recordings were made distally in the limbs. For this reason the conduction time in peripheral nerves is very short in turtle experiments and the CSP latency mainly reflects the central processing time. The latency of CSPs in turtle experiments is closely similar to central processing time for the CSP in humans, about 20 ms (Shefner and Logigian 1993).

In conclusion, cutaneous stimuli evoke CSPs in proximal hind limb motor pool in the turtle and the CSP involves direct postsynaptic inhibition of motoneurons.

4.2. Protracted scratch initiation

It is known that the spinal network "remembers" the somatosensory information for a few seconds so that subsequent stimuli may induce a scratch episode (Sherrington 1906, Crowe and Linnartz 1985, Currie and Stein 1988, Currie and Stein 1990). The mechanism causing sub-second temporal integration of somatosensory input was aimed to determine in this study.

A long-lasting (>1 s) increase of pre-motor network activity during temporal integration was found. Moreover, excitatory and inhibitory synaptic activity in motoneurons increases with each sub-threshold stimulus prior to the onset of fictive scratching. However, this synaptic activity did not induce noticeable lasting changes in the excitability of motoneurons.

The persistent activity of neurons to brief stimuli observed in many regions of CNS is believed to serve as a working memory mechanism (Major and Tank 2004). Intrinsic plateau properties of individual neurons (Russo and Hounsgaard 1994, Bennett, Hultborn et al. 1998, Di Prisco, Pearlstein et al. 2000) or recurrent networks (Li, Soffe et al. 2006) are likely mechanisms underlying long lasting spiking after short stimuli.

Intrinsic properties of motoneurons during protracted scratch initiation

If the intrinsic response properties of motoneurons select for "useful" response patterns (Delgado-Lezama and Hounsgaard 1999) they would seem an appropriate target for regulation. Metabotropic facilitation of L-type Ca^{2+} channels increase excitability in turtle motoneurons (Perrier, Alaburda et al. 2002). Glutamate released during scratch network activity produces robust facilitation of motoneurons that outlasts the scratch by tens of seconds (Alaburda and Hounsgaard 2003). In transverse slices a similar effect is produced even with a brief train of action potentials in glutamatergic (DLF – dorsolateral funiculus) axons in the absence of network activity (Delgado-Lezama, Perrier et al. 1997). Nevertheless, the experiments reported here did not reveal changes in the excitability of motoneurons prior to the onset of scratch episodes. This suggests that the intensity of glutamate release during protracted activation of scratch is limited despite the increase of synaptic activity. The undetectable increase in conductance during protracted scratch initiation supports this possibility, particularly compared with the dramatic increase in synaptic conductance during scratch network activity (Alaburda, Russo et al. 2005, Berg, Alaburda et al. 2007, Berg, Ditlevsen et al. 2008). It is also possible that DLF and afferent nerve stimulation activate different groups of glutamatergic synapses with and without the ability to facilitate L-type Ca^{2+} channels.

Pre-motor network activity during protracted scratch initiation

The time scale of increased spinal network excitability following sub-threshold stimulation (Currie and Stein 1988) is compatible with the duration of increased pre-motor network activity reported here. It is also similar to the frequency range in which temporal summation is observed in long afterdischarge interneurons in spinal segment D4 (Currie and Stein 1990). It is tempting to hypothesize that temporal summation in long afterdischarge interneurons as the common parsimonious underlying cause for synaptic wind up and protracted activation of scratch episodes. In support, temporal summation in long afterdischarge interneurons and protracted scratch initiation is reduced by NMDA receptor antagonists in vivo (Currie and Stein 1992). However, the lowered responsiveness may be due to non-specific reduction of synaptic excitation rather than elimination of the mechanism for the multisecond storage of excitability. Plateau generating spinal interneurons in the turtle (Hounsgaard and Kjaerulff 1992, Russo and Hounsgaard 1996) provide a candidate for the long afterdischarge interneurons. In response to brief depolarization these cells generate a long afterdischarge of action potentials due to activation of a plateau potential. In addition, the plateau mechanism promoted by voltage and transmitter dependent facilitation (Russo and Hounsgaard 1994, Russo and Hounsgaard 1996, Russo, Nagy et al. 1997, Perrier, Alaburda et al. 2002), displays temporal summation on a timescale appropriate for a unifying common explanation. Wind up of this kind is evoked by NMDA receptors activating L-type Ca^{2+} channels in plateau generating wide dynamic range neurons during central pain sensitization (Fossat, Sibon et al. 2007). It is possible that these mechanisms in wide dynamic range neurons also account for temporal integration of non-nociceptive sensory input observed here.

Intracellular recordings from the interneurons that generate long afterdischarges to brief sensory stimuli could help to uncover the mechanism underlying temporal integration of somatosensory input. However, direct experimental test awaits conditional knock-outs targeted to L-type Ca^{2+}

channels or NMDA receptors in relevant subpopulations of spinal interneurons. This approach will be needed to test a) if the activity of the pre-motor network is part of the network mechanism that leads to the particular behavioural form in response to stimulation or b) just an unspecific arousal that merely serves to reach threshold for the "real" scratch mechanism.

In conclusion, findings reported here suggest that increased activity of the pre-motor network may contribute to scratch initiation but leaves the excitability of motoneurons unchanged at the onset of scratching.

4.3. The role of mid-thoracic spinal segments during pocket scratch generation

It was thought that neural network generating pocket scratch reflex resides in lumbar enlargement (Mortin and Stein 1989). Here we showed that mid-thoracic segments of spinal cord are highly involved in generating pocket scratch reflex. Scratch motor activity changes after removal of mid-thoracic segments. It seems that these segments does not have a particular role in the scratch generation but increases the excitability of network generating scratch response.

Intracellular recordings of neurons from D4 spinal segments revealed that about 90% of neurons are activated during pocket scratch. Some neurons receive tonic input during pocket scratch. Most likely these inputs come from lumbar enlargement generating scratch motor output. However, the possibility that sensory afferents spread to D4 segments and activate these neurons cannot be excluded.

About 50 % of neurons recorded were rhythmically activated during pocket scratch. The rhythmicity in D4 neurons was tightly coupled to motor output, HF nerve activity. The phase lock of D4 neurons to HF nerve activity confirm that D4 neurons during pocket scratch are activated by network located in lumbar enlargement and generating scratch reflex. Phase analysis of D4

neurons showed distributed activation of neurons. D4 neurons are activated in all phases during the scratch cycle. There is no clear phase preference of activity of D4 neurons. The same distribution of activity of spinal interneurons was observed in cats during scratch reflex (Berkinblit, Deliagina et al. 1978).

The isolation of D4 segment demonstrates that somatosensory processing in local D4 segment network do not show any rhythmic activity in neurons (Currie and Stein 1990). This sugests that rhytmic activity observed in this study appears somewhere outside D4 segment. Most likely it originates in lumbar enlargement. All observations suggest that there is a strong ascending drive from lumbar enlargement to mid-thoracic segments.

It was found, that about 50% of rhytmically activated D4 neurons send their axons back towards lumbar enlargement. This demonstrates that D4 neurons not only receive input from lumbar enlargement but could contribute to the motor activity generation.

Interconnectivity between lumbar enlargement and mid-thoracic segments suggests that the real network distribution generating motor output is much wider than it was thougt before.

Mid-thoracic segments contain motoneurons inervated intrinsic back muscles (Tortora and Derrickson 2006). The function of these muscles is to keep body balance during locomotion (Thorstensson, Carlson et al. 1982, Zomlefer, Provencher et al. 1984). However, turtles do not have neither back muscles nor motoneurons in mid-thoracic segments (Nieuwenhuys, Donkelaar et al. 1998). Moreover, intrinsic back muscles show double burst activity during one step cycle (Thorstensson, Carlson et al. 1982, Zomlefer, Provencher et al. 1984). D4 neurons always are activated with a single burst activity during scratch cycle.

The importance of mid-throracic segments in motor activity generation is increasing during the last few years. It was shown that synaptic connections in these segments is important for interlimb coordination and transmission of

information from higher brain centers (Cowley, Zaporozhets et al. 2008, Cowley, Zaporozhets et al. 2010). Moreover, the plasticity in these spinal segments could restore voluntary movements after spinal lesions (van den Brand, Heutschi et al. 2012). The wide distribution of neural network generating hind limb movements could explain findings presented here.

Conclusions

- Temporal integration of somatosensory input can occur without change in excitability of motoneurons;
- The activity of pre-motor neurons increases during temporal integration of somatosensory input;
- Inhibition of motor activity occurs after stimulation of somatosensory nerves. Temporal characteristics of this inhibition match characteristics of cutaneous silent period observed in humans;
- Motoneurons are inhibited during cutaneous silent period;
- Removal of thoracic segments change pocket scratch generation;
- Most of neurons in thoracic segments receive synaptic input from lumbar enlargement and some of these neurons send axons back towards lumbar enlargement.

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Santrauka (Summary in Lithuanian)

Kasymosi refleksas – tai motorinis atsakas siekiant pašalinti dirgiklį nuo kūno paviršiaus. Priklausomai nuo stimulų, pasiekiančių kūno paviršių, kasymosi refleksas sukeliamas arba ne. Jeigu stimulas yra pakankamai stiprus, tai po stimulo seka kasymosi refleksas. Tačiau stimulo stiprumui mažėjant, sprendimas apie atsako generavimą gali būti priimamas atsižvelgiant į stimulo pasikartojamumą. Parodyta, kad nugaros smegenų neuronų tinklas geba integruoti somatosensorinius įėjimus, įvykusius rečiau nei viena sekundė. Iki šiol nebuvo aiškus mechanizmas, leidžiantis nugaros smegenims saugoti informaciją apie somatosensorinį stimulą ilgiau nei sekundę. Šiame darbe parodoma, kad somatosensorinių stimulų integracija galima ir be motorinių neuronų sužadinamumo kitimo. Somatosensorinių stimulų laikinės integracijos metu padidėja priešmotorinių neuronų aktyvumas. Šis priešmotorinio tinklo aktyvumo padidėjimas galėtų būti viršsekundinės somatosensorinių stimulų integracijos mechanizmas.

Lokomocija ir refleksai yra organizuojami nugaros smegenų neuronų tinkle. Tiksli šio tinklo lokalizacija ir jo išplitimas stuburiniuose nėra visiškai aiškus. Daugelis tyrimų teigia, kad neuronų tinklas, valdantis užpakalines galūnes, yra išsidėstęs strėnų srities išplatėjime. Šiame tyrime parodoma, kad krūtininiai nugaros smegenų segmentai taip pat prisideda prie užpakalinių galūnių judesių organizavimo – jų pašalinimas keičia užpakalinių galūnių motorinius atsakus. Taip pat parodyta, kad neuronai, esantys krūtininiuose segmentuose, yra stipriai aktyvuojami neuronų tinklo, esančių strėnų srities išplatėjime. Šiame darbe imunohistocheminiais metodais parodyta, kad dalis aktyvuojamų neuronų savo aksonus siunčia atgal link strėnų srities išplatėjimo. Apibendrinant galima teigti, kad neuronų tinklas valdantis užpakalinių galūnių judėjimą nėra apribotas strėnų srityje. Jis išplitęs gerokai labiau, nei manyta iki šiol.

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- Robertas Guzulaitis, Aidas Alaburda, Jorn Hounsgaard; Scratch generation beyond the lumbar enlargement; 1st European neuroscience conference by doctoral students (ENCODS 2013), Bordeaux, France, 2013;
- Robertas Guzulaitis, Aidas Alaburda, Jorn Hounsgaard; Scratch generation beyond the lumbar enlargement; 10th Gottingen Meeting of the German Neuroscience Society, Gottingen, Germany, 2013;
- Robertas Guzulaitis, Aidas Alaburda, Jorn Hounsgaard; Scratch generation beyond the lumbar enlargement; Annual INF Conference, Denmark, 2013;
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- Robertas Guzulaitis, Aidas Alaburda; Spinal network generating swim-like activity; Motoneuron meeting, Paris, France, 2010;
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- Robertas Guzulaitis, Aidas Alaburda; Motoneuronų aktyvavimas kasymosi ir plaukimo metu; Lietuvos Neuromokslų asociacijos konferencija "Nervų sistemos tyrimai Lietuvoje" Vilnius, Lithuania, 2009;

- Robertas Guzulaits, Aidas Alaburda; Plaukimo ritmo priklausomybė nuo elektrinio stimuliavimo dažnio; Virtualūs instrumentai biomedicinoje, Klaipėda, Lithuania, 2009;
- Robertas Guzulaits, Aidas Alaburda; Increased conductance of spinal motoneurons during swim-like activity; 13th international conference Biomedical engineering, Kaunas, Lithuania, 2009;

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