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TRANSMITTER PHENOTYPES OF COMMISSURAL INTERNEURONS IN THE LAMPREY SPINAL CORD

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Abstract—The fundamental network for locomotion in all vertebrates contains a central pattern generator or CPG that produces the required motor output in the spinal cord. In the lamprey spinal cord different classes of interneuron's forming the core CPG circuitry have been characterized based on their morphological and electrophysiological features. The commissural interneuron's (C-INs) represent one essential component of CPG that have been implicated in controlling left–right alternation of the motor activity during swimming. However, it is still unclear if the C-INs displays a homogenous neurotransmitter phenotype and how they are distributed. In this paper we investigated the segmental distribution of glycine, glutamate and GABA-immunoreactive (ir) C-INs by combining retrograde Neurobiotin tracing with specific antibodies for these transmitters. The C-INs were more abundant in caudal and rostral segments adjacent to the injection site and their number gradually decreased in more distal segments, suggesting that these interneurons project over a short distance. The glycine-ir neurons represented around 50% of the total C-INs, while glutamate-ir neurons represented only 29%. Both types of C-INs were homogenously distributed over different segments along the spinal cord. Finally, no Neurobiotin labeled C-INs displayed GABA-ir, although many interneurons were ir to GABA, suggesting that GABAergic interneurons are not directly responsible for controlling left–right alternation of activity during locomotion in lamprey. Overall, these results show that the C-INs display a gradual rostrocaudal distribution and consist of both glycine- and glutamate-ir neurons. The difference in the proportion of inhibitory and excitatory C-INs represents an anatomical substrate that can ensure the predominance of alternating activity during locomotion.

Key words: central pattern generator, locomotion, spinal cord, retrograde tracer, neurobiotin, interneurons.

Various regions in the CNS are capable of generating orchestrated rhythmic activity underlying different motor behaviors. Muscle contractions responsible for locomotor movements such as swimming and walking are governed by neuronal networks located in the spinal cord (Grillner, 2003; Selverston, 2005; Kiehn, 2006). Although these networks are capable of generating rhythmic motor activity in the absence of sensory and descending inputs from the brain, their activity is initiated by specific regions in the brainstem and regulated by sensory feedback (Shik and Orlovsky, 1976; El Manira et al., 1997a,b; Jordan, 1998; Grillner and Wallen, 2002; Dubuc et al., 2008). One common feature of the locomotor pattern is the left–right alternation of motor activity that is mediated by inhibitory commissural interneurons (Buchanan, 1982; Roberts et al., 1998; Grillner, 2003; Kiehn, 2006). Blockade of this reciprocal inhibition induces synchronous activity in left and right motoneuron pools, suggesting the existence of excitatory interneurons that couple the two sides of the spinal cord.

In the lamprey, the locomotor network is composed of ipsilateral excitatory and crossed inhibitory interneurons (Grillner and Wallen, 1980; Buchanan, 1982; Cohen and Harris-Warrick, 1984; Buchanan and Grillner, 1987; McPherson et al., 1994; Buchanan and McPherson, 1995; Grillner, 2003). Rhythmic motor activity is generated by a network of excitatory interneurons in hemicord preparations, while crossed inhibitory interneurons seem to be only responsible for producing the left–right alternation during locomotion (Cohen and Harris-Warrick, 1984; Buchanan, 2001; Cangiano and Grillner, 2005). These commissural interneurons (C-INs) project both rostrally and caudally in the contralateral side and display a substantial morphological diversity with regard to their soma size and the extent of their axonal projections (Buchanan, 2001). Both small and large diameter C-INs has been shown to mediate glycinergic inhibition to contralateral neurons and undergo rhythmic membrane potential oscillations during locomotor activity (Ohta et al., 1991; Buchanan, 2001). Blockade of glycinergic inhibition transforms the alternating motor pattern into synchronous activation on both sides of the spinal cord that is likely to be mediated by an interaction via crossed excitatory interneurons (Cohen and Harris-Warrick, 1984). However, little is known about the relative proportion of inhibitory versus excitatory commissural interneurons and their distribution along the spinal cord.

In this study, we have used retrograde tracing experiments in combination with immunohistochemistry to determine the transmitter phenotype of both rostrally and caudally projecting commissural interneurons and their distribution along the spinal cord. Our results show that the majorities (50%) of these C-INs are glycine-immunoreactive (ir) and only 29% are glutamate-ir, while none of them display GABA-ir. These results provide evidence for the existence of a difference of the transmitter composition of crossed interneurons in the lamprey spinal cord that might

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Abbreviations: C-INs, commissural interneurons; CPG, central pattern generator; HEPES, N-hydroxyethylpiperazine-N-ethane sulfonic acid; ir, immunoreactive; PB, phosphate buffer; SD, standard deviation; SEM, standard error of the mean; VGLUT, vesicular transporter for glutamate.

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constitute a substrate for the prevalence of alternating activity during locomotion.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on adult river lampreys (*Lampetra fluviatilis*, $n=29$, body length: 30–35 cm). The animals were kept in fresh aerated water maintained at 8 °C. All experimental procedures were approved by the Ethical Committee, Stockholm according to the Swedish regulations for the care and use of laboratory animals. Prior to dissection, the animals were deeply anesthetized through immersion in fresh water containing tricaine methane sulfonate (MS 222, 100 mg/L; Sigma Aldrich, Stockholm, Sweden). Care was taken to minimize the number of animals used and their suffering.

Retrograde tracing

The spinal cord with the underlying notochord was exposed by removing tissue covering the dorsal side of the cord. The preparation was then transected behind the gill region up to the beginning of the dorsal fin. The preparation was pinned down in a Sylgard-lined (Dow Corning, Midland, MI, USA) dish and perfused with cooled (8–12 °C) Ringer's solution with the following composition (in mM): 138 NaCl, 2.1 KCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, four glucose, and two N-hydroxyethylpiperazine-N-ethane sulfonic acid (HEPES). The solution was bubbled with O_2 for 15–20 min and the pH adjusted to 7.4 with NaOH. A vertical cut was made with the tip of a thin bored needle from near to the midline up to lateral aspect of the ipsilateral side of spinal cord in the middle of a 20 segment long spinal cord. The level of the ventral root was taken as a segmental starting point so that around 10 segments long spinal cord could be analyzed simultaneously both rostrally and caudally. Each ventral root was used to delineate the segmental boundary. The length of each segment were measured and noted for future reference when cells were counted after scanning in confocal microscopy. The mean segment length of 2.5 mm was used for our calculation. Care was taken so that the cut did not extend all the way to the edge of the spinal cord or cross to the contralateral side. A Vaseline border was made around the cut so that the tracer solution would not be taken up by the cut muscle fibers and eventually by the ventral roots. To retrogradely label the commissural interneurons that project contralaterally (C-INs), small crystals of Neurobiotin (Vector, Burlingame, CA, USA) was picked up at the tip of an edged tungsten pin and immediately applied at the injection site after cutting. The rest of the spinal cord was covered with tissue soaked in ringer to prevent it from drying up. Tissue was then washed with Ringer's solution to remove excess tracer and to wash away soaked tissue. The preparation was then kept in Ringer's solution for 48–72 h in a cold aerated room for the tracer to travel a considerable distance. At least two experiments could be performed on each animal because of the relatively large size of the spinal cord (20 segments) used in each experiment. After the transport of tracer three animals were used for whole-mount preparation and rest was processed for combined immunohistochemistry and tracer labeling.

Whole mount histochemistry

After the tracer transport, the meninx primitiva were removed from the spinal cord, which was isolated from the underlying notochord and pinned down in the Sylgard and immediately fixed in 4% formalin and 14% of a saturated solution of picric acid in 0.01 M phosphate buffer (PB, pH 7.4) for 2 h and washed 3×15 min in PB. The spinal cord was then incubated with streptavidin–Alexa 488 (dilution 1:200; Molecular Probes, Stockholm, Sweden) overnight to detect the Neurobiotin. The tissue was then dehydrated in ascending ethanol series (50, 70, 90, and 100%, 10 min each), transferred onto a microscope slide and cleared and embedded in methyl salicylate.

Immunohistochemistry and tracer labeling

Forty–eight to Seventy–two hours after tracer injection, the spinal cord was fixed for 12–14 h in appropriate fixatives depending on the antigen to be detected. Seven animals were used for detection of glycine- and glutamate-ir and 10 animals for GABA-ir. For glycine detection we used 4% formalin, 2% glutaraldehyde and 14% of a saturated solution of picric acid in PB. For glutamate and GABA detection we used 0.5% and 0.25% glutaraldehyde, respectively, with the same concentration of formalin and picric acid as for glycine. The different percentage of glutaraldehyde used here gave the best signal-to-noise ratio for unambiguous detection of each antibody. Samples were then cryoprotected with 30% sucrose in 0.01 M PB overnight or until the tissue sank in the bottom of sucrose solution. To facilitate cryosectioning each 20 segments long spinal cord were cut into five segments long pieces rostral and caudal to the neurobiotin injection site. To maintain the same experimental condition pieces from the same half (i.e. rostral or caudal) were embedded together in Tissue Tek (Sakura, Torrance, CA, USA) and 14 μm thick horizontal sections were cut with the cryostat and collected on Super Frost® (Menzel–Gläser) glass slides. Sectioned spinal cord were then used immediately or kept at −20 °C and processed later. All the slides were used within 1 week after sectioning. For co-localization of retrograde tracer Neurobiotin with glycine, glutamate or GABA antibody, the sections were first washed with PB, followed by preincubation in 1% bovine serum albumin (BSA), 0.3% Triton X-100 in PB (all antisera were diluted in this solution) for 30 min in order to block nonspecific binding of the secondary antibody. The primary antibody, either rabbit polyclonal anti-glycine (code number, 290; dilution, 1:800) (Dale et al., 1986; Ottersen et al., 1988; Davanger et al., 1991; Shupliakov et al., 1996), rat polyclonal anti-glycine (IG1002 ImmunoSolutions, Jesmond, New South Wales, Australia; dilution, 1:5000), rabbit polyclonal anti-glutamate (catalog No. AB133; Chemicon, Temecula, CA, USA; dilution, 1:600), rabbit polyclonal anti-GABA (catalog No. AB131; Chemicon, Temecula, CA, USA; dilution 1:1000), or mouse monoclonal anti-GABA (mAb 3A12; dilution 1:1000) (Matute and Streit, 1986) was then applied to the sections for 24–30 h. After thorough rinsing in PB, the sections were incubated overnight at 4–8 °C in a cocktail of streptavidin–Alexa 488 (dilution 1:400) for Neurobiotin and donkey anti-rabbit IgG conjugated to Rhodamine (Jackson ImmunoResearch; dilution 1:200) for rabbit polyclonal anti-glycine, anti-glutamate or anti-GABA. For mouse monoclonal anti-GABA, donkey anti-mouse conjugated to Rhodamine was used. For triple immunofluorescence, sections were incubated as before with streptavidin conjugated to Cy3 (Jackson ImmunoResearch; dilution, 1:200) for Neurobiotin, donkey anti-rabbit conjugated to Cy2 for glutamate and donkey anti-rat conjugated to Rhodamine for glycine. The sections were then washed three times in PB and, except for triple immunofluorescence, they were incubated with Neuro Trace® fluorescent Nissl's stain (N-21483, Molecular probes; dilution. 1:1000) for 2 h at room temperature to visualize neurons. They were subsequently washed in PB and mounted in glycerol-gelatin and analyzed under confocal microscopy.

Antibody specificity

Previous studies have shown that the primary antibodies used in our studies recognize exclusively glycine (Dale et al., 1986; Ottersen et al., 1988; Davanger et al., 1991; Shupliakov et al., 1996), glutamate (Billig et al., 2003) or GABA (Sigurjonsson et al., 2005). As a further specificity control the primary antibody, they were omitted from control sections and no immunoreactivity was de-