

Serotonin-Related Enhancement of Recovery of Hind Limb Motor Functions in Spinal Rats after Grafting of Embryonic Raphe Nuclei

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ABSTRACT

Recently, we demonstrated improvements in hind limb locomotor-like movements following grafting of embryonic raphe nuclei cells into the spinal cord below the level of total transection in adult rats. The purpose of the present study was to clarify whether this improvement was due to newly established serotonergic innervation between the graft and the host. Two months after intraspinal grafting of the embryonic raphe nuclei, the spinalized rats, when put on a treadmill, could be induced to walk with regular alternating hind limb movements with the plantar contact with the ground during the stance phase, and ankle dorsiflexion during the swing phase of each step cycle. In the same situation the spinal rats, that did not receive the graft, were not able to initiate the dorsiflexion of the ankle joint during the swing phase and very often the dorsal surface of the foot was dragged along the ground. Intraperitoneal application of directly acting 5-HT₂ antagonist Cyproheptadine (1 mg/kg) impaired reversibly the hind limb locomotor-like movements in grafted rats. This impairment lasted for 2–3 h. The same procedure in control rats did not markedly alter the hind limb locomotor-like movements. The effect of Cyproheptadine in grafted rats was reversed by i.p. injections of the 5-HT₂ agonist Quipazine (0.5 mg/kg). These results show that the graft-induced restitution of hind limb locomotor abilities in adult spinal rats is brought about by the new serotonergic innervation of the host spinal cord circuitry from the grafted neurons and is mediated by 5-HT₂ receptors.

Key words: EMG; graft; locomotion; rats; serotonergic innervation; spinal cord

INTRODUCTION

IN MAMMALS, extensive spinal cord injury induces severe loss of motor and sensory function in the part of the body controlled by the central nervous system below the lesion. These deficits could be reduced in

adult rats by intraspinal grafting of embryonic raphe nuclei. Animals that, 1 week after total spinal cord transection, received such grafts below the place of lesion demonstrated significant improvements in hind limb locomotor-like movements (Yakovleff et al., 1995; Feraboli-Lohnherr et al., 1997; Ribotta et al., 2000). More-

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over, even when the grafting was carried out several weeks after spinal cord injury, the grafts encouraged the recovery of hind limb motor function in the adult rats (Sławińska et al., 2000a).

Our previous results showed that intraspinal transplantation of embryonic tissue containing a defined group of neurons into the spinal cord below the level of total transection was able to influence its residual circuitry (which includes CPGs for locomotion and motoneurons to hind limb muscles) to improve the recovery of motor function of hind limbs (Sławińska et al., 2000a). The exact mechanisms by which grafted neurons influence the spinal CPG and also how the host neurons act on the grafted neurons are still unknown. The purpose of the present study was to clarify the contribution of the serotonergic innervations supplied by the grafted cells to the host spinal cord in the improvement of hind limb motor function. In order to establish the nature of the interaction between the graft and the host spinal cord, we examined the effects of agents that interfere with serotonergic transmission. We examined the effects of serotonergic agonists and antagonists on the hind limb locomotor functions of spinal rats that had received a graft of embryonic raphe nuclei into the spinal cord below the level of total transection 1 month after injury.

The crucial roles of serotonergic innervations in recovery of locomotor hind limb movements in spinal animals have been demonstrated in many studies. It might be that this effect of serotonergic innervation is mediated by 5-HT₂ receptors. These receptors play a major role in the direct depolarization of motoneurons (Elliot and Wallis, 1992; Jackson and White, 1990; Lindsay and Feldman, 1993; Wang and Dun, 1990), and 5-HT₂ agonists potentiate ventral root reflexes in acute spinal rats (Yamazaki et al., 1992) and cats (Miller et al., 1996). Moreover, systemic administration of 5-HT₂ agonist (Quipazine) increases the step length, the amplitude of EMGs from hind limb extensor and flexor muscles in cats spinalized as adults (Barbeau and Rossigol, 1990, 1991; Edgerton et al., 1997), and long-term treatment with Quipazine (subdural) clearly induces a progressive improvement of locomotor capabilities in spinal rats (Antri et al., 2002).

In a previous study (Ferraboli-Lohnherr et al., 1997) concerning the role of transplanted serotonergic cells in enhancing the restoration of hind limb movement, it was demonstrated that the use of re-uptake inhibitors of serotonin (zimelidine) increases the amplitude of action potentials in sciatic nerve branches during fictive locomotion. This is consistent with the hypothesis that reestablished serotonergic innervation is able to increase the excitability of spinal cord network. However, there is still no evidence whether blocking serotonergic trans-

mission will alter the graft-induced restored function. We therefore examined the action of a 5-HT₂ receptor antagonist (Cyproheptadine) and agonist (Quipazine) on the locomotor function of hind limbs that had been restored by a graft of serotonergic cells. Thus, the goal of our investigation was to test the possibilities that the restoration of locomotor-like hind limb movements in spinal rats that have received a graft of serotonergic cells was mediated by the 5-HT₂ receptor subtype. The behavioral investigations were combined with simultaneous electromyographic recordings from the extensor and flexor muscles of the hind limb ankle joint. Preliminary results from these experiments have been presented in abstract form (Sławińska et al., 2000b).

MATERIALS AND METHODS

The project was approved by Local Ethics Committee at the Nencki Institute and followed EU guidelines of animal care. Adequate care was taken to minimize pain and discomfort.

Host Surgery

The experiments were performed on 11 3-month-old Wistar rats in which, under deep Equithesine anesthesia (3 mL/kg, i.p.) and sterile conditions, the spinal cord was completely transected at low thoracic level (T9). To prevent possible axonal regrowth through the cavity of lesion, 2–3 mm of spinal cord tissue was gently aspirated using a glass pipette. Then the fascia overlying the paravertebral muscles was closed using sterile sutures (MERSILK 6/0), and the skin was closed with stainless-steel surgical clips. An adequate level of anesthesia during surgery was ensured by regular testing for the lack of cutaneous withdrawal reflexes of the forelimbs. After surgery, the animals received a non-steroidal anti-inflammatory and analgesic treatment (Tolfedine 0.4 mg/100 g). All animals recovered from anesthesia 2–3 h after surgery. During the following 10 days, the animals were routinely given antibiotics (Gentamicin 0.2 mg/100 g and Oxacilline 0.3 mg/100 g). During this postoperative period, the bladder was emptied manually twice a day until the voiding reflex was re-established.

Dissection of Embryonic Raphe Nuclei

Embryos were obtained from the time-mated female rats of the same inbred strain as the hosts (Wistar). Fourteen-day-old embryos (E14; the day following mating is designated as embryonic day zero—E0) were removed by cesarean section from anesthetized pregnant rats and transferred to Hanks' buffered solution enriched with

0.5% glucose. This time period corresponds to the stage when most neurogenesis in the raphe nuclei is completed. For transplantation, a small piece of the raphe region was dissected under the microscope by cutting out a rectangular block of neural tissue 0.3 mm to either side of the midline (Fig. 1).

Grafting

One month after spinal cord transection, five animals selected randomly were anaesthetized with Equithesin (0.3 mL/100 g, i.p.), and the spinal cord was exposed by a small laminectomy at T12 level, making a small opening in the dura mater to perform the embryonic tissue transplantation (Fig. 1C). A needle connected to a Hamilton microsyringe containing a solid piece of embryonic tissue (approximately 2 μ L) was inserted with a micromanipulator into the distal stump of the spinal cord below the section. The embryonic tissue was injected into the spinal cord medially, 1 mm below the pial surface. After the end of pressure injection of the embryonic tissue, the needle was slowly withdrawn to avoid the graft movement. The muscles of the back and the skin were

then sutured. Six other spinal animals were sham operated. Their spinal cords were exposed, and a needle attached to a micromanipulator was inserted into the distal stump of the spinal cord below the section, but no graft was injected. These rats were kept for further investigation as a control group. To prevent infection, all the animals were treated with antibiotics for the following 7 days (Gentamicin 0.2 mg/100 g and Oxacilline 0.3 mg/100 g).

Implantation of EMG Recording Electrodes

Two months after spinal cord transection, the bipolar EMG recording electrodes were implanted in the soleus and tibialis anterior muscles of both hind limbs. The electrodes were made of Teflon-coated stainless-steel wire (0.24 mm in diameter; AS633, Cooner Wire, Chastworth). The tips of the electrodes with 1–1.5 mm of the insulation removed were pulled through a cutaneous incision at the back of animal, and each of the hook electrodes was inserted into the appropriate muscle, where it was secured by a suture (Hnik et al., 1978; Sławińska et al., 1995, 1998). The distance between the tips of electrodes was 1–2 mm. The ground electrode was placed under the skin on the back of the animal at some distance from hind limb muscles. The connector with the other ends of the wires fixed to it, covered with dental cement (Spofa Dental) and silicone (3140 RTV, Dow Corning), was secured to the back of the animal. A wire loop left under the skin on the back of the animal prevented the electrodes from being pulled out from the muscles during movements. EMG recordings started 3–5 days after electrode implantation. Electrode position in the muscles was verified visually after the animals were sacrificed.

Testing of Recovery of Motor Function

To study recovery of motor function, the locomotor pattern of hind limb movements was investigated 3 months after spinal cord transection (i.e., 2 months after grafting in spinal grafted animals or sham grafting in spinal control animals). This time was chosen because, after this interval, no further improvement in hind limb locomotor function of paraplegic rats with intraspinal grafts can be observed (K. Maleszak, unpublished observation).

The hind limb locomotor abilities were examined in animals with their forelimbs placed on a special platform above the treadmill (Letica Scientific Instruments, Barcelona) and its hind limbs touching the moving belt. Under such experimental conditions, an additional stimulation (pinching of the rat tail) elicited in spinal rats (particularly in those with a graft) hind limb locomotor-like movements. Locomotor abilities of hind limbs were scored

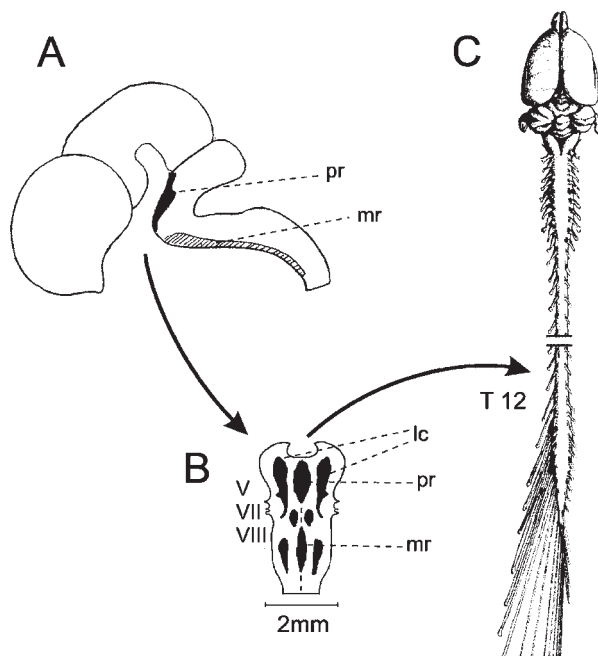


FIG. 1. Schematic presentation of raphe nuclei dissection and method of embryonic tissue transplantation into the spinal cord. (A) The upper part of embryonic nervous system. (B) The embryonic brain stem. (C) The spinal cord of adult rat and the place of transplantation. pr, pontine raphe nucleus; mr, medullary raphe nucleus; lc, locus coeruleus; V, VII, VIII, consecutive cranial nerves; T12, the thoracic spinal cord level where the graft was injected.

relatively to those obtained in the normal adult rats; however, it must be noted that hind limb locomotor-like movements were not occurring spontaneously but were induced by tail pinching. Recently, the BBB (Basso, Beattie, Bresnahan) scale has become the most frequently used method for analyzing the functional performance of animals after spinal cord injury during spontaneous open field locomotion (Basso et al., 1995, 1996a,b; Muir and Webb, 2000; Antri et al., 2002, 2003). The BBB test was designed to evaluate motor deficits following partial lesions of the spinal cord. The original BBB scale ranges from 0 (no observable hind limb movements) to 21 points, which includes fore hind limb coordinated plantar walking with trunk stability and erected tail position. However, the assessment of fore hind limb coordination, trunk stability, and tail position is not applicable in animals with complete spinal cord transection, and locomotor tests should involve hind limb movements only. Moreover, in our experiments, the forelimbs were kept motionless on a platform above a moving treadmill belt, and the locomotor-like movements of hind limbs were initiated by tail pinching. Therefore, in order to analyze the hind limb locomotor-like movements we used the modified BBB scale that was developed by Antri et al. (2002, 2003). Here we present only a brief description, as it was presented in a previous paper (Antri et al., 2003). Motor performance is scored on a 22-point scale overlapping four distinct levels. Scores of 0–1 correspond to either the absence of any movement or some weak movements, scores of 2–9 indicate an improvement in right-left alternation with weak or large amplitude movement, score 10 describes a clear large amplitude movement and a small capability for body weight support, and finally, scores of 11–22 correspond to an increase in body weight support and an improvement in plantar foot placement and right-left alteration.

All the experimental animals were observed independently and scored by two observers (one was “blind”). Care was taken to score the most stable dominant behavior during the 2-min locomotor test. The scores obtained in animals of both groups were compared using nonparametric Wilcoxon-Mann-Whitney test.

Drug Injections

After completing the data concerning the level of locomotor-like hind limb recovery induced by intraspinal grafting of serotonergic embryonic tissue, the effect of the drugs on the walking pattern was evaluated. To avoid any cumulative effect of pharmacological treatment, the 5-HT₂ antagonist (Cyproheptadine) or agonist (Quipazine) was applied in each animal in a single experiment. Before drug application, the hind limb locomotor abilities were tested on a treadmill to determine the predrug baseline performance, which might vary with time after the

lesion. Then the evaluation of hind limb motor performance was carried out at 30 min after drug injection (i.e., when the maximal effect of the drugs administration was usually observed) and was continued for 3 h. The effect of the antagonist drugs generally lasted for at least 4–5 h. The effects were assessed at three various speeds (5, 10, 20 cm/sec) of the moving belt at various time intervals after drug application. In the case of testing the effects of the antagonist and agonist in the same experiment, the evaluation of locomotion was performed following the injection of the second drug, 30 min after the injection of the first drug. Generally, each pharmacological experiment performed on a single animal was separated by at least 72 h to prevent interaction between drugs.

Electromyographic Analysis

During the hind limb locomotor-like movement testing, the EMG activity of two muscles, extensor-soleus (Sol) and flexor-tibialis anterior (TA) of both hind limbs, was simultaneously recorded on the V-store Racal magnetic recorder (0.1–1 kHz band pass) and evaluated later off-line after digitization on the computer (2 kHz sampling frequency). The amplification of the recorded EMG signal was kept at the same level during the whole experiment. This enabled us to compare the amplitude of the raw EMG signals recorded during various experimental conditions when the locomotor abilities were altered by the 5-HT antagonist or agonist treatment. In order to analyze the differences obtained during these experiments, the mean of the maximal amplitude of the burst EMG activity taken from approximately 50 consecutive step cycles for the soleus muscles was compared before and after pharmacological treatment using Student's *t*-test. The maximal amplitude of the EMG burst was established as a difference between the maximum and minimum of the raw EMG signal.

For the analysis of EMG activity pattern of the extensor and flexor muscles during locomotor-like movement, the following parameters were assessed semiautomatically: burst duration (BD), defined as the time between the onset and the offset of an EMG burst; and step cycle duration (SCD), defined as the time elapsed between onsets of two consecutive EMG bursts of the same muscle.

The relations between the burst duration and the step cycle duration were examined using the method of linear regression (least-squares method). The significance of the correlation coefficients was tested using Pearson test.

RESULTS

None of the rats with the spinal cord totally transected was able to support the body weight during spontaneous

movement around the home cage, irrespective of whether or not it received a graft of embryonic raphe nuclei containing serotonergic cells into the spinal cord below the level of injury. They all moved around using their forelimbs, while the body and hindquarters were dragged behind them. However, there was a difference in the position of hind limbs in spinal rats with or without a graft. Spinal rats without a graft, when sitting motionless, kept their hind limbs extended, while spinal rats with a graft kept their hind limbs often in flexion in the knee and ankle joints. Although, in rats with a graft, the plantar surface of both hind limbs contacted the ground, the body weight support and the standing posture was generally absent. It is important to note that, in all animals that have received intraspinal graft, the survival of the serotonergic neurons and the presence of their axons in the distal part of the transected spinal cord was verified with the immunocytochemical staining for serotonin (Sławińska et al., 2000a).

The locomotor-like movements were tested in animals with their hind limbs touching the moving belt of a treadmill, while their forelimbs and forequarters were kept on a platform about 3 cm above the belt. In grafted rats, unlike in spinal control animals (without a graft), pinching of the tail evoked the extension in hind limbs, putting them into convenient position to initiate locomotion. The continued pinching of the tail induced the rat hind limbs to carry out the prolonged episodes of well-coordinated alternating movements with the pelvis lifted off the

ground. None of the spinal animals without a graft was able to do this. The hind limb movements were tested at various speeds of the moving belt: 5, 10, 20 cm/sec. The behavioral observation indicated that, in all experimental animals, the pattern of locomotor-like hind limb movements depended on the speed of the moving belt. The best locomotor performances were obtained for 5 and 10 cm/sec, while at the speed of 20 cm/sec even the grafted animals were not able to present the proper plantar walking. Figure 2 shows consecutive video frames taken during locomotion of spinal control animal (A) and a spinal grafted animal (B) on the moving treadmill belt at the speed of 10 cm/sec. Visual inspection of the consecutive video frames taken during hind limb locomotor behavior of spinal grafted rats allowed us to recognize stance and swing phases within each step cycle in locomotion on the basis of the contact of the paw with the ground (Fig. 2B). Unlike spinal grafted rats, the spinal control animals without a graft were unable to dorsiflex the ankle joint before lowering the paw on the ground (Fig. 2A). Most of the time their hind limb locomotor-like movements were limited to the proximal joints (hip and knee) while the ankle joint was not involved, so the feet were dragged with the dorsal surface of the paw touching the ground.

Pharmacological Interference

Hind limb locomotor-like movements of spinal grafted rats were markedly impaired after i.p. injections of the

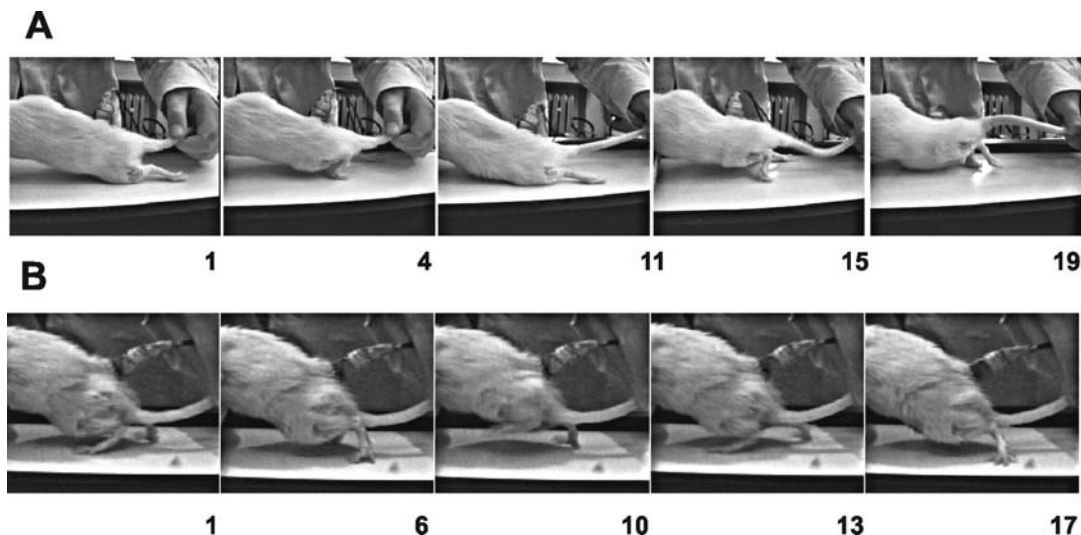


FIG. 2. A consecutive video frames (the number of each frame out of a consecutive series at 30 frames/sec is shown) taken during locomotor-like hind limb movements on a treadmill (the speed of moving belt, 10 cm/sec) 3 months after spinal cord transection of one spinal rat without (A) and one spinal with (B) a graft. Note the landing of the paw on the ground. The locomotor-like hind limb movements in the spinal rat are limited only to proximal joints (hip and knee), while in the spinal grafted rat all three joints are involved and plantar walking of hind limbs is well pronounced.

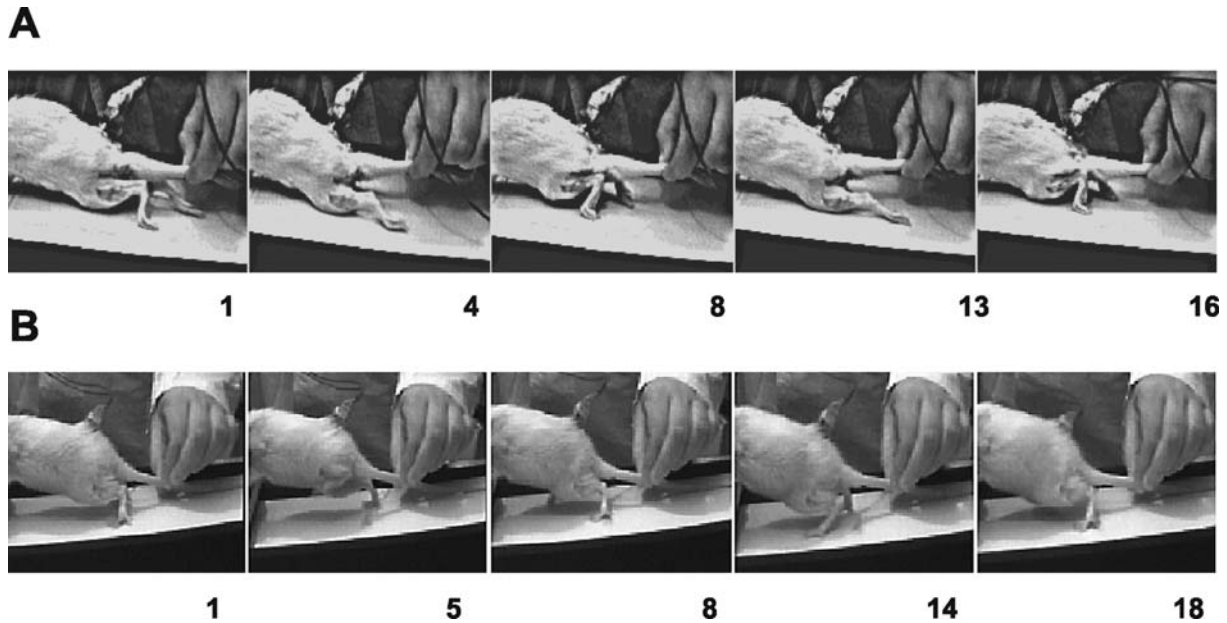


FIG. 3. A consecutive video frames taken from spinal rat with a graft (the number of each frame out of a consecutive series at 30 frames/sec is shown) during locomotor-like hind limb movements on treadmill with the moving belt at the speed of 10 cm/sec altered by Cyproheptadine (0.5 h after treatment) (A) and restored by Quipazine (1 h after Cyproheptadine and 0.5 h after Quipazine treatment) (B).

5-HT₂ antagonist Cyproheptadine (1 mg/kg) (Fig. 3A). The impairment lasted 2–3 h. Figure 4 illustrates the time course of changes in the scores of the hind limb locomotor-like movements after Cyproheptadine treatment. Before pharmacological treatment, the spinal grafted rats could be induced to walk with a regular alternating plantar walking pattern, and in the majority of animals the score was 16 or more. At 30 min after Cyproheptadine administration, a significant deterioration of the locomotor movement was visible (Wilcoxon-Mann-Whitney test; $p < 0.05$). In most animals, the score dropped from 16 or more to 3–5. The hind limb movement was limited to proximal joints (hip and knee), and the plantar walking was absent. In some animals, such alterations were present even 3 h after treatment. In spinal control animals, i.p. injection of Cyproheptadine did not have any significant effect on the hind limb locomotor movements, and in most animals, the score remained almost at same level as before pharmacological treatment, that is, 5–7 (Wilcoxon-Mann-Whitney test; $p > 0.05$). Although the locomotor-like movements in grafted rats significantly deteriorated after Cyproheptadine treatment, in some animals scores were still higher compared to those seen in control ungrafted animals, suggesting that the 5-HT system may not be the only one engaged in restoration of hind limb motor functions.

In the next experiment, the Cyproheptadine administration was followed 30 min later by an i.p. injection of

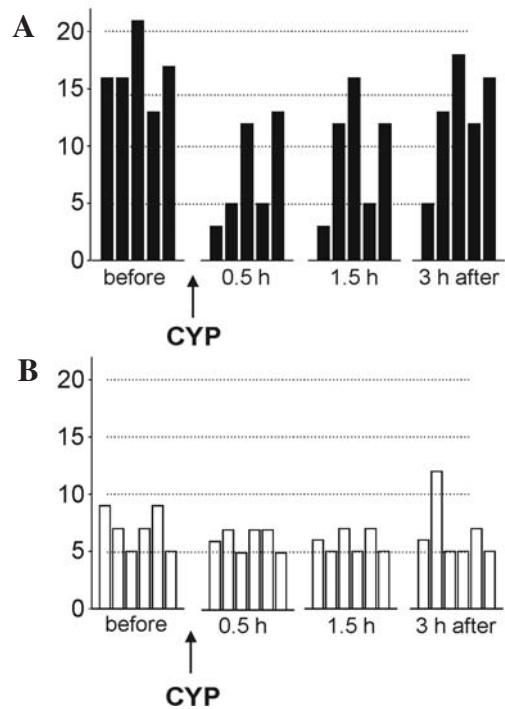


FIG. 4. Time course of changes in scores describing hind limb locomotor-like movements after Cyproheptadine (1 mg/kg) treatment. (A) Black bars show results from the spinal rats that received the graft of embryonic raphe nuclei. (B) White bars show results from the spinal-control rats. Each bar shows the results obtained in one rat.

Quipazine (0.5 mg/kg), an agonist of 5HT₂ receptors. Before pharmacological treatment, the spinal grafted rats could be induced to walk with regular alternating planar walking pattern, and in all of the animals, the score was higher than 15 (Fig. 5A). At 30 min after Cyproheptadine administration, a significant alteration of the locomotor movement was visible (Wilcoxon-Mann-Whitney test; $p < 0.05$). In all the grafted animals, except for one rat, the score of locomotor-like hind limb movements did not exceed the score of 9 (Fig. 5A). Injection of Quipazine reversed the motor performances of the animals, so that in most of the grafted rats the score was higher than 14 (Wilcoxon-Mann-Whitney test; $p < 0.05$). The Quipazine treatment improved the locomotor performance (Fig. 3B) that had been previously impaired by Cyproheptadine, so that the animals could walk as before any pharmacological intervention (Wilcoxon-Mann-Whitney test; $p > 0.05$). In spinal rats without a graft, no significant effect of pharmacological treatment on hind limb movements was obtained (Wilcoxon-Mann-Whit-

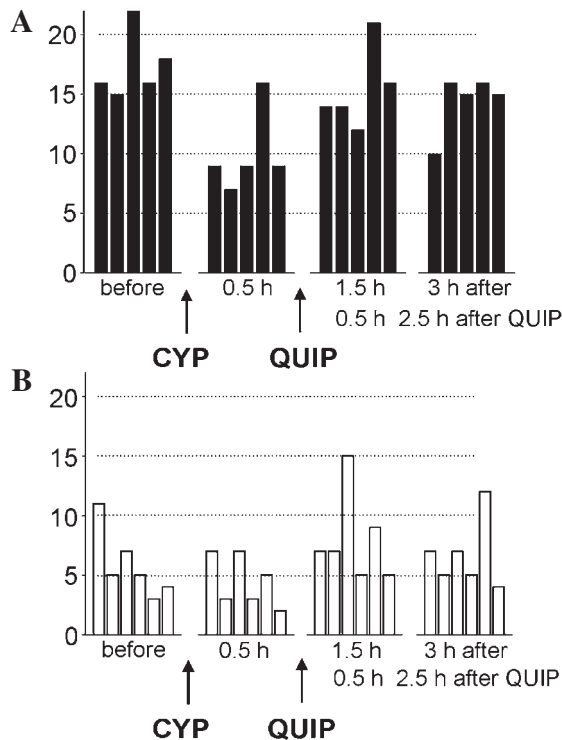


FIG. 5. Time course of changes in scores describing hind limb locomotor-like movements after pharmacological treatment with Cyproheptadine (1 mg/kg) and 0.5 h later Quipazine (0.5 mg/kg). (A) Black bars show results from the spinal rats that received the graft of embryonic raphe nuclei. (B) White bars show results from the spinal-control rats. Each bar shows the results obtained in one rat.

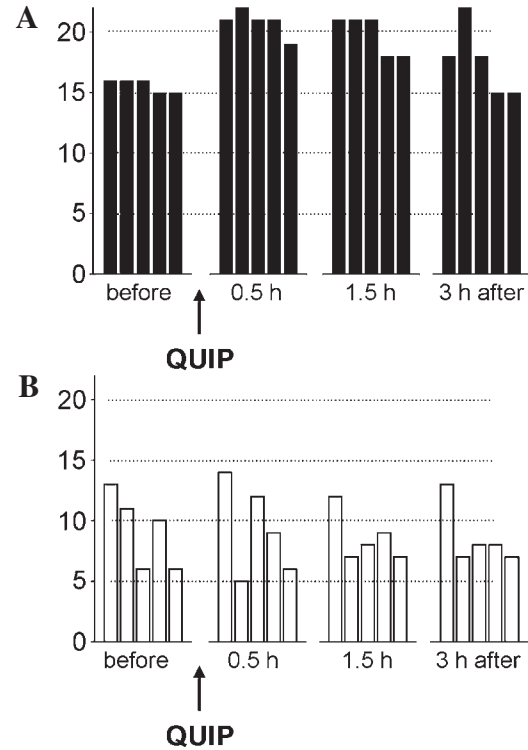


FIG. 6. Time course of changes in scores describing hind limb locomotor-like movements after Quipazine (0.5 mg/kg) treatment. (A) Black bars show results from the spinal rats that received the graft of embryonic raphe nuclei. (B) White bars show results from the spinal rats. Each bar shows the results obtained in one rat.

ney test; $p > 0.05$) and the score remained in all animals, except one, within the range of 4–7 points.

In a separate experiment, the effect of the administration of the 5HT₂ agonist (Quipazine) indicated that there was an improvement (Fig. 6) of locomotor function of hind limbs in the group of spinal animals with a graft (Wilcoxon-Mann-Whitney test; $p < 0.01$). In animals without a graft, only in two rats did the Quipazine application improve the locomotor-like hind limb movements, but the statistical verification performed for the whole control group did not show significant changes (Wilcoxon Mann-Whitney test; $p > 0.05$).

Electromyographic Investigation

The behavioral investigations were combined with simultaneous recording of EMG from the Sol and TA muscles of both hind limbs. The EMG recordings allowed us to use the objective criteria such as step cycle duration and the burst activity duration to describe the hind limb motor performance of the respective muscles moving the ankle joint. Figure 7 shows examples of EMG activity recorded

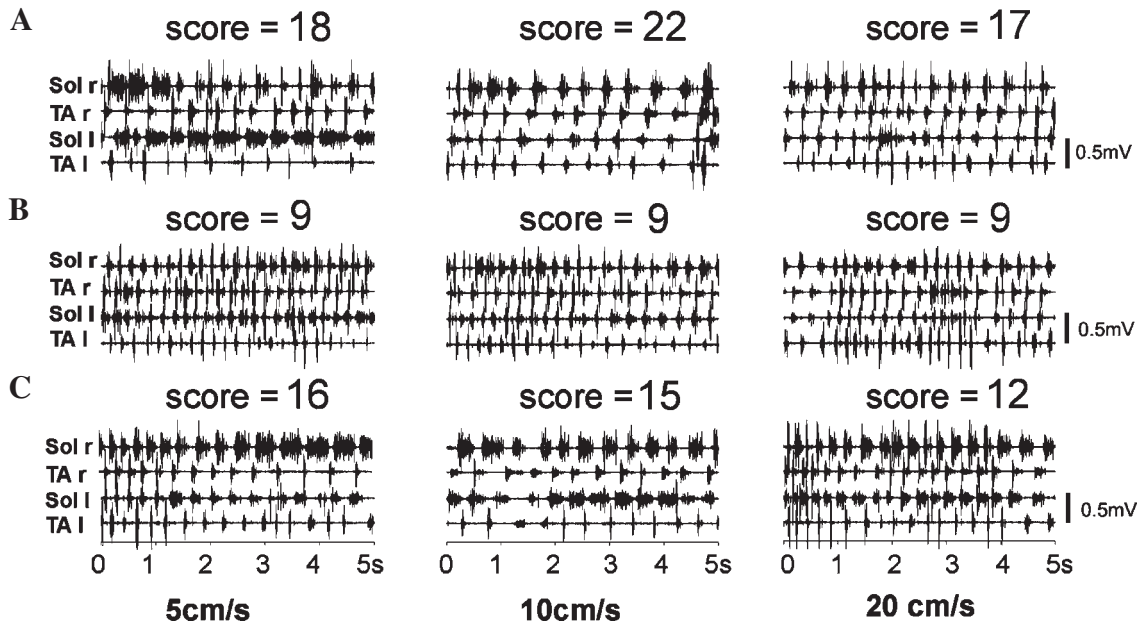


FIG. 7. Examples of EMG activity of two muscles, soleus (Sol) and tibialis anterior (TA) of both hind limbs (L/r: left/right), recorded during locomotor-like movements with graft-enhanced recovery of hind limb motor function when the hind limbs were put on the moving belt of a treadmill (at various speed: 5, 10, 20 cm/sec). The motor-like movements was induced by pinching the tail. The scores indicate the level of correctness of the hind limb locomotor-like movements. (A) Before pharmacological treatment. (B) At 0.5 h after Cyproheptadine treatment. (C) At 1 h after Cyproheptadine and 0.5 h after Quipazine treatment. Above each trial of EMG recordings, the score of corresponding locomotor-like hind limb movements is indicated. On the bottom, the various speeds of the moving belt are indicated.

during locomotor-like activity of hind limbs at three speeds of the treadmill (5, 10, 20 cm/sec) of one rat in which the recovery of motor functions was enhanced by grafting of embryonic tissue. The proper alternating pattern of EMG activity demonstrates the long burst of extensor muscle activity as well as a short burst of flexor muscle activity of both hind limbs that illustrate the plantar walking pattern that was obtained in this animal during the test performed before treatment with pharmacological agents (the score 18, 22, or 17 for various speeds of the moving treadmill belt; Fig. 7A). After Cyproheptadine treatment, the locomotor-like hind limb movements had deteriorated (the score was 9). This deterioration was related to the shortening of the extensor burst duration (Fig. 7B). Moreover, in some animals the deterioration of the locomotor pattern was associated with a decrease of the step cycle duration, which implicates an increase of the step cycle frequency (statistically significant only in two animals). Figure 8A shows that, before pharmacological treatment in grafted rats, the burst duration of EMG activity of extensor muscle (Sol) was positively correlated with the step cycle duration, while the burst duration of flexor muscle EMG activity (TA) was almost constant for various step cycle duration. After Cyproheptadine treatment, the relationship

between the burst duration of the Sol muscle activity and the step cycle duration was altered (Fig. 8B). This alteration was due to a significant shortening of the burst duration of Sol muscle and was confirmed by the analysis of variance that indicated that the slopes of the regression lines established after pharmacological treatment were statistically different from those of the pre-drug analysis (analysis of variance $p < 0.001$). The Quipazine treatment reversed the burst duration as well as the step cycle duration to the level obtained before pharmacological treatment (Fig. 8C). Figure 9A illustrates the shortening of Sol burst duration in four (out of five) rats. In three of them, the shortening of the bursts in the Sol was associated with lengthening of burst duration of the TA (Fig. 9B). This indicates that Cyproheptadine treatment reduced dramatically the extensor activity, although the step cycle duration was shortened significantly only in two rats (data not shown). Moreover, the analysis of changes in EMG amplitude after the pharmacological treatment demonstrated that, in most of the animals in which the recovery of motor functions was enhanced by grafting, the maximal amplitude of the burst EMG activity recorded during locomotor-like movements from Sol muscles was significantly reduced after Cyproheptadine administration (Fig. 10). All

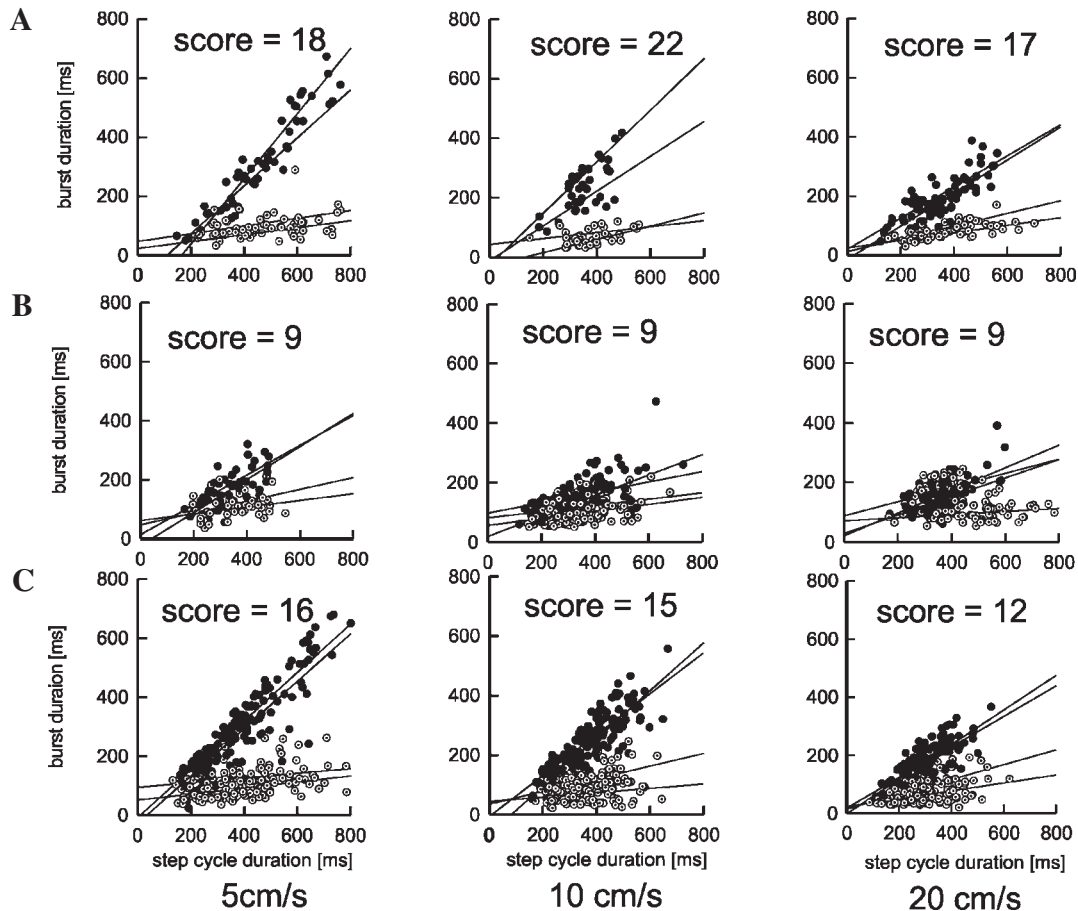


FIG. 8. Plots presenting the relationships between the EMG burst duration of two muscles, soleus (Sol) (●) and tibialis anterior (TA) (○), of both hind limbs and the step cycle duration calculated from the EMG activity recorded from one rat, as in Figure 7. (A) Before pharmacological treatment. (B) At 0.5 h after Cyproheptadine treatment. (C) At 1 h after Cyproheptadine and 0.5 h after Quipazine treatment. The scores indicate the correctness of the corresponding locomotor-like hind limb movements.

these changes in EMG activity were associated with the changes in motor performance that were illustrated by the scoring system (Fig. 5). This effect was reversed by Quipazine treatment (Fig. 5).

All the results of the EMG analysis were consistent for various speeds of the moving belt. Even at the speed of 20 cm/sec where some limitations in hind limb locomotor-like movements were noted, the EMG pattern and relationships between burst and step cycle duration were well pronounced (compare Figs. 7 and 8).

DISCUSSION

This study confirms our earlier findings that the recovery of hind limb locomotor functions after complete spinal cord transection is much better in rats that received a graft of embryonic brainstem raphe nuclei into the

spinal cord below the lesion than that without a graft. The graft of the raphe nuclei contained serotonergic cells. The EMG pattern of the flexor (TA) and extensor (Sol) muscles and the temporal correlations of the bursts of activity were dependent on the speed of locomotion and similar to those described in intact (Sławińska et al., 2000a; Sławińska and Kasicki, 2002) or decerebrate (Nicolopoulos-Stournaras and Iles, 1984) rats. However, in contrast to intact rats, the spinal rats had difficulty in adapting the step cycle duration to the fastest velocity of the moving belt (20 cm/sec). This finding confirms our behavioral observations that the grafting procedure was unable to support the full recovery of motor control in hind limbs of spinal animals. The present results also confirm our previous work (Sławińska et al., 2000a) that, even if carried out a long time after spinal cord injury (1 month post-injury), the transplantation of the neural cells from raphe nuclei improves the recovery of hind limb

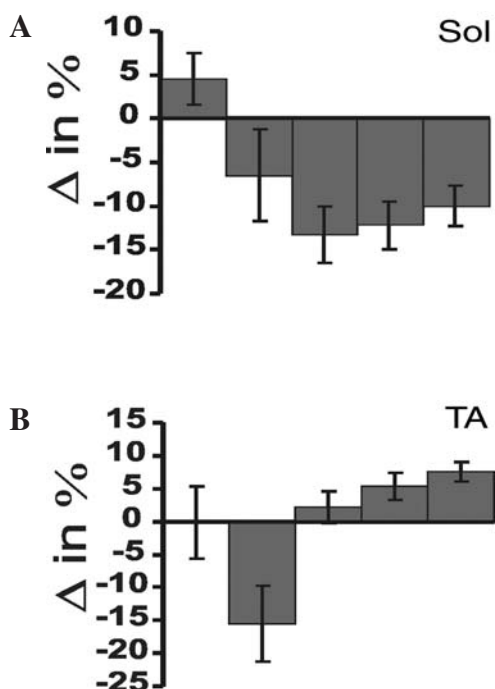


FIG. 9. Changes in the burst duration of Sol (A) and TA (B) muscles after Cyproheptadine administration calculated as $\Delta = \frac{BD_{after}}{SCD_{after}} [\text{in}\%] - \frac{BD_{before}}{SCD_{before}} [\text{in}\%]$ (i.e., a difference of the burst duration (BD) after and before treatment expressed as a percentage of the step cycle duration (SCD) in rats ($n = 5$) that have the recovery of hind limb motor function enhanced by a graft of embryonic raphe nuclei).

motor function. At 1 month after total transection of the spinal cord, the neuronal circuitry below the lesion is already reorganized, and changes in the effectors (i.e., motoneurons) are well advanced (Thompson et al., 1992). This indicates that, even a long time after spinal cord injury when the advanced changes in neural circuitry are present, the grafted serotonergic cells are able to become integrated into the host neuronal circuitry.

The improvements in hind limb motor functions were associated with the presence of serotonergic cells in the isolated part of the spinal cords of grafted animals (for a detailed description of the method of immunocytochemical verification used in all our animals, see Sławińska et al., 2000a). Thus, the grafted embryonic serotonergic cells are responsible for the enhancement of recovery of hind limb locomotor functions. This effect might be due to establishment of connections made by the grafted serotonergic cells with the spinal cord network of the host, particularly those involved in the control of hind limb locomotion, and such hypothesis was tested pharmacologically in the present paper.

The potential role of 5-HT in locomotor system activation, modulation, and functional recovery after spinal cord lesions has been intensively investigated over the past 30 years. It is well known that 5-HT increases the amplitude of lumbar monosynaptic reflexes (Anderson and Shibuya, 1966; Clineschmidt et al., 1971; White and Neuman, 1980). Moreover, specific agonists for serotonin applied directly depolarize motoneurons (Houngaard et al. 1988; White and Fung, 1989; Berger et al., 1992; Elliot and Wallis, 1992; Larkman and Kelly, 1992; Lindsay and Feldman, 1993; Miller et al., 1996; Takahashi and Berger, 1990; Wang and Dun, 1990). Stimulation of raphe-spinal pathways can induce plateau properties in motoneurons (Browstone and Hultborn, 1992). 5-HT may also indirectly influence motoneuron excitability by attenuation of both excitatory and inhibitory synaptic inputs to motoneurons (Wu et al., 1991; Yomono et al., 1992; Lindsay and Feldman, 1993; Umemiya and Berger, 1995). Moreover, transmissions from group I and/or group II muscle afferents and low-threshold skin afferents to various types of ascending tract neurons are modulated by serotonin and noradrenaline (Jankowska et al., 1997; 2000; Hammar et al., 2002).

In the present study, we have pinpointed the receptors through which these effects might be mediated. Our results demonstrated the impact of agonist and antagonists (acting directly at 5-HT₂ receptors) on the hind limb motor functions that had been restored due to the grafted cells. The axons of grafted serotonergic cells were probably growing and innervating those neural structures that express serotonergic receptors in the spinal cord and are

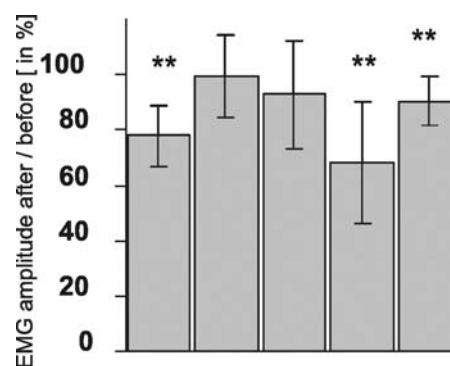


FIG. 10. The mean amplitude (\pm SD) of the max EMG burst activity recorded from the soleus muscles during locomotor-like hind limb movements after Cyproheptadine administration, expressed as a percentage of the mean amplitude of the max EMG burst activity of the same muscle before treatment in various rats ($n = 5$) that had the recovery of hind limb motor function enhanced by a graft of embryonic raphe nuclei (**Students *t*-test, $p < 0.001$).

responsible for modulation of locomotor activity. It was demonstrated by others that transplanted serotonergic neurons are able to reinnervate specifically the main target areas in the sublesional spinal cord with an innervation pattern similar to that of intact animals (Rajaofetra et al., 1992; Yakovlev et al., 1995; Dumoulin et al., 2000). The electron microscopic study showed 5-HT immunoreactive fibers establishing axodendritic and axosomatic synapses in transplanted spinal cord. It is likely that their effect on the locomotor behavior of the animal is mediated through these contacts. On the other hand it is also possible that the spontaneous release of serotonin from the grafted cells has a diffuse effect on the spinal cord circuitry. In either case, our results show that functional improvement induced by the graft is related to the action of serotonergic cells by the 5-HT₂ receptors on the neural circuitry present in the spinal cord of our grafted animals.

The descending serotonergic projections from raphe nuclei in normal rats are topographically arranged and innervate the dorsal and ventral horns of rat spinal cord from the cervical to the lumbar segments (Skagerberg and Björklund, 1985; Sur et al., 1996). Such distribution of 5-HT fibers in the spinal cord provides an anatomical basis for the involvement of serotonin in the control of nociception (Ruda, 1988) and motor neuron activity (Crick and Wallis, 1991; Elliot and Wallis, 1992). The serotonin recognized sites in the brain and spinal cord can be divided into a number of receptor subtypes: 5HT₁ family, 5HT₂ family, 5HT₃ and others (Peroutka, 1994, 1995). In the spinal cord the distribution of various 5HT receptor subtypes has a high specificity. The 5-HT_{1a} and 5-HT_{1b} are mainly located in the dorsal horn while their levels in the ventral horn is rather low (Thor et al., 1993; Kia et al., 1996). The 5-HT_{2c} receptors are mainly located in the ventral horn (Fone et al., 1991; Thor et al., 1993; Sur et al., 1996). The 5-HT₃ receptors were found in high density in the dorsal horn as well as in motor neuron nuclei (Kia et al., 1995; Morales et al., 1996). In addition, the subtype of 5-HT receptor expressed by the target neuron determines whether 5-HT display excitatory or inhibitory effects. In animal models, the functional correlates for a number of these receptor subtypes can be identified. There is now evidence to suggest that some motor behavior (the head shakes in rats, the head twitch in mice) is mediated by 5-HT₂ receptors (Berendsen et al., 1991). Moreover, the 5-HT₂ receptors are involved in the recovery of hind limb locomotor movement in the chronic spinal rat (Antri et al., 2002). Recently it was demonstrated that also 5-HT_{1A} receptors might play a significant role in processes responsible for locomotor function recovery (Antri et al., 2003). However, the 5-HT_{1A} receptor activation of the spinal network responsible for hind limb locomotor movement is

controversial because it has been shown that application of antagonists of this receptor subtype could block fictive locomotion in the spinal cord neonatal preparation (Cazalets et al., 1992).

Although it is well known that the 5-hydroxytryptophan (5-HTP; precursor of 5-HT) potentiates spinal reflexes in both intact (Ahlman et al., 1971) and spinal animals (Grillner, 1981; Nozaki et al., 1977), the effects of serotonergic drugs on locomotion are less clear. In the low spinal decerebrate cat 5-HTP failed to elicit rhythmic activity but markedly increased the tonic activity in all muscles (Grillner and Shik, 1973). In chronic spinal adult cats that are capable of maintaining a stable and well-coordinated locomotor pattern due to locomotor training on a treadmill, the administration of serotonergic drugs (5-HTP and 5-HT agonist Quipazine) increased the step length and augmented the amplitude of hind limb extensor and flexor muscles (Barbeau and Rossignol, 1990, 1991). In chronic spinal rats, the same dose (0.5 mg/kg) of Quipazine produced only the small but significant effects on hind limb locomotor movements (Feraboli-Lohnherr et al., 1999). In spinal grafted rats, Quipazine administration also resulted in significant improvement of locomotor-like hind limb movements. The difference between the action of a 5-HT agonist (Quipazine) and the serotonin releasing graft can be attributed to the long modulatory effects of serotonin (Hochman et al., 2001; Svensson et al., 2001) in the case of grafted spinal rats but a single bolus of agonist in the case of ungrafted rats may not cause a sufficient stimulation of serotonergic receptors. Although it was demonstrated that chronic treatment using a serotonergic agonist (Quipazine), delivered continuously to the spinal cord below the transection, induced the partial restoration of hind limb motor functions in spinal rats (Antri et al., 2002); it is difficult to compare acute and chronic effects 1 month after grafting. Some other factors can play important roles in the control of the optimal hind limb locomotor-like movements. Among those factors the various types of receptors should be mentioned (Schmidt and Jordan, 2000; Garraway and Hochman, 2001; Jordan and Schmidt, 2002). For example, it is well known that the cell bodies and axons primarily considered serotonergic contain also at least two peptides: substance P and thyrotropin releasing hormone (TRH), and moreover, they can be identified in the same dense core vesicles of terminals surrounding motoneurons (Pelletier et al., 1981). Thus, these peptides may participate in the control of locomotor movements (Bedard et al., 1987; Clarke and Kirby, 1994; Barthe and Clarac, 1997). Further work is needed to explore the role of these peptides in our experimental model. Moreover, whether the observed effects on locomotor-like hind limb movements in our ex-

perimental rats result from an action on motoneurons or also interneurons remains to be determined.

It has been demonstrated that 3 months after intraspinal grafting of embryonic cell suspensions containing serotonergic cells, the 5-HT-immunoreactive fibers from grafted cells were selectively distributed in the ventral horn and the intermediolateral cell column where they established conventional synaptic contacts (Rajaofetra et al., 1992). These results suggest that the grafted cells were able to reestablish connections with the motoneurons in the spinal cord network of the host. We have investigated this possibility by evaluating the effects of agonist and antagonist on 5-HT₂ receptor on the modulation of hind limb locomotor abilities in adult rats that had received the graft of serotonergic cells after spinal cord total transection. We demonstrated that in grafted rats the i.p. administration of 5-HT₂ antagonists (Cyproheptadine) dramatically altered the hind limb locomotor-like movements and this effect was reversed by administration of the 5-HT₂ agonist Quipazine. Moreover, Quipazine administration alone produced also improvement of the hind limb locomotor like movements, while in spinal control rats that did not receive a graft, it resulted in small, if any changes in locomotor performances. Thus, the grafted cells probably modulate the locomotor behavior by acting on the postsynaptic 5-HT₂ receptors of the spinal cord. In the absence of a graft the spinal cord network responsible for the control of hind limb locomotion was not sufficient to produce a pattern of plantar walking and moreover did not respond to pharmacological manipulation. It means that the reestablished innervations of the 5-HT system in grafted rats was crucial for recovery of hind limb locomotor-like movements.

In conclusion, our study shows that the recovery of hind limb locomotor function after complete spinal cord transection was much better in rats that received a graft of a part of embryonic raphe nuclei into the spinal cord below the lesion. The results of pharmacological manipulation using the antagonist and agonist of 5-HT₂ receptors showed unequivocally for the first time that the improvement of motor function was related to functional connections between the grafted serotonergic cells of embryonic raphe nuclei and the host spinal cord circuitry. It means that the grafted serotonergic cells after integration with the host nervous system were able to activate spinal circuitry below the lesion and enhance the significant recovery of hind limb locomotor function.

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