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MORPHOLOGICAL STUDY OF LAST-ORDER PREMOTOR INTERNEURONS AND COMMISSURAL INTERNEURONS IN THE LUMBAR SPINAL CORD OF RATS

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The Examination takes place at the Lecture Hall of the Department of Physical Medicine and Rehabilitation, Medical and Health Science Center, University of Debrecen
at 10 a.m. 22 November, 2012.

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1. INTRODUCTION

Central pattern generator

The basic motor patterns underlying rhythmic limb movements during locomotion are generated by neuronal networks located within the spinal cord. These networks - capable of generating and shaping the rhythmic activity in the absence of descending pathways and sensory feedback - are called as central pattern generators (CPGs). In two non mammalian vertebrate species, the lamprey and the Xenopus tadpole, the critical neuronal elements of the CPG for swimming have been identified and characterized in great detail. Less is known about the CPGs generating walking in limbed animals including mammals.

In swimming animals, the CPGs are distributed along the entire spinal cord. In the rodent, the cervical enlargement of the spinal cord contains CPG networks involved in movements of forelimbs, whereas the CPSs in lower thoracic and lumbar spinal segments are concerned to movements of hind limbs. Labeling studies suggesting that the crucial elements of the locomotor circuits are located in the ventromedial area of the mammalian spinal cord in laminae VII, VIII and X. The key features of walking CPG are:

1.) the rhythm generation: ipsilaterally projecting glutamaterg neurons are most likely the source the rhythm generation.

2.) ipsilateral coordination of flexors and extensors across the same or different joints in a limb: ipsilaterally projecting inhibitory premotor interneurons are the dominant elements.

3.) left-right coordination: commissural interneurons - whose axons cross the midline via the ventral commissure- play a crucial role in coordination of motor activities between the right and left sides of the spinal cord.

In our study two fundamental elements of the hind limb locomotor CPG: the last-order premotor interneurons and commissural interneurons were investigated.

Last-order premotor interneurons (LOPIs)

There is a general agreement that neural activities generated by spinal neural mechanisms are transmitted to spinal motoneurons by last-order premotor interneurons (LOPIs) that establish excitatory and inhibitory monosynaptic connections with motoneurons. In addition to a strong drive from the spinal motor apparatus, LOPIs also receive sensory inputs from the periphery and descending commands from supraspinal motor centers. Therefore, LOPIs may integrate the activities generated by the spinal motor apparatus with the sensory information and the volleys arising from higher motor centers.

Simultaneous extra- and intracellular recordings have revealed that LOPIs receive monosynaptic inputs from groups I and II muscle afferents, from low threshold cutaneous afferents and various supraspinal centers. Despite the wealth of studies the synaptic input to these neurons has not been hitherto accurately investigated from the morphological point of view. On this basis we wished to visualize close appositions of primary afferent terminals on LOPIs in the lumbar spinal cord of rats by using double label neuronal tracing method.

Commissural interneurons (CINs)

Conveying neuronal signals from one side of the spinal cord to the other, CINs are essential elements of neural circuits underlying left-right coordination. It has previously been demonstrated that neurons are involved in generation of left-right alternation during locomotion are located in the ventromedial area of the lumbar spinal cord in neonatal rats. It has also been established that many of these neurons possess axons that cross the midline and terminate in the contralateral gray matter.

Studies in Xenopus tadpole and lamprey indicated that CINs in swimming CPGs produce inhibition of rhythm generating interneurons and motoneurons on the contralateral side of the spinal cord by using glycine as a neurotransmitter. This inhibition causes termination of motoneuronal firing on the opposite side during the time while motoneurons on the side of CINs are active and plays a key role in alternating motoneuron discharge during swimming.

In quadruped animals coordinated movements during walking are produced by alternating rhythmic activities of flexor and extensor muscles of the fore and hind limbs. Thus, CINs involved in generation of the hind limbs rhythm have to coordinate alternating activities of motoneurons on the left and right sides of the same lumbar segments and synchronous activities of flexor and extensor motoneurons located in different lumbar segments of the spinal cord. Results obtained from previous studies suggest that CINs in the mammalian spinal cord represent a heterogeneous population with respect their axonal projection, synaptic connections and neurotransmitter content. In the present study we investigated the synaptic connections and neurotransmitter phenotypes of CINs in the lumbar spinal cord of neonatal rats.

2. OBJECTIVES

We investigated two elements of the spinal motor system in newborn and adult rats:

1. Last-order premotor interneurons that convey sensory and descending information and rhythmic signals from spinal CPG to the motoneurons.

By using double label neural tracing methods, we aimed to visualize and analyse the possible contacts between primary afferents and LOPIs.

2. The **commissural interneurons** are involved in generation alternating activities of motoneurons on the sides of the spinal cord.

By using anterograde and retrograde tracing methods we wanted

- to describe the location and distribution of CINs in the spinal gray matter
- to identify direct contacts between CINs and CINs and between CINs and motoneurons.

With the help of immunochemistry and confocal microscopy we tried

- to determine the neurochemical properties of the CINs.

3. MATERIALS AND METHODS

3.1. LAST-ORDER PREMOTOR INTERNEURONS

Labeling of last-order premotor interneurons in the spinal cord of newborn rats

Under deep anesthesia newborn (1-4 days) Wistar rats were decapitated and eviscerated. The spinal cords were exposed by ventral laminectomy and transferred to a recording chamber superfused with artificial cerebrospinal fluid (ACSF). At the level of the L2-L4 segments 10% of biotinylated dextran amine (BDA, MW: 10 000, Molecular Probes) was injected iontophoretically into the lateral motor column of the spinal gray matter. After tracer application, the spinal cords were kept in the recording chamber for 4-8 hours and then transferred into a fixative containing 2,5% glutaraldehyde, 0,5% paraformaldehyde and 0,2% picric acid in 0,1 M phosphate buffer. The lumbar segments of the spinal cord were sectioned horizontally at 60 µm on a vibratome. For histochemical detection of the BDA the free-floating sections were incubated with avidin-biotinylated peroxidase complex (ABC, 1:100, Vector Labs) overnight at 4^{o}C. The reaction was completed with a nickel-intensified diaminobenzidine reaction (DAB, SIGMA).

Labeling of last-order premotor interneurons in the spinal cord of adult rats

Under deep anesthesia dorsal laminectomy was performed to expose the L3-L5 segments of the lumbar spinal cord of adult Wistar rats. The LOPIs were labeled

1.) by iontophoretic injection of 5% Neurobiotin (Vector Labs.) into the lateral motor column in three animals,

2.) or by iontophoretic injection of 10% biotynilated dextran amine into the lateral motor column in 11 animals.

Labeling of primary afferents in the spinal cord of adult rats

The labeling of primary afferents was made according to the following two protocols:

1.) After staining of LOPIs with Neurobiotin, the ipsilateral dorsal root of the same spinal segment was cut and introduced into a glass tube filled with horseradish peroxidase (HRP, 10%, Sigma). HRP was visualized using a nickel-intensified diaminobenzidine reaction.

2.) After labeling of LOPIs with BDA, 1% solution of cholera toxin subunit B (CTb, List Biological) was pressure-injected into the central cut end of the sciatic nerve. The CTb was revealed with the help of immunocytochemical reaction by using rabbit anti CTb (1:10 000, List Biological) as a primary antibody and biotinylated goat anti-rabbit IgGs (1:200, Vector Labs.) as a secondary antibody. The sections were incubated in ABC and the histochemical reaction was completed with DAB chromogen reaction.

3.2. COMMISSURAL INTERNEURONS

Labeling of commissural interneurons and motoneurons in the spinal cord of newborn rats

Newborn (1-4 days) Wistar rats were decapitated and eviscerated under deep anesthesia. The spinal cords were exposed by ventral laminectomy and carefully dissected sparing the ventral and dorsal roots. The cords were then transferred to a recording chamber superfused with artificial cerebrospinal fluid (ACSF). 10% solution of BDA was injected iontophoretically into the ventromedial area of the gray matter at the level of the L2-L4 lumbar segments in order to label the CINs. Following BDA injection, the motor neurons contralateral to the injection side were also labeled by placing biocytin crystals (Sigma) on the L2-L5 ventral roots. After the tracer application, the spinal cords were kept in the recording chamber for 4-8 hours then transferred into a fixative containing 2,5%

glutaraldehyde, 0,5% paraformaldehyde and 0,2% picric acid. The lumbar segments of the spinal cord were sectioned horizontally at 60 µm on a vibratome. For histochemical detection of BDA and biocytin, free-floating sections were incubated with ABC (1:100, Vector Labs) and with nickel intensified DAB.

Detection of CIN-motoneuron connections

The distribution and morphology of the retrogradely labeled CINs were investigated on serial sections of the spinal cord. The dendritic trees of labeled CINs were reconstructed by using a camera lucida. Presumed contacts between CINs and motor neurons were identified with 100x oil immersion objective. In order to verify the synaptic connections on electronmicroscopic level, the sections were treated with 1% osmium tetroxide, dehydrated and flat-embedded into Durcupan ACM resin (Sigma). Ultrathin (60 nm) sections were cut and collected on Formvar-coated single-slot nickel grids, counterstained with uranyl acetate and lead citrate and examined in a Jeol 1010 electron microscope.

Identification of neurotransmitters of labeled CINs

Neurotransmitters of labeled CIN axon terminals were investigated by incubating the sections with a cocktail of primary antibodies against vesicular glutamate transporters VGLUT1 (1:5000, Chemicon) and VGLUT2 (1:5000, Chemicon) raised in guinea pig or with a mixture of antibodies against glutamic acid decarboxylase (GAD65/67, 1:10 000, Sigma) raised in rabbit and against glycine transporter (GLYT2, 1: 5000, Chemicon) raised in sheep. The sections were then incubated in species-specific secondary antibodies (goat anti-guinea pig IgG conjugated with Alexa Fluor 546, or donkey anti-rabbit IgG conjugated with Alexa Fluor 646 and donkey anti-sheep IgG conjugated with Alexa Fluor 546, all diluted 1:1000, Molecular Probes). Finally, the sections were mounted on glass slides, coverslipped with Vectashield (Vector Labs.) and examined in a Zeiss LSM 510 confocal laser scanning microscope. In each case ten 0,5m-thick optical

sections from the ventral horn (including lamina VII and IX) were obtained through 60x oil-immersion lens by using Zeiss LSM software. The same area of the spinal cord presented labeled CIN terminals were scanned sequentially with 488 nm, 658 nm or 647 nm of lines of laser. The BDA labeled CIN terminals in the ventral horn were revealed by using 488 nm line of laser. The VGLUT1,2 and GLYT2 immunoreactive terminals were observed with 568 nm of line and GAD 65/67 were examined by using 647 nm line of laser. The confocal z-series were then analysed by using Zeiss LSM Image Browser program. All BDA labeled terminals in the selected area of the spinal cord were counted. Afterwards the labeled CIN terminals presented immunoreactivity against transmitter specific proteins were identified on merged images of corresponding fields of the spinal cord. Co-localization of anterograde BDA-labeling with immunoreactivity in each axon terminal was verified by observing the double labeled bouton in 0,5 nm-thick consecutive slices along the Z-axis.

4. RESULTS

4.1. CONNECTIONS OF LAST-ORDER PREMOTOR INTERNEURONS WITH PRIMARY AFFERENTS

Labeling of last-order premotor interneurons in the spinal cord of newborn rats

Following iontophoretic injection of BDA into the lateral motor column at the level of L2 or L4 spinal segments, most of the labeled neurons were located ipsilateral to the injection site (83%), although stained neurons were also recovered in substantial numbers in the contralateral gray matter (17%). Ipsilateral to the site of injections, the labeled neurons were mostly confined to laminae V-VIII. In contrast to this, neurons in the contralateral gray matter were concentrated in the ventromedial gray matter corresponding mostly to lamina VIII and partly to lamina VII, X. In case of injections at L2, neurons were recovered in substantial numbers both rostral and caudal to the injection site. In case of injections at L4, however, neurons were almost exclusively found rostral to the site of BDA delivery.

Labeling of last-order premotor interneurons in the spinal cord of adult rats

Both Neurobiotin and BDA injection at the level of L3-L5 spinal segments resulted in relatively large number of retrogradely labeled neurons. Most of the labeled cells were located ipsilateral to the injection site, and only 2-6% of them were observed in the contralateral gray matter. Reconstruction of spinal serial sections showed that labeled interneurons were confined to laminae V-VII within three or four segments of the spinal cord, with the highest density at the level of the tracer application. The cell body was in average 20-50 µm in diameter and gave rise to two-five stem dendrites that extended to the spinal gray matter over a distance of 300-400 µm in various directions.

Projections of primary afferent fibers to last-order premotor interneurons

Application of HRP into the dorsal root and injection of CTb into the sciatic nerve resulted in intense labeling of primary afferents in the laminae V-VII. Quantitative analysis of the sections showed that only a minor proportions of LOPIs (2,5%-9,2%) were contacted by these labeled primary afferents and These postsynaptic LOPIs were concentrated in spinal segments located close to the injection site (i.e. close to the innervated motoneurons) and they were distributed throughout the mediolateral extent of laminae V-VI and in the dorsal portion of lamina VII of the spinal gray matter.

4.2. SYNAPTIC TARGETS AND NEUROTRANSMITTER SYSTEMS OF COMMISSURAL INTERNEURONS

Labeling, morphology and distribution of commissural interneurons

Injection of BDA into the ventromedial area of the spinal cord at the level of the L4 segment labeled CINs via two different ways. Those cells which were located close to the injection site took up BDA through their cell bodies and dendrites and transported the dye into their axons (iCINs). The axons of these anterogradely labeled iCINs crossed the midline in the ventral commissure and extensively arborized in the contralateral gray matter. The other group of CINs (cCIN) took up BDA through their axon terminals extending into the injection site and transported it retrogradely to their somata and dendrites.

Depending on the size and location of the injection site, the numbers and distribution of the retrogradely labeled cells varied in a wide range. Most of the cells were located within the segments of the injection, however, some of them were scattered in one or two segments rostral or caudal to the BDA application. We focused our attention to the cells located contralateral to the side of injection. These retrogradely labeled cCINs were located in the ventromedial area of the spinal gray matter (lamina VII, VIII and X). Forty-seven of the investigated 112

contralateral CINs were located in the medial part of the lamina VII and presented pyramidal or multipolar cell bodies, the diameter of which varied in the range of 10-20 µm. Sixty-five neurons were located in the medial extent of the ventromedial gray matter. These cells had fusiform cell bodies with diameters of 20-30 µm giving rise to three or four stem dendrites that arborized once or twice near the cell body.

Commissural interneuron-commissural interneuron connections

Following BDA injections into the ventromedial gray matter large numbers of retrogradely labeled neurons were recovered contralateral to the injection site. Thorough investigation of the somata and dendritic trees of labeled contralateral CINs showed that many of them were contacted by labeled iCIN axon terminals. Of the 35 neurons examined in this way 17 were apposed by labeled axons. Individual neurons received 1 to 4 contacts and these appositions were found exclusively on proximal dendrites.

Commissural interneuron-motor neuron connections

BDA injections into the ventromedial gray matter labeled a strong axon bundle that crossed the midline in the ventral commissure and extensively arborized in the contralateral gray matter. Many of the axons could be followed as far as the lateral motor column where they formed close appositions with somata and dendrites of retrogradely labeled motoneurons. We traced the cell body and dendritic tree of 632 motor neurons located at the lumbar spinal cord. Careful analysis of the sections revealed close appositions between labeled axon terminals and cell bodies and/ or dendrites of motor neurons in 291 cases. Individual motor neurons received 1-3 contacts from labeled axon terminals. Nearly one-fourth of the appositions were found on somata, whereas the others were revealed on proximal dendrites. The electron microscopic examinations verified that the close

appositions were identified at LM level represented synaptic connections between CIN axon terminals and somata or dendritic trees of motoneurons.

Neurotransmitters of commissural interneurons

The axon terminals of CINs were revealed by delivering BDA unilaterally into the ventromedial area of the spinal gray matter. The labeled iCIN axons crossed the midline in the ventral commissure and branched extensively in the contralateral side of the spinal cord. Large numbers of anterogradely labeled CIN terminals were encountered in the lateral part of lamina VII and in the lateral motor column of the spinal gray matter.

It has been demonstrated that glutamate transporter proteins (VGLUT) convey the glutamate into synaptic vesicles and the antibodies raised against these transporters are regarded as specific markers for glutamatergic axon terminals. Therefore the excitatory characteristic of CINs was tested by applying antibodies to VGLUT1 and VGLUT2 which are concentrated in the axon terminals. The co-localization of BDA-labeling with immunoreactivity for VGLUT was investigated in the ventral horns of four spinal cords. About one-fourth (158) of the 590 identified CIN axon terminals presented VGLUT1 and/or VGLUT2 immunoreactivity.

It has been generally accepted that GABA and glycine are the main inhibitory neurotransmitters in the spinal cord. In GABAergic spinal neurons two different isoforms of glutamate decarboxylase enzyme (GAD65 and GAD67) have been identified that transform glutamate into GABA and accumulate relatively high concentration in GABAergic axon terminals. Hence, CIN axon terminals with putative GABAergic phenotype were revealed by applying antibody recognize the GABA-synthesizing enzymes. We investigated 1146 BDA-labeled CIN axon terminals in the ventral spinal cord of five animals and 350 terminals showed immunostaining for GAD65/67 antibody indicating that about one third of total population of labeled CIN boutons may use GABA as inhibitory neurotransmitter.

The glycine-containing axon terminals include high-affinity transport proteins (GLYT1 and GLYT2) in the plasma membrane to remove glycine from the synaptic cleft. It has been proved that glycine is accumulated in GLYT2-immunoreactive boutons. In our studies 1146 BDA-labeled CIN axon terminals in five animals were investigated and 502 (44%) were immunoreactive for GLYT2.

Several morphological and physiological evidences indicated that GABA and glycine could be present in the same axon terminal and could act as co-transmitters at spinal synapses. We also demonstrated co-localization of markers for inhibitory amino acid neurotransmitters in CIN terminals in the spinal cord. In total 189 terminals were double labeled for both GAD 65/67 and GLYT2 antibodies, which constitute 17% of all BDA-labeled CIN terminals and about one third of putative inhibitory CIN boutons (189 of 663).

Our results showed that CINs in the spinal cord of neonatal rat represent a heterogeneous population with respect to their neurochemical properties. The immunoreactivity of BDA-labeled CIN terminals for specific transmitter-associated proteins indicated that about one forth of the CIN terminals could be excitatory and might use glutamate whilst more than half of the CIN boutons (58%) assumed to be inhibitory. The majority of the BDA-labeled putative inhibitory terminals (502 out of 663) contained glycine transporter protein and half of them (350 out of 663) assumed to be GABAergic phenotype. About one third of GLYT2-positive terminals (189 out of 502) presented also GAD 65/67 immunoreactivity whereas one half of GAD positive terminals (189 out of 350) were also GLYT2-positive.

5. DISCUSSION

5.1. PROJECTIONS OF PRIMARY AFFERENT FIBERS TO LAST-ORDER PREMOTOR INTERNEURONS

Labeling of last-order premotor interneurons and primary afferents in adult rats

In our study, BDA and Neurobiotin injection into the lateral motor column resulted in large number of labeled LOPIs. The application of HRP into the dorsal root and CTb injections into the sciatic nerve labeled a substantial number of thick myelinated primary afferents that terminated in laminae V-VII, where the retrogradely labeled LOPIs were concentrated. Therefore, the double labeling technique provided a valuable tool for analysis of connections between primary afferents and LOPIs. With the help of morphological methods we demonstrated for the first time close appositions between primary afferents and LOPIs.

LOPIs receiving monosynaptic inputs from primary afferent fibers

It has been shown that groups of LOPIs receive monosynaptic inputs from groups I and II muscle afferents and from low threshold cutaneous afferents. In support of these electrophysiological data, here we showed that terminals of primary afferents do indeed establish contacts with LOPIs. However, our results also suggest that a minor proportion of LOPIs receives monosynaptic inputs from primary afferents, and even the ones that are innervated establish only a few contacts with primary afferent fibers in the lumbar spinal cord of the rat.

This sparse input, however, may provide an effective sensory drive for LOPIs. Physiological experiments have demonstrated that stimulation of muscle and cutaneous nerves evoked disynaptic EPSPs and IPSPs on spinal motoneurons indicating that the limited number of primary afferent contacts that we found can

generate large postsynaptic potentials on LOPIs, and in certain conditions can even make them fire.

Our study provided the first evidence of segmental and laminar distribution of LOPIs that receive monosynaptic inputs from primary afferents. We demonstrated that LOPIs which are contacted by primary afferents distributed in laminae V-VII ipsilateral to the site of innervated motoneurons within two or three segments rostrally and caudally from the site of the tracer application.

5.2. COMMISSURAL INTERNEURONS

Labeling, distribution and morphology of commissural interneurons

Injection of wheat germ agglutinin into the muscles of the lower limb in cats or BDA into the motor column of adult rat spinal cord showed that a proportion of commissural interneurons in the ventromedial aspect of the spinal gray matter project directly to motoneurons and can be regarded as last-order premotor commissural interneurons. Our present findings are consistent with this previous observation. After injection of BDA into the lateral motor column at the level of L2 and L4 segments of the neonatal rat spinal cord, retrogradely labeled neurons contralateral to the site of the tracer application were confined to the ventromedial aspect of the gray matter. We also presented morphological evidence that axon terminals of CINs in the ventromedial gray matter established close appositions with somata and proximal dendrites of motoneurons. These synaptic contacts were also verified by electron microscopic investigation.

Puskár and Antal reported that after injection of BDA into the lateral motor column at the level of L1-L2 and L4-L5 segments of the adult rat spinal cord labeled LOPCINs were encountered in a three-to-four segments-long compartment of the lumbar cord. Here we demonstrated that the rostrocaudal distribution of LOPCINs in the neonatal rat is wider than in adult animals. After injecting BDA into the lateral motor column at the level of L2 or L4 spinal segments LOPCINs

were found throughout the entire length of the lumbar spinal cord. However, most of the LOPCINs are located at or very close to the segmental level of the innervated motor neurons, although same may establish positions three to four segments away and innervate the contralateral motor neurons with long ascending and descending axons. This notion is strongly reinforced the findings that the ventromedial area of the neonatal rat contains CINs that possess ascending and/or descending axons that extend as long as four to five spinal segments.

Although LOPCINs can be found throughout the lumbar segments their distribution appeared to be uneven along the rostrocaudal axis of the spinal cord. After injecting BDA into the motor column either the level at L2 or L4 spinal segments, LOPCINs were labeled in large numbers at the level of L1-L4, but were recovered only in limited numbers at L5-L6. This observation indicates that while L1-L4 segments contain a substantial number of LOPCINs with ascending and/or descending axons L5-L6 segments contain only a few LOPCINs with ascending axons. However, following BDA injection into the ventromedial gray matter at L4, in addition to neurons at L1-4, a substantial number of stained cells were also found in the ventromedial gray matter of the L5-6 segments. These findings suggest that the ventromedial gray matter at L5-L6 contains CINs with ascending axons in considerable numbers, but these ascending axons terminate primarily in the contralateral ventromedial gray matter with few monosynaptic projections to contralateral motoneurons.

In adult rat it was demonstrated that LOPCINs were labeled in substantial numbers in animals in which BDA was injected into the medial motor column. However, after lateral motor column injections LOPCINs were recovered only in limited number; that represented no more than 2-6% of the total population of the retrogradely labeled neurons. After delivering BDA at the level of L2 and L4 into the lateral motor column in newborn rat, higher number (17%) of retrogradely labeled cells proved to be LOPCINs. This difference between the adult and

neonatal rats indicates that LOPCINs projecting to motor neurons in the lateral motor column may undergo a substantial postnatal developmental reorganization.

Reciprocal connections between commissural interneurons in the ventromedial gray matter

A number of physiological studies carried out on aquatic vertebrates have shown that ventromedial CINs on the two sides of the spinal cord form inhibitory reciprocal interactions with each other. This reciprocal CIN-CIN integrative system has been shown to be a key element in the coordination of the swimming behavior.

With the application of morphological methods, we show for the first time that a similar arrangement appears to be present also in the mammalian spinal cord. We found that following injection of BDA into the ventromedial area of the spinal cord the labeled contralateral CINs established monosynaptic appositions with CINs located ipsilateral to the injection site. The finding that CINs contacted by commissural axons were recovered not only at the segmental level of the tracer application, but also in adjacent segments both rostral and caudal to the site of BDA injections indicates that CINs may form monosynaptic contacts with each other not only at the segmental level but intersegmental direct CIN-CIN interactions may also exist. Our results also suggest that these reciprocal interactions may be powerful, since a substantial proportion (46%) of the investigated CINs was found to be apposed by axon terminals of contralateral CINs.

Neurotransmitters of commissural interneurons

Locomotor activity in vertebrates is produced by activation of spinal neuronal networks located on both sides of the spinal cord. In lower vertebrate species, the lamprey and Xenopus tadpole, the ipsilateral interneurons maintain the excitatory drive to motoneurons while commissural interneurons cross the midline

and inhibit all types of neurons in the contralateral CPG. Pharmacological studies indicate that inhibitory effect of CINs is mediated by glycinerg transmission in both species. This inhibition causes termination of motoneuronal firing on the contralateral side during the time while network interneurons and motoneurons on the side of CINs are active. Therefore, the reciprocal inhibition mediated by CINs between the sides of the spinal cord plays a significant role in generation of alternating motor pattern during swimming.

In the present study we have carried out immunocytochemistry with antibodies raised against specific transmitter-related proteins correspond to glutamate, GABA and glycine to determine the neurotransmitter phenotypes of CINs in the spinal cord. The immunoreactivity of the BDA labeled CIN terminals for the applied markers indicates that CINs in the ventromedial area of the lumbar spinal cord may utilize various neurotransmitters.

Excitatory commissural interneurons

In locomotor network of lamprey and Xenopus tadpole CINs proved to be glycinergic and carry reciprocal inhibition between the two sides of the spinal cord during swimming. Here we demonstrated that substantial proportion (27%) of CIN axon terminals were immunoreactive for VGLUT1 and VGLUT2, suggesting that CINs in the ventromedial gray matter of the spinal cord of neonatal rat may use glutamate as neurotransmitter.

Previous physiological studies indicated that crossed glutamatergic pathways exist in the spinal cord of neonatal rat which directly activate the contralateral motoneurons. By coupling ipsilateral flexor and extensor networks with contralateral motoneurons, the glutamatergic CINs assist to provide activities for proper coordination of limb muscles during locomotion.

It appears that normal left-right coordination of the spinal cord depends on the balance between excitatory and inhibitory components of the transverse coupling system. In recent experiments using knockout mice, a group of ipsilateral

excitatory CPG interneurons was identified in the ventromedial area of the spinal gray matter. In mutant mice the axons of these cells aberrantly crossed the midline and increased the level of excitation on the contralateral side of the spinal cord. The change in normal balance of excitation and inhibition between the left and right sides of the spinal cord induced synchronous rhythmic activation of motoneurons accompanied by synchronous movements between the limbs.

Inhibitory commissural interneurons

Our present study showed that three-forth of the inhibitory CIN axon terminals use glycine supporting that crossed glycinergic transmission plays a powerful action in the mammalian spinal cord. Physiological data indicated that descending intersegmental CINs in the L2 lumbar segment gave glycinergic inhibiton to L4 motoneurons on the contralateral side of the spinal cord. In cat, the axons of glycinerg CINs in the L4-L5 spinal segments inhibit contralateral interneurons and motoneurons located in the L7 segment. These evidences strongly suggest that glycinergic inhibitory CINs are directly involved in alternation of activities of motoneurons in the mammalian spinal cord.

We showed that 14% of the examined CIN axon terminals were labeled for GAD65/67 antibody. The same result was found in examination carried out on newborn mouse where also 14% of the CIN terminals proved to be GABAergic. These data suggest that in addition to the glycinergic CINs that constitute the core of the left-right coordinating circuits in locomotor models of aquatic vertebrates, the rodent CPG may encompass a strong GABAergic left-right coordinating circuit.

It has been well established that the two major inhibitory neurotransmitters GABA and glycine could be co-expressed in the same nerve terminal in the mammalian spinal cord. Similarly to these previous studies, our data also show that 17 % of CIN axon terminals may accumulate both amino acids. Co-expression of GABA and glycine was also verified in the lumbar spinal cord of mouse where

10% of the CINs contained both inhibitory neurotransmitters. It has been demonstrated that axon terminals releasing both GABA and glycine produce fast-decaying glycine-receptor mediated and slow-decaying $GABA_A$-receptor mediated components. Selective suppression of glycinergic and GABAergic synapses by applying low concentrations of strychnine and picrotoxin indicated that GABA and glycine play different roles in coordination of rhythmic activities between the two sides of the spinal cord. GABA regulates the onset and duration of rhythms, whereas glycine stabilizes the pattern of alternating rhythms.

6. SUMMARY

The basic motor patterns underlying rhythmic limb movements during locomotion are generated by neuronal networks located within the spinal cord. These networks are called central pattern generators (CPGs). We investigated two elements of the CPG: the last-order premotor interneurons and commissural interneurons.

After BDA injection into motor column of the lumbar spinal cord of newborn rats at the L2 or L4 spinal segments about 80% of the labeled last–order premotor interneurons (LOPI) were located ipsilateral to the injection site and were confined to laminae V-VIII. We also recovered substantial number (18%) of labeled cells in the contralateral gray matter that we could define as last-order commissural interneurons (LOPCINs). We demonstrated that the distribution of LOPCINs is uneven along the rostrocaudal axis of the lumbar spinal cord. LOPCINs were labeled in large numbers at the level of L1-L4, but were recovered only in limited numbers at L5-L6. First in the literature we gave an account for the segmental and laminar distribution of LOPIs that receive monosynaptic inputs from primary afferents (PA). It was revealed that LOPIs contacted by PA terminals tend to be concentrated at the segmental level of the innervated motoneurons, and are evenly distributed along the mediolateral extent of laminae V-VI and in the dorsal portion of lamina VII. We concluded that a minor proportion (<10%) of LOPIs received close appositions from stained primary afferents and we could encounter 1-5 terminals on each postsynaptic neuron.

We demonstrated that CINs established monosynaptic contacts with motor neurons on the opposite side of the spinal cord and direct reciprocal connections between CINs on the two sides of the spinal cord also exist. The immunoreactivity of BDA labeled CIN terminals for specific transmitter-associated proteins indicated that about one forth of the CIN terminals could be excitatory and may use glutamate as a neurotransmitter whilst more than half of the CIN boutons assumed to be inhibitory. It was also found that three forth of the inhibitory CIN axon terminals may use glycine supporting that crossed glycinergic transmission plays a powerful function in the mammalian spinal cord. We also demonstrated that about half of the inhibitory terminals in neonatal spinal cord were labeled for GAD 65/67 antibody indicating that GABAerg neurotransmission also be involved in left-right coordination.

7. LIST OF PUBLICATIONS

Candidate: Ildikó Wéber

Neptun ID: I1BVJX

Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Wéber, I.**, Veress, G., Szűcs, P., Antal, M., Birinyi, A.: Neurotransmitter systems of commissural interneurons int he lumbar spinal cord of neonatal rats.
   Brain Res. 1178, 65-72, 2007.
   DOI: http://dx.doi.org/10.1016/j.brainres.2007.06.109
   IF:2.218

2. **Wéber, I.**, Puskár, Z., Kozák, N., Antal, M.: Projections of primary afferent fibers to last-order premotor interneurons in the lumbar spinal cord of rats.
   Brain Res. Bull. 71 (4), 337-343, 2007.
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   J. Comp. Neurol. 461 (4), 429-440, 2003.
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List of other publications

4. Stelescu, A., Sümegi, J., **Wéber, I.**, Birinyi, A., Wolf, E.: Somato-dendritic morphology and dendritic signal transfer properties differentiate between fore- and hindlimb innervating motoneurons in the frog Rana esculenta.
BMC Neurosci. 13 (1), 68, 2012.
DOI: http://dx.doi.org/10.1186/1471-2202-13-68
IF:3.042 (2011)

Total IF: 10.875

Total IF (publications related to the dissertation): 7.833

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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Abstracts related to this study

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Other Abstracts:

I. Wéber, A. Birinyi, O. Shupliakov, M. Antal. 2004. Colocalization of zinc with GABA and glycine in the spinal cord. Clinical Neuroscience 57/1: 72.

E. Wolf, A. Stelescu, **I. Wéber**, A. Dityatev, A. Birinyi. 2004. Postsynaptic factors that may control size of propriospinal single fibre EPSPs in lumbar motoneurons of frogs. SYMBIONIC, Computational Systems Biology of the Neural Cell, Trieste, Italy.

E. Wolf, A. Stelescu, **I. Wéber**, A. Dityatev, A. Birinyi. 2004. Non-linear summation of postsynaptic potentials and morphoelectrotonic differences in positions of synaptic contacts differentiate propriospinal synapses with small and large amplitudes of EPSPs in lumbar motoneurons of frogs. FENS Abstr. A058.19, Lisbon.

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