Ph.D. THESIS

COMBINED ELECTROPHYSIOLOGICAL AND MORPHOLOGICAL INVESTIGATION OF SUBSTANCE P (SP) – NEUROKININ 1 (NK1) RECEPTOR INTERACTION IN THE SPINAL CORD AND DORSAL ROOT GANGLIA OF THE RAT

Dr. Szűcs Péter

Supervisor: Prof. Dr. Antal Miklós

University of Debrecen
Medical and Health Science Centre, Faculty of Medicine
Department of Anatomy, Histology and Embryology
2003

INTRODUCTION

The tachykinin substance P (SP) plays an important role in nociceptive information processing in the spinal cord. Upon peripheral noxious stimuli SP is released from the central terminals of Aδ- and C- type primary afferents and activates its high affinity neurokinin 1 (NK1) receptor, expressed mainly in lamina I, III and IV of the spinal dorsal horn. In chronic pain (e.g. pain in inflammation) the excessive SP release is responsible for the so-called "central sensitization" through the continuous activation of neurons expressing NK1 receptor in the dorsal horn. So far three types of NK receptors have been identified, from which SP binds with the highest affinty to the G protein coupled NK1 receptor. Activation of NK1 receptors on secondary sensory neurons result in an elongated depolarisation.

Experiments conducted on dorsal root ganglia (DRG) of adult rats and guineapigs confirmed that besides spinal cord neurons SP can directly activate certain primary afferents.

In the last few decades the distribution of NK1 receptors and SP have been thoroughly investigated in all areas of the CNS of adult animals. However our knowledge is very limited concerning the same distribution in the spinal cord and DRG of young animals. Rapidly developing electrophysiological techniques in the last twenty year made investigation of young animals more and more available. Spinal cord slice preparations and other electrophysiological methods are more stable on preparations from young animals. Slices from older animals are usually hard to visualise with the commonly used DIC potics due to myelinisation.

These methods combined with morphological techniques allowed us to perfom experiments on young rats in order to shed more light on the distribution and function of the NK1 receptor in the spinal cord and the DRG.

Objectives:

1. Using combined electrophysiological, morphological and pharmacological methods we aimed to investigate the NK1 receptor expression in DRG neurons of young rats and their response to SP application. We wanted to know whether SP has any effect on DRG neurons and if there is any difference between certain DRG cell population concerning their response to SP.

2. Applying immunohistochemical reactions we wanted to sutdy the NK1 recepor distribution pattern in the lumbar spinal cord of young rats. We also wanted to compare the expression pattern with that of the adult animals, to see whether the results obtained from young animals can be fitted into the data accumulated from adult experiments.

3. Previous expereiments of other laboratories as well as our immunohistocehmical results confirmed the presence of pale but definitive somatic NK1 receptor immunolabelling in certain neurons of laminae V-VI and the intermedier gray matter of the spinal cord. Although these cells do not take place directly in nociceptive information processing, they can be direct postsynaptic targets of primary sensory neurons. Therefore we studied these neurons with combined physiological and morphological techinques to shed more light on the inputs and connections of local interneuronal circuits of the spinal cord.

4. We investigated if SP has any direct / indirect effect on the NK1 receptors (shown to be expressed by others and by our immunocytochemical reactions) of neurons in the deeper laminae (V-VI) and the intermediate gray matter of the spinal cord.

MATERIALS AND METHODS

Preparation

DRGs along with their 10-13mm peripheral nerve and dorsal root were obtained from 14 day old Wistar rats, anaesthetised with enflurane. The preparation was continously perfused with ice-cold arteficial cerebrospinal fluid (ACSF) bubbled with an O_2/CO_2 mixture (95%/5%). Composition of the ACSF was (mM): NaCl, 130; KCl, 3,5; NaH_2PO_4, 1,25; $NaHCO_3$, 24; $CaCl_2$, 1,2; $MgCl_2$ 1,2, glucose 10 (pH: 7,4). Spinal cords for immunochytochemistry were taken from anaesthetised animals perfused transcardially with phisiological saline and subsequently 4% paraformaldehyde (in 0,1M phostphate buffer (PB) pH:7,4). For the slice preparations we used neonatal rat pups (P0-8). Animals were decapitated under deep anaesthesia and the lumbar spinal cord segment removed. All steps of th epreparation procedure were carried out in ice-cold ACSF bubbled with a O_2/CO_2 (95%/5%) gas mixture. The L4 segments of the spinal cord were cut out, embedded in agar and than 3-400 micrometer thick transversal sections were cut with a Vibratome.

Immunocytochemistry and morphological evaluation

Following paraformaldehyde fixation DRGs were treated with 50% alcohol for 45 minutes. Blocking was performed with 1% normal goat serum (NGS, Vector) for 50minutes. Sections were than incubated in the polyclonal anti-NK1 receptor antibody raised in rabbit (#11886-5, a gift of Dr. S. Vigna, diluted 1:40000) for 72 hours. Subsequently sections were transferred into biotinylated goat anti-rabbit serum (biot-GAR, Vectorm diluted 1:200) and than into avidin-biotinylated peroxidase complex (ABC, Vector, diluted 1:100) for 1 hour. The immunreaction was finally visualized with a nickel-enhanced diamino-benzidine (DAB) chromogen reaction (0,05% DAB and 0,01% H_2O_2 in 0,05M Tris buffer, pH: 7,4) reaction. Sections were mounted on gelatin coated slides, dried, lightly counter-stained with cresyl-violet, dehydrated and covered with DPX (Fluka, Buchs, Switzerland) mounting medium. Labelled neurons were investigated and drwn by a Camera Lucida and the numerical data was analysed by the NeuroBuild software.

Spinal cord sections after cut from the L4 segments were treated similarly to the DRGs. The percentage of immunopositive neurons were calculated from the total

cell number obatined from Nissl-stained transversal sections of 14 day old Wistar rat lumbar spinal cords.

Following the electrophisiological registration spinal cord slices were transferred into fixative (4% paraformaldehyde, 1,25% glutaraldehyde and 0,2% picric acid in 0,1M PB) for 1-4 days, and later re-sectioned to 50 micrometer thick sections with a Vibratome.qw

In order to visualize the biocytin diffused from the recording electorde into the cell durnig the recording, sections were treated according to the avidin-biotinylated peroxidase method (Extravidin, 1:1000, Vector, Burlingame, CA, USA) and the immunoreaction was visualized with a DAB (Sigma, St. Louis, MO, USA) chromogen reaction. Sections were counterstained with toluidin blue to help laminar orientation, dehydrated, mounted with DPX medium and subsequently reconstructed in 3D with the Neurolucida system (Microbrightfield Inc., Villiston, VT, USA).

Electrophysiology

DRGs intented for electrophysioological recordings were pinned to the Sylgard bed of a recording chamber and perfused with oxygenated ACSF (rate: 1,5ml/min). Dorsal roots and peripheral nerves (25-28mm) were inroduced into recording and stimulating suction electrodes, respectively. Extracellular potentials were amplified with an Axoclamp 2A high-impedance amplifier (Axon Instruments, Union City, CA, USA). Substance P (1µmol/l, Sigma) was superfused to DRGs for 30 seconds. SP applications in these experiments were repeated during continuous superfusion of the selective NK1 receptor antagonist RP67580 (1µmol/l, Rhone Poulenc Rorer Inc., France) applied 15 minutes prior to SP application.

During the intracellular recordings DRGs were treated with collagenase for 5 minutes, to remove the capsule covering the ganglia. The peripheral nerve was introduced into a suction electrode and DRG cells were stimulated by single pulses (0-100V, 20-200µs, ST-02 stimulator, Experimetria, Budapest, Hungary). Intracellular recordings were obtained using glass microelectrodes filled with K-acetate (2mol/l; 60-80MΩ). Voltage signals were amplified with the Axoclamp 2A amplifier (Axon Instruments, Union City, CA, USA). Conduction velocity was calculated by dividing the distance between the stimulating and recording electrodes by the delay time between the stimulus artefact and the onset of the action potential.

Neurons located in laminae V-VII were visually identified with a Zeiss Axiskop FS microscope equipped with a x40 water immersion objective, DIC filter and infrared CCD camera system (Hamamatsu, Japan). Conventional whole-cell patch clamp recordings were performed in current clamp mode using Axopatch 1D, Axopacth 200B and Axoclamp 2B amplifiers (Axon Instruments, Union City, CA, USA) and pipettes witha a resistance of 4-7MΩ. The electrode filling solution contained (mmol/l): K-gluconate, 126; KCl, 4; ATP, 4; GTP, 0,3; phosphocreatine, 10; HEPES, 10 and 0,5% biocytin (Sigma, St. Louis, MO, USA) (pH:7,2). Firing patterns were obtained by applying 800ms long incrementing current steps ranging from -30pA to 100pA. Capacitance and series resistance were not compensated. All recordings were performed on room temperature.

All drugs used during the experiments were added to the ACSF and were transported to the recording chamber with the help of either a peristaltic pump or a gravitational perfusion system (rate 1,5-2 ml/min).

Data were recorded on an IBM PC, filtered at 5 kHz, digitized with a Digidata 1320A A/D board (Axon Instruments, Union City, CA, USA). Recordings were analysed with Clampfit 8.0, Origin (Microcal Software, Northampton, MA, USA), Whole Cell Program and Electrophysiology Data Recorder (Dr. J. Dempster, University of Strachlyde, Glasgow, UK) software packages. All curve fittings were performed using the built-in iterative Levenberg-Marquadt algorithm in Clampfit (Axon Instruments, Union City, CA, USA). All numerical data are presented as mean±SEM. Statistical significance was assessed using Student's *t*-test and ANOVA.

RESULTS

SP evoked responses of DRG neurons

NK1 receptor immunoreactivity could be observed in the whole depth of all the randomly taken DRG sections. There was no obvious organization of the immunopositive neurons, however, occasionally small clusters of immunoreactive cells were seen. All immunoreactiove neurons were stained in their plasma membrane, however, several cells showed immunostaining in the cytoplasm too. |The immunostaining int he plasma membrane was usually discontinuous and ocasionally immunoreactive stem processes and nerve fibers were seen to emerge from the soma. Some small oval-shaped cells in the epi-, peri- and endoneurium also showed NK1 receptor immunopositivity. In contrtol sections, no immunopositive cells were observed.

Cell counting on randomly taken sections revealed that 32 ± 1,5% (22,2–37,6%) of the DRG neurons were NK1 receptor immunopositive. The majority of the immunolabelled neurons were of small and medium diameter range (10-30μm). The mean diameter of the NK1 receptor immunopositive cell population was 28 ± 0,3 μm (n=799).

Superfusion of SP (1μmol/l) for 30 seconds to the DRG evoked a reversible, long-lasting depolarization (mean amplitude: 320 ± 49,7μV, range: 200-600μV; mean duration: 248 ± 9,7s, range: 162-297s; n=18). No desensitization to consecutive SP applications was observed during the experiments. The lowest concentration of the non-peptide NK1 receptor antagonist RP 67580 producing significcant reduction in the amplitude of the SP-evoked response was 1μmol/l. The mean reduction was 23 ± 2,5% volt (n=4). Increasing the concentration of the antagonist did not produce further reduction.

Stable intracellular recordings suitable for studying the actions of SP were obtained from 29 neurons, Conduction velocity was used to characterise the recorded DRG neurons. Cells were identified as Aα/β-type if the conduction velocity was faster than 2,6 m/s, Aδ-type if the conduction velocity was between 0,8 m/s and 2,6 m/s, and C-type if the value was below 0,8 m/s. Based on these criteria, 10 Aα/β-, 11 Aδ- and 8 C-type cells were tested for SP sensitivity. The active and passive

membrane parameters of these neurons were characteristic for each group of DRG cells and similar to the membrane properties of DRG neurons described previously.

Application of SP (1μmol/l) had no effect on the membrane properties of Aα/β cell (n=10), while four out of 11 Aδ-type neurons and 2 of the eight C-type cells responded to SP application with either depolarization or spike discharge. The mean amplitude of the depolarization was 5,6 ± 1,3 mV (n=4) and the mean duration was 158 ± 3,9s (n=4).

NK1 receptor distribution in the spinal cord of young rats

The heaviest immunoractivity int he dorsal horn was observed in lamina I. Both perikarya and fibers in this layer were intensively stained. Thick dendrites running paralell with the dorsal surface of the gray matter were characteristic of lamina I. The great majority of the immunoreactive perikarya in this lamina belonged to two cell types. The round and small immunopositive perikarya represented fusiform cells (76,1%). About 20% of lamina I NK1 receptor immunoreactive neurons with long paralell dendrites along the gray/white matter border belonged to the multipolar type.

Immunoreactivity in lamina II showed inhomogenity. While the lateral part was almost empty, the middle part showed pale immunopositivity. Altogether 2-3% of the total neuronal population of this lamina showed immunoreactivity and about 80% of these cell bodies were elongated and small. Theseperikarya represented stalked cells based upon their morphological characteristics. Approximately 10% of the immunoreactive neurons belonged to cells resembling iselt cells. They had a round cell body without stem dendrites in the transverse plane. Altough the number of other types of immunopositive neurons was low they were characteristic of lamina II. These cells had large, multipolar perikarya. Their dendrites could usually be followed far away from the soma. They either ran in a mediolateral or dorsoventral direction entering laminae I, III and IV. These cells resembled Cajal’s "transverseoux" and Beal’s large central cells.

Lamina III and IV contained a rich network of immunoreactive fine fibers, particularly int he medial part of lamina IV. About 4% and 5% of neuron sin laminae III and IV were immunopositive, respectively. Two different morphological types of neurons were immunoreactive in these laminae. In lamina III the majority of them

(about 90%) was rounded or multipolar in shape with dendrites running towards lamina II. However they never entered the more superficial laminae. These cell bodies represented central cells. About 10% of the immunopositive cells in lamina III were antenna cells. They had multipolar perikarya and long dorsally oriented dendrites which always penetrated laminae I and II. In lamina IV central cells wre about 65% while antenna cells about 35% of the total number of immunoreactive cell bodies.

The immuorecativity in laminae V-VII was uniform and relatively weak. In lamina V a small group of immunopositive cell bodies was found bot hat the very medial part and very lateral part of the lamina. In lamina VII large immunorecative cell bodies were clustered in a zone just lateral to the central canal. These cell bodies were usually multipolar. Neurons in the medial part of lamina VII had large cell bodies and had mediolaterally oriented dendrites.

Lamina VIII contained the weakest immunoreactivity in the ventral horn, and immunopositive perikarya were encountered only very rarely.

Despite the strong immunoreactivity of fibers forming a network around neurons of lamina IX, perikarya were stained only very weakly.

Lamina X contained lightly labelled neurons and strongly labelled fine fibers forming a rich network around the central canal. Immunopositive cells were small, multipolar and fusiform. They were rather encountered in the dorsal part of this lamina.

Physiological and morphological characteristics of neurons in laminae V-VI and the intermediate gray matter

Seventy neurons were recorded. Both electrophysiological recordings and the bioctyin staining were successful in 47 cases. The average resting membrane potential of the recorded cells were -51,71 ± 1,52 mV. On the basis of their firing patterns evoked by suprathreshold depolarising current pulses the recorded neurones were divided into four groups that we classified as (i) "phasic" neurones (10 cells), (ii) "repetitive" neurones (15 cells), (iii) "single" neurones (9 cells), and (iv) "slow" neurones (8 cells). Five neurones could not be classified according to this scheme; their firing patterns showed similarities partly to the "phasic" partly to the "repetitive" neurones.

"Phasic" neurones discharged a continuous train of action potentials during suprathreshold depolarising current pulses. In addition, they showed a marked spike

accommodation including a progressive increase in interspike intervals and attenuation of spike amplitude during the 800 ms long depolarising current step. Short duration monophasic AHPs were also observed after the spikes. The average ratios between the first and last spike amplitude and interspike interval were 5.56 ± 0.72 and 0.44 ± 0.03, respectively.

Similar to “phasic” cells, “repetitive” neurones also discharged a continuous train of action potentials during suprathreshold depolarising current pulses. In contrast to the “phasic” cells, however, we did not observe any obvious sign of spike accommodation or attenuation, but spikes were always followed by a marked slow monophasic AHP.

In contrast to all of the other cell types, “single” neurones fired a single (in some cases two) action potential upon suprathreshold depolarisation. Consecutive spikes could be evoked only after repolarization to resting membrane potential**.** In these cells only a small AHP could be detected after the single spike.

Similar to the “repetitive” cells, “slow” neurones also discharged a continuous train of action potentials during suprathreshold depolarisation. There was no obvious sign of spike accommodation or attenuation, and spikes were always followed by a long-lasting monophasic AHP. However, the average spike frequency of "slow" neurones (8.9±0.6Hz) was significantly lower than that of the “repetitive” cells (21.83±1.5Hz), (P<0.001).

Statistical analysis revealed that the values of resting membrane potentials, input time constants, action potential overshoots and half-widths did not differ significantly among the neurons with different firing patterns. However, certain parameters made the “single” and “slow“ cells unique and obviously different from the “phasic” and “repetitive” neurones. Neuron sin the „single” group had the smallest AP amplitudes (54,44 ± 4,14 mV) and overshoot (16,84 ± 6,16 mV), buta t the same time had thge highest threshold (-40,94 ± 5,95 mV) among the recorded cells. The average amplitude of AHP was also significantly smaller in the “single” cell population. The current-voltage curves appeared to be flat in “slow” and “single” neurones, while they were steeper in “repetitive” and steepest in the “phasic” cells, indicating that the input resistance of “slow” and “single” cells is lower than that of neurones in the “repetitive” and especially in the “phasic” groups. Indeed “slow” cells presented the smallest input resistance among the recorded neurones.

Of the 47 reconstructed neurons, 13 were located within laminae V-VI and 34 in lamina VII. Similarly to membrane parameters, the distribution and morphology of “”phasic” and “repetitive” neurons were similar to each other, while “single” and “slow” cells showed unique characteristics.

Most of the “phasic” (8 out of 10) and “repetitive” (13 out of 15) neurons presented multipolar perikarya with 4-6 primary dendrites, and were distributed equally in laminae V-VII. Of the 9 “single” neurons 7 were recovered in the lateral aspect of laminae V-VI, and only two were located in lamina VII. In these 7 neurons the small multipolar perikarya gave rise to 3-5 stem dendrites, and presented an extensive but poorly arborising dendritic tree with a preferred orientation in the dorso-ventral direction. In 5 cells out of the 7 the axon was also labelled. After arising from the cell body the axons turned ventrally and terminated in the ventral gray matter. From the neurons investigated in this study, “slow” neurons presented the largest and most elongated perikarya. Six out of the 8 recovered “slow” neurons had 6-8 primary dendrites were arranged in two groups that arose from the opposite poles of the elongated somata and formed a richly arborising bushy dendritic arbor. In contrast to the other cells, “slow” neurons were confined to the ventromedial part of lamina VII. In case of 7 neurons the axon was also labelled. The labelled axon arose from the cell body and extended either towards the midline or turned laterally. Of the 5 medially oriented axons, 4 crossed the midline in the anterior commissure and terminated in the contralateral ventral gray matter. The laterally oriented axons targeted the lateral motor column.

Analysis of branch point distribution histograms obtained from dendrograms of neurones with different firing patterns showed that in this regard the dendritic arborisation pattern of “phasic”, “repetitive” and “single” neurones were quite similar to each other. However, “slow” neurones presented dendritic structures that were substantially different from the dendritic arbores of cells in the other groups. The long, slender dendrites of “phasic”, “repetitive” and “single” neurones branched infrequently, in a way that most of the branching points were scattered within a distance of 100-150μm from the soma. In contrast to this, “slow” neurons formed a richly arborising bushy dendritic arbor in which the dendrites continuously branched as they receded away from the cell body.

Modulation of the synaptic inputs to the neurons of the intermediate gray matter by SP

The typical response after SP (1µmol/l) application in the 34 recorded neurons was an increase in the spontaneous EPSP frequency (7 out of 11). Consecutive application of SP produced a similar but somewhat smaller response.

The observed SP-evoked response was effectively blocked by the non-peptide NK1 receptor antagonist SR140333 (10nmol/l) in 7 out of 11 neurons. Following the wash out of the antagonist SP application alone could not produce a marked EPSP number increase. 9 out of 9 cells showed a large increase of EPSP number upon application of [Sar9,Met(O_2)11]-SP (50-200nmol/l), a potent NK1 receptor agonist. This response was very much similar to that observed with SP.

Th increased number of EPSPs can also be due to an increased spontaneous release of transmitters from presynaptic terminals (mini EPSPs) independent of action potentials. To test this hypothesis we repeated the SP application (1µmol/l) int he presence of 500nmol/l TTX. In 3 out of 3 cells after decreasing the inintial EPSP frequency TTX inhibited the SP derived frequency increase of EPSPs.

After dividing our recorded neurons into the upon described firing pattern groups, we found that upon application of SP (1µmol/l) the input resistance and thus the thrsehold of the cells decreased in all groups (n=11). However, while „phasic” and „slow” cells presented a reversible change, the input resistance of neurons in the „repetitive” group has been altered inrreversibly.

DISCUSSION

Responses of DRG neurons to SP application

Results of the present experiments demonstrate that a sub-population of DRG neurons expresses functional NK1 receptor in 14-day-old rats. Previous electrophysiological studies on in vitro DRG preparations have shown thath intracellular recordings are more stable on young than adult animals. To allow comparison between the electrophysiological and immunohistochemical data, all experiments int he present study were performed on two-week old animals.

The NK1 receptor antibodies used int he present study are specific for the 15 amino acid sequence at the C-terminal region of the rat NK1 receptor. This antibody has been previously used to investigate adult rat spinal cord, caudal trigeminal nucleus, DRG and peripheral nerve. While a great number of NK1 receptor immunopositive neurons have been identified in the spinal cord, no NK1 receptor staining has been found in DRGs of the adult rat. There may be several reasons for the discrepancy between these results and ours. NK1 receptor expression in DRG neurons may decrease during poistnatal developement, as has been demonstrated int he spinal cord for different receptors including the NK1 receptor. Reduction in immunostaining whitin DRGs may also occur as a result of NK1 receptor translocation away from the soma towards the central or peripheral terminals during developement.

In the present study 1/3 of the total DRG neuronal population showed NK1 receptor immunopositivity while 1/5 of the intracellularly recorded cells could be activated by SP. Our immunohistochemical findings are in agreement with the majority of previous data showing that 30-40% of DRG neurons and primary afferent fibers were sensitive to SP.

The majority of the NK1 receptor immunopositive neuron sin this study belonged to the small and medium size cell population. Correlative morphological and physiological studies suggest that small and medium size DRG neurons are associated predominantly with Aδ- and C-type neurons. In the present experiment we observed SP-induced membrane effects in Aδ- and C-type cells.

The highly selective and potent NK1 recepotor antagonist RP67580 produced only an 25% reduction on dorsal root depolarization induced by SP application. It has been shown that a sub-population of DRG neurons could be activated through all

types of neurokinin receptors. Although SP has the highest affintiy to the NK1 receptor, it also activates NK2 and NK3 receptors. Thus, the part of the SP-evoked response which could not be further reduced with high concentration of RP67580 may represent the NK2 and/or NK3 mediated activity of DRG cells.

The presence of functional NK1 receptors on neuron sin sensory ganglia raises the question of their possible physiological significance. One possibiltiy is that NK1 receptors whithinsensory ganglia themselves play a role in sensory signal transmission. An alternative to the above possibility is that NK1 receptors may only be expressed transiently on the perikarya of DRG neurons prior to their transport to peripheral and central terminals. Although other laboratories failed to show NK1 receptor immunoreactivity of primary afferents in the spinal cord, our findings that NK1 receptors are expressed by DRG cells with Aδ- and C-characteristics, and the fact that many of the Aδ- and C-fibers terminate in lamina I of the spinal cord where NK1 receptor immunoreaction shows the highest intensity, suggest that further studies are needed to resolve this issue.

NK1 receptor immunoreactivity int he lumbar spinal cord of young rats

In general, we found that the staining pattern of NK1 receptor immunostaining in young rat spinal cord is almost identical to that found previously in adult rats. The majority of the immunostained neurons in the young rat spinal cord belong to the same type as those found in adult rats, although in some laminae there are some differences.

Interestingly in the deeper laminae and in the intermediate gray matter we observed pale but definitely immunopositive cell bodies. Previous experimental data showed NK1 receptor immunopositivity mainly on motoneurons in the adult rat spinal and human spinal cord. In the present study, the membrane of most motoneurons was lightly labelled.

Since we have very limited information about the function of these NK1 receptors expressed by these neurons we wanted to characterise the neurons in laminae V-VII using electrophisiological and combined morphological techniques.

Electrophysiological and morphological characteristics of neurons in lamina V-VII and in the intermediate gray matter

It is generally accepted that neurones in the ventral spinal gray matter, a substantial proportion of which can be regarded as constituents of the spinal motor apparatus, receive and integrate synaptic inputs arising from various peripheral, spinal and supraspinal sources. Thus, a profound knowledge concerning the integrative properties of interneurons in the spinal ventral gray matter appears to be essential for a fair understanding of operational principles of local spinal neural assemblies.

In this study we have documented that intrinsic membrane properties as well as the morphology of neurones vary widely in the ventral gray matter of the neonatal rat lumbar spinal cord. Based on their firing patterns in response to depolarising current steps, we have classified the recorded neurones into four categories, and distinguished “phasic”, “repetitive”, “single” and “slow” neurones. Neurones with firing properties characteristic of the “phasic”, “repetitive” and “single” cells have previously been reported in the superficial and deep dorsal horn of the spinal cord as well as among cultured spinal neurones. However, this is the first account in the literature in which “slow” neurones have been recovered and described in the spinal cord.

Although we have frequently observed spontaneous activity in the network which might modify the discharge patterns of the recorded neurons, it is likely that this external influence had little, if any effect on our results, since “phasic, “repetitive” and “single” neurons have previously been identified also among cultured spinal neuron, where this network effect can obviously be excluded. Thus we assume that the different discharge patterns of neurons that we have demonstrated here are primarily determined by the intrinsic membrane properties of the recorded neurons.

„Phasic” and „repetitive” neurons dis not show any characteristic morphological nor electrophysiological parameters in lamnia V-VII. Neurons with the “phasic” and “repetitive” properties are scattered all over the entire cross-sectional area of the spinal gray matter. In addition to laminae V-VII, where we located them in the present study, neurons with identical firing patterns were identified also in the superficial and deep spinal dorsal horn. Studying the physiological properties of these neurons in lamina I, Prescott and De Koninck (2002) concluded that “repetitive” (tonic in their classification scheme) and “phasic” cells respond in a graded fashion over a wide range of stimulus intensities and their slow synaptic events and slow

membrane time constant promote temporal summation or integration of synaptic inputs.

In contrast to the above statements „single” neurones are incapable of encoding stimulus intensity through firing frequency but could follow high frequency stimulation that may arise from various sources including burst firing or rhythmically active neurones. Thus they can be also regarded as onset detectors. In our sample most of "single" neurons were distributed in lamina V-VI. Neurons with similar firing pattern were reported also in the superficial and deep dorsal horn, where various types of primary afferents terminate. These observations which show an overlapping between the distribution of primary afferent terminals and "single" neurons suggest, that "single" neurons among many other possible sources may receive innervation also from primary sensory neurons. This notion is reinforced by the finding that Ia inhibitory interneurons, that are known to be heavily innervated by primary afferents, have also been classified as "single"-spiking neurons in the adult cat spinal cord.

"Slow" neurones showed unique physiological (eg. low input resistance and firing frequency) and morphological characteristics (e.g. large cell body and the rich bushy dendritic arbour) that made them substantially different from the other cell types. It also appears to be important that all "slow" neurones recorded in this study were confined to the ventromedial gray matter of the spinal cord, where previously our immunohistochemical experiments showed NK1 receptor immunopositive cell large cell bodies with dendrites in a ventro-medial orientation. Taking all of this together, we assume that "slow" neurones may represent essential constituents of rhythm and/or pattern generating spinal motor neural assemblies that are responsible for the generation of flexor-extensor and left-right alternation.

SP sensitivity of neuron sin the intermediate gray matter

The fact that we did not observe any „single" firing cells int he intermediate gray matter is in good agreement with our previous findings where we concluded that „single" cells are mainly confined to lamina V-VI.

Our knowledge about the role of NK1 receptors int he intermediate gray matter is very limited. Limited number of experiments showed that the use of NK1 receptor antagonists (eg. SR140333, RP67580) could effectively block the dorsal root stimulation-evoked depolarisation in the ventral root and individual intracellularly recorded motoneurons. This could be indirect evidence for functional NK1 receptors int he ventral horn.

Furthermore, there is published data proving the presence of a rich network of SP immunoreactive fibers around certain neurons in the intermediate gray matter at the L2-4 segments of the rat, monkey and human spinal cord.

However there is little direct evidence for the functionality of the NK1 receptors showed immunohistochemically in the intermediate gray matter of the rat lumbar spinal cord.

The results of the above study allow us to draw three conclusions:

1. Neurons in the intermediate gray matter receive a substantial input from SP sensitive neurons, thus increasing the activity of these presynaptic neurons result ina n increased number of EPSPs on the membrane of the intermediate gray matter neuron. This effect is likely to be mediated by the NK1 receptor, since antagonists of this receptor could effectively block the response, while NK1 receptor agonists could reproduce the effect of SP. The exact location of these suggested presynaptic, SP sensitive cells remain a question to be answered. Possible locations include the dorsal horn and the intermediate gray matter itself.
2. Based ont he results of the experiments with SP int he presence of TTX we can exclude the contribution of miniEPSP number increase to the overall EPSP number increment. Although we can not fully exclude the possibility of presynaptic NK1 receptors, the results of the immunohistochemical experiments showed exclusively somatic NK1 receptor labelling in this region and we did not see any immunopositive fibers.
3. Among the three firing pattern group recorded int he intermediate gray matter SP only altered the membrane properties of the „repetitive" cells in an irreversible fashion. This observation further augments the hypothesis of the presence of

fucntional somatic NK1 receptors on these neurons. The question whether these NK1 receptor immunopsoitive neurons overlap with the „repetitive” cell population and the lack of long lasting depolarization (the characteristic SP response in dorsal horn and DRG neurons) requires further investigation.

Taking all this together one can assume that SP apart from its „classical” role in nocicpetive information processing may also integrate the nociceptive information into other fucntions of the spinal cord by affecting individual neurons, participating in local neuronal networks, through the NK1 receptor. This suggestion is in agreement with the observation that the nociceptive flexor reflex can be sensitized by application of SP or C-type primary afferent stimulation, and that this senzitization can be effectively blocked by NK1 receptor antagonists.

SUMMARY

The results of the present theses demonstrate that a sub-population of DRG neurons expresses functional NK1 receptors in young rats. These neurons belong to the small and medium size cell populations, that previously have been shown to posess type C and Aδ fibers, majority of which are terminating in lamina I of the spinal dorsal horn.

We used immunohistochelmical methods to investigate the distribution of NK1 receptor immunorceativity in the spinal cord of young rats. We found strong immunolabelling in the superficial laminae, especially in lamina I where mainly fibers appeared to be immnunopsitive. This distribution pattern was similar to that reported in older animals. We found NK1 receptor immunopostitve cell bodies in laminae V-VI and in the intermedier gray matter of the lumbar spinal cord. We applied both morphological and electrophysiological techniques in order to characterize these neurons.

Upon the basis of their firing pattern (response to 800ms long suprathreshold depolarising current steps) we distinguished four groups of the recorded neurons, three of which have been previously reported in different areas of the spinal cord. In our experiments we described a fourth group of neurons with a so-called "slow" firing pattern, characteristic morphological features and specific laminar distirbution. To our best knowledge this is the first time that "slow" firing neurons have been reported in the spinal cord.

We observed a large increase in the number of EPSPs reaching the neurons of the intermedier gray matter during SP application suggesting a presynaptic effect of SP. In certain cells we documented changes in the membrane resistance and friring threshold that are likely to be connected to the presence of fiunctional NK1 receptors.

We hope that our results provide more data for a better understanding of the role of tachykinins in nociceptiv information processing and reflexes.

LIST OF PUBLICATIONS

Peer-reviewed articles related to this study

1. **P. Szucs**, E. Polgar, I. Spiegelman, R. Porszasz and I. Nagy: Neurokinin 1 receptor expression on young rat dorsal root ganglion neurons. *J. Periph. Nerv. Sys.*, **4:** 270-278, 1999. IF: **1,038**
2. E. Polgar, **P. Szucs**, L. Urban, K. Matesz and I. Nagy: Immunohistochemical localization of neurokinin-1 receptor in the lumbar spinal cord of young rats: morphology and distribution. *Somatosensory & Motor Research*, **16(4)**: 361-368, 1999. IF: **0,931**
3. **P. Szucs**, F. Odeh, K. Szokol and M. Antal: Neurons with distinctive firing patterns, morphology and distribution in laminae V-VII of the neonatal rat lumbar spinal cord. *Eur. J. Neurosci.*, **17**, 537-544, 2003. IF: **3,919**

Cumulative IF: 5,888

Other peer-reviewed articles

1. A. Kulik, E. Polgar, C. Matesz, **P. Szucs**, S. Kothalawala and I. Nagy: Sub-population of capsaicin sensitive primary afferent neurons in thoracic, lumbar, and sacral dorsal root ganglion in young rats revealed by stimulated cobalt uptake. *Acta Biol. Hung.*, **47 (1-4):** 385-394, 1996. IF: **0.291**
2. I. Nagy, J. Croxford, E. Polgar, **P. Szucs**, A. Dray and L. Urban: GAP-43 immunoreactivity is enhanced after UV irradiation in the peripheral nervous system of the rat. *Primary Sensory Neuron*, **2**:43-63, 1997. IF: **-**
3. E. Polgar, **P. Szucs**, L. Urban and I. Nagy: Substance P immunoreactivity in rat spinal dorsal horn after ultra violet irradiation induced hind paw inflammation. *Brain Res.*, **786:** 248-251, 1998. IF: **2.526**
4. G. Maccaferri, J. D. B. Roberts, **P. Szucs**, C. A. Cottingham, P. Somogyi: Cell surface domain specific postsynaptic currents evoked by identified GABAergic neurones in rat hippocampus *in vitro*. *J. Physiol.*, **524:** 91-116, 2000. IF: **4.450**
5. P. Ganter, **P. Szűcs**, O. Paulsen and P. Somogyi: Properties of horizontal axo-axonic cells in stratum oriens of the hippocampal CA1 area of rats *in vitro*. *Hippocampus*, 2003 (in press) IF: **4.330**
6. I. V. Melnick, S. Santos, P. Szűcs and B. V. Safronov: Ionic basis of tonic firing in spinal substantia gelatinosa neurons of rat. *J Neurophysiol*, 2003 (submitted).

Cumulative IF: 11,597

LECTURES, ABSTRACTS

1. **P. Szucs**, E. Polgar, I. Nagy: Activation of dorsal root ganglion cells with Substance P. Worldwide Hungarian Medical Academy, Pécs, Hungary, July 4-6, 1996.
2. **P. Szucs**, E. Polgar, I. Nagy: NK1 receptor expresssion in dorsal root ganglion cells of young rats. MAT'IX., Szeged, Hungary, April 4-6, 1997.
3. **P. Szucs**, E. Polgar, L. Urban, S. Jeftinija and I. Nagy: Spinal cord neurons in which interaction between N-methyl-D-aspartate and neurokinin-1 receptors may occur. *Soc. Neurosci.* 156.5 (1995).
4. I. Nagy, E. Polgar, **P. Szucs**, L. Urban and J.N. Wood Plastic changes in neurokinin-1 receptor expression of spinal cord neurons in inflammatory hyperalgesia. *Soc. Neurosci.* 44.7 (1995).
5. I. Nagy, E. Polgar, **P. Szucs**, A. Matisz and L. Urban: Neurokinin 1 receptor expression by dorsal root ganglion neurons in young rats. *IASP Publications*, Congress Abstracts, 121 (1996).
6. **P. Szucs**, E. Polgar and I. Nagy: Spinal cord neurons in which interaction between N-methyl-D-aspartate and neurokinin-1 receptors may occur. *Neurobiology*, 4 (1996) 281-282.
7. **P. Szucs**, E. Polgar, I. Spiegelman, R. Porszasz, I. Nagy: Dorsal root ganglion cells expressing the NK1 receptor in young rats. *Neurobiology*, 5 (1997).
8. E. Polgar, **P. Szucs**, K. Matesz, L.A. Campbell, L. Urban and I. Nagy: Locally injected nerve growth factor increases substance P synthesis in dorsal root ganglion neurons in all segments. *European Neuropeptide Club, Abstracts*, P10 (1997)
9. P. Somogyi, P. Ganter, N. Kogo, G.M. Maccaferri, C. Paspalas, O. Paulsen, J.D. Roberts, R. Shigemoto, **P. Szucs**: Compartmentalisation and properties of synapses and receptors in a feedback circuit of the cerebral cortex. *J. Physiol.* (1999), **518P**, *pp.* 22S *Proceedings of the scientific meeting held at UCL, 20-22 April, 1999*
10. **P. Szucs**, F. Odeh, K. Szokol and M. Antal: Electrophysiological and morphological characteristics of neurons in the intermediate gray matter (laminae V-VII) of the neonatal rat lumbar spinal cord *in vitro*. IBRO CEER, Neurobiology (*in press*, 2002)
11. **P. Szucs**, F. Odeh, K. Szokol, M. Antal: Electrophysiological and morphological characteristics of neurones in the intermediate gray matter (laminae V-VII) of the neonatal rat lumbar spinal cord in vitro. FENS, July 2002, Paris.
12. **P. Szucs**, M. Antal, K. Szokol, F. Odeh, P. Poisbeau: Modulation of neuronal excitability by substance-P in laminae V-VII of the neonatal rat lumbar spinal cord. MITT, January 2003, Balatonfured, HUNGARY