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Direct Agonists for Serotonin Receptors Enhance Locomotor Function in Rats that Received Neural Transplants after Neonatal Spinal Transection

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We analyzed whether acute treatment with serotonergic agonists would improve motor function in rats with transected spinal cords (spinal rats) and in rats that received transplants of fetal spinal cord into the transection site (transplant rats). Neonates received midthoracic spinal transections within 48 hr of birth; transplant rats received fetal (embryonic day 14) spinal cord grafts at the time of transection. At 3 weeks, rats began 1–2 months of training in treadmill locomotion. Rats in the transplant group developed better weight-supported stepping than spinal rats. Systemic administration of two directly acting agonists for serotonergic 5-HT_2 receptor subtypes, quipazine and (+/−)-1-[2,5]-dimethoxy-4-iodophenyl-2-aminopropane), further increased weight-supported stepping in transplant rats. The improvement was dose-dependent and greatest in rats with poor to moderate baseline weight support. In contrast, indirectly acting serotonergic agonists, which block reuptake of 5-HT (sertraline) or release 5-HT and block its reuptake (D-fenfluramine), failed to enhance motor function. Neither direct nor indirect agonists significantly improved locomotion in spinal rats as a group, despite equivalent upregulation of 5-HT_2 receptors in the lumbar ventral horn of lesioned rats with and without transplants. The distribution of immunoreactive serotonergic fibers within and caudal to the transplant did not appear to correspond to restoration of motor function. Our results confirm our previous demonstration that transplants improve motor performance in spinal rats. Additional stimulation with agonists at subtypes of 5-HT receptors produces a beneficial interaction with transplants that further improves motor competence.

Key words: spinal cord injury; transection; fetal transplant; serotonin agonists; locomotion; kinematics

Transplanting neural tissue into the site of a spinal lesion can improve motor function in cats and rats (Kunkel-Bagden and Bregman, 1990; Iwashita et al., 1994; Howland et al., 1995; Cheng et al., 1996; Li et al., 1997; Miya et al., 1997; Deiner and Bregman, 1998a). We (Miya et al., 1997) found that fetal spinal cord grafted into the site of complete spinal transections in neonatal rats (transplant rats) increased the likelihood that the rats develop weight-supported locomotion compared with those with transection alone (spinal rats). The degree of improvement varied with the difficulty of the task, and even within tasks transplant rats displayed a wide range of function, with some performing poorly. One of our goals, therefore, has been to develop adjunctive treatments to enhance the motor function mediated by transplants in spinal animals.

Serotonergic pathways arise primarily from the caudal raphe and innervate the spinal cord, including α motoneurons and interneurons (Bowker et al., 1981). 5-HT increases motoneuron excitability (Jackson and White, 1990; Ziskind-Conhaim et al., 1993; Cowley and Schmidt, 1997), facilitates generation of plateau potentials (for review, see Kiehn and Eken, 1998), which may be particularly relevant for motoneurons innervating postural muscles (Lee and Heckman, 1998), and can modulate spinal central pattern generators (Sillar et al., 1997). Extracellular 5-HT is increased in dialysates from spinal cord in adult rats during locomotion on a treadmill (Gerin et al., 1995). Because transection eliminates serotonin (5-HT) innervation caudally, treatment with drugs that stimulate mechanisms mediated by serotonin should improve motor function. Systemic administration of 5-HT agonists, such as quipazine, increased step length, the amplitude of EMGs from hindlimb extensors and flexors, and the activity of axial muscles in cats spinalized as adults (Barbeau and Rossignol, 1990, 1991; Edgerton et al., 1997). The serotonergic agonist ±-1-[2,5]-dimethoxy-4-iodophenyl]-2-aminopropane (DOI) produced similar results (Miller et al., 1996). Among their pharmacological properties, quipazine and DOI are agonists at 5-$HT_{2A/2B/2C}$ receptors (Conn and Sanders-Bush, 1987; Sanders-Bush and Breeding, 1991; Berg et al., 1994; Wainscott et al., 1996). 5-HT_2 sites are present in the spinal cord, with a dense concentration of these receptors in the ventral horn (Marlier et al., 1991; Pranzatelli et al., 1993; Thor et al., 1993; Sharma et al., 1997) and are the likely site of action for these drugs. Fetal grafts promote growth of serotonergic axons (Bregman, 1987; Howland et al., 1995; Yakovleff et al., 1995; Feraboli-Lohnherr et al., 1997; Miya et al., 1997; Deiner and Bregman, 1998b) and also improve

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motor function, although the relationship between the distribution of serotonergic axons and recovery remains undefined.

In this study, we examined the effects of quipazine and DOI on weight-supported hindlimb stepping during conditioned locomotion on a treadmill in spinal and transplant rats. Quipazine and DOI act directly at 5-HT_2 receptors to mimic serotonergic neurotransmission. Therefore, both transplant and spinal rats might be expected to respond to the motor actions of these agents. Spinal transection would be expected to increase the density of postsynaptic 5-HT receptors. Transplants should reduce that upregulation by promoting regeneration of serotonergic axons that reinnervate these receptors. Thus rats with transplants might be less sensitive to the action of directly acting 5-HT agonists than spinalized rats without transplants. In contrast, indirectly acting 5-HT agonists should enhance motor activity in transplant but not spinal rats. We therefore examined the actions of sertraline, a selective inhibitor of 5-HT reuptake (Koe et al., 1983), and D-fenfluramine, which releases endogenous 5-HT and blocks its reuptake (Borroni et al., 1983), in transplant and spinal rats tested as adults.

We report that directly acting agonists at 5-HT_2 receptors increase weight-supported stepping in transplant rats. In contrast, indirectly acting 5-HT agonists did not improve function. 5-HT_{2C} binding was upregulated in lumbar cord in both spinal and transplant rats. None of the drugs improved weight-supported stepping in spinal rats as a group, despite the upregulation of 5-HT_2 receptors.

MATERIALS AND METHODS

Animals and surgical procedures. Sprague Dawley pups were obtained within 48 hr of birth. Littermates were assigned to unoperated control (control, $n = 19$), spinal transection (spinal, $n = 20$), and transection plus transplantation (transplant, $n = 40$) groups. Surgical procedures, described in detail in a previous study (Miya et al., 1997), were performed under the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of MCP Hahnemann University. All operated and unoperated pups were handled and treated identically except for the surgical procedures.

Spinal transection. Pups were anesthetized by hypothermia. They were wrapped in a cotton pad and placed in a bed of ice during the surgical procedures. The spinal cord was exposed by partial laminectomy at the T8–T9 level and transected with iridectomy scissors followed by aspiration, which removed up to two segments of spinal cord. The gap between the spinal stumps was filled with sterile Gelfoam. The site of the transection was covered with Durafilm, and the muscle and skin were sutured in layers with 5–0 silk sutures. Animals were warmed and returned to the mother and littermates when they became active.

Fetal transplantation. Spinal cord from embryonic day 14 (E14) fetuses was obtained from timed pregnant Sprague Dawley rats. The dams were anesthetized with an intraperitoneal injection of a cocktail of ketamine hydrochloride (95 mg/kg), xylazine (10 mg/kg), and acepromazine (0.7 mg/kg), laparotomized, and the fetuses removed. Fetal spinal cord was dissected, the meninges stripped, and a portion of thoracic cord was removed and cut transversely into 2 mm pieces and placed in a covered Petri dish containing DMEM on ice. The spinal cord was transected in the neonatal host animals, using the method described above but omitting the insertion of Gelfoam. One or two pieces of fetal spinal cord were inserted to fill the space between the spinal stumps, and the dura was replaced over the transplantation site and covered with Durafilm. Muscle and skin were sutured.

Behavioral training and testing on treadmill locomotion. At 3 weeks, pups were weaned and housed three per cage under a 12 hr light/dark cycle, and for those used in the behavioral studies treadmill training was begun. Baseline data were obtained for control ($n = 13$), spinal ($n = 16$), and transplant ($n = 36$) rats. From these animals a complete series of observations of effects of either directly or indirectly acting agonists was obtained from 13 control, 12 spinal, and 27 transplant rats. Animals were water-deprived overnight (~16 hr). They were weighed daily, and those gaining <5 gm/d were given water *ad libitum* and supplemental diet (Nutri-cal) until they again gained at least 5 gm/d. During the training period of 4–6 weeks, animals were trained to walk on the treadmill; they received a reward of 10% sucrose solution through a drinking tube at one end of the treadmill for a total of 9 min/session. Rats were trained at three treadmill speeds (2 cm/sec, 5 cm/sec, and 10 cm/sec). These training sessions took place once a day, 5 d/week, during the late morning. The rats did not require external support to negotiate the treadmill. Because the rats could receive the water reward by locomotion using only their forelimbs, they were not penalized for failing to use their hindlimbs. When performance had stabilized after several weeks of training, baseline weight-supported stepping for each animal was determined after administration of 0.9% saline (1 ml/kg, i.p.). The treadmill performance was videotaped in the lateral view at 30 Hz using a Panasonic video camera (shutter speed 1/1000 sec, 30 Hz frame rate at 60 fields/sec) from a distance of 12 feet, which minimized distortions related to perspective.

Directly acting 5-HT_2 agonists. One cohort of animals (control, $n = 9$; spinal, $n = 8$; transplant, $n = 20$) was assigned to be tested with the serotonergic agonists quipazine dimaleate (quipazine) and DOI, purchased from Research Biochemicals (Natick, MA). Both quipazine and DOI were dissolved in filtered distilled/deionized water and injected in a volume of 1 ml/kg. Testing with drugs began the day after measurement of baseline performance. On each testing day, the animals received an intraperitoneal injection of saline or quipazine (0.15, 0.3, and 0.6 mg/kg), and treadmill testing began 5 min later. Animals were tested for 3 min at each speed, and all testing was completed within 15 min of the injection. In preliminary studies, some animals given 1.2 mg/kg of quipazine developed severe hypermetria that disrupted posture and interfered with motor performance. The treatments were randomized such that each rat received each of the doses over the course of the experiment. At least 2 d separated consecutive testing sessions. There was no apparent carryover effect of the drug on later testing. The observers were blind to the surgical and pharmacological treatments of the individual rats. The testing sessions were videotaped and analyzed. On completion of the quipazine study, the animals were tested with two doses of DOI (0.075 and 0.15 mg/kg, i.p.). Higher doses of DOI (0.3 mg/kg) were toxic in some animals.

Indirectly acting 5-HT agonists. Another cohort (control, $n = 4$; spinal, $n = 4$, transplant, $n = 7$) of rats was treated with the selective serotonin reuptake inhibitor sertraline hydrochloride (a gift from Pfizer Central Research, Groton, CT) and with the reuptake inhibitor/releasing agent D-fenfluramine hydrochloride (Research Biochemicals). Both drugs were dissolved in filtered distilled/deionized water and injected in a volume of 1 ml/kg. These animals were prepared, trained, and tested similarly to the other group except that animals were tested on the treadmill beginning 30 min after drug injection, and testing was completed within the next 15 min. Randomized doses of saline, 1.0 and 3.3 mg/kg sertraline were injected intraperitoneally into each animal followed by the highest dose of sertraline (10 mg/kg). Animals were then tested with D-fenfluramine (0.5 and 1.0 mg/kg). Two days separated each testing session within the same drug treatment, but 1 week separated the sertraline and D-fenfluramine tests.

Behavioral analysis. The locomotor performance of the rats was evaluated quantitatively from the videotapes by two observers who were unaware of the surgical history or drug treatment of the individual rats. The quantitative analyses were confined to locomotion at a treadmill speed of 5 cm/sec. This speed was used because at least some rats from each surgical condition demonstrated weight-supported stepping at this speed, whereas spinal rats displayed virtually no weight support at a treadmill speed of 10 cm/sec. Transplant rats that showed weight-supported stepping at 5 cm/sec were able to make some, although fewer, weight-supported steps at the higher speed. Control rats showed continuous weight-supported stepping at all three treadmill speeds. For consistency we chose to quantify stepping during a 1 min segment of the tape beginning 15 sec after the start of the treadmill locomotion. The observer recorded the number of step cycles displayed during that 1 min period. A step cycle was defined as flexion and extension of the hindlimb. The inter-rater reliability for counting step cycles was 0.91 (Pearson correlation coefficient), determined from assessments of step cycles made by two observers on the same segments of videotapes. Not all step cycles on a treadmill involve weight-supported stepping. Thus, we distinguished weight-supported step cycles, in which the hindlimb-supported the hindquarters sufficiently so that the hindquarters were seen on the videotape to be elevated above the surface of the treadmill, from non-weight-supported cycles in which the hindlimbs flexed and extended, but the

knee remained in contact with the treadmill, and the hindquarters were not elevated above the surface of the treadmill. Weight-supported steps included lift-off, swing, touch-down, and stance. In controls ($n = 9$) and the subsets of transplant ($n = 20$) and spinal ($n = 8$) rats that developed some weight-supported stepping, we measured the duration of weight-supported step cycles and the time in stance and swing. Stance was measured from foot contact to the onset of forward movement of the foot; swing was defined as the period from the onset of forward movement to the next contact (Belanger et al. 1996). We also recorded the number of weight-supported steps that were followed immediately by another weight-supported step. These steps were counted as linked weight-supported steps (steps interrupted by a stationary period were not counted). This provided an index of continuous weight-supported locomotion.

Kinematic analysis. Videotapes of transplant rats that received quipazine were evaluated before and after the drug administration. Only the hindlimb on one side (the right side) was analyzed; there were no systematic marked asymmetries in animals that developed weight-supported locomotion. The records were digitized by stepping through single video fields on a Panasonic AG 7355 editing deck. Individual video fields were acquired using an Omnicomp (Dallas, TX) MM basic frame grabber, and the image was digitized on-line using a pointing device. Six points along the dorsal body axis were digitized to assess axial posture. Hip, knee, ankle, pad, and toe tip in hindlimb, and shoulder, elbow, wrist, and toe in forelimb were selected from the captured frame and digitized. Skin markers were not used because of the problems of slippage. The knee, in particular, is difficult to identify precisely. The important distinctions in weight-supported locomotion, that the knee not be in contact with the treadmill and that the trunk be elevated above the treadmill surface, could readily be recognized. The software used to acquire, digitize, and display the data were written in the C^{2+} language and customized to views of the rat (S. Giszter, unpublished observations).

Statistics. The effects of pharmacological treatment on locomotion were analyzed parametrically for each drug in mixed, two-factor ANOVAs with surgical condition the between-group variable and dose of drug the within-subjects variable. One-way repeated measures ANOVAs were used, where appropriate, to test the significance of effects in individual groups (transplants, spinal, control). *Post hoc* comparisons of specific pairs of treatments were made using the Newman–Keuls test. The threshold for significance for all tests was $p < 0.05$. All analyses were conducted using the Sigma Stat version 1.0 statistical program (Jandel Scientific, San Raphael, CA).

Anatomical analysis. Animals were killed after the completion of behavioral testing, 2–4 months postoperatively (postnatally). Animals were anesthetized deeply and perfused intracardially with 0.9% physiological saline followed by 4% paraformaldehyde with 0.3% picric acid fixative in 0.1 M phosphate buffer. The spinal cord was removed, and blocks were prepared for cryostat sectioning. Blocks rostral and caudal to the area of the lesion were cut in serial transverse 20 μm sections; blocks containing the lesion/transplant were cut in serial, sagittal 20 μm sections. In all animals, adjacent spinal cord sections through the lesion site were stained with cresyl violet to verify the lesion and assess the morphological characteristics of the transplant. The completeness of the spinal transection was assessed by the absence of continuity between rostral and caudal stumps in serial sections through the lesion site. In rats with transplants, the transplanted tissue was recognized as cellular tissue that did not show the laminar organization of normal spinal gray matter or organized myelinated tracts and which often contained cysts. The area of integration between the transplant and the host was quite variable, but was usually demarcated by a region of small cells. The cells within the transplant were identified as neurons by morphological criteria, supplemented in some cases by staining with antibodies to MAP2, which recognizes neurons (Miya et al., 1997).

5-HT immunoreactivity. Antibodies to 5-HT were used to visualize descending serotonergic axons that have grown into or through the transplant. Adjacent sections from regions rostral and caudal to the lesion site were stained with a Nissl stain and with antibodies to 5-HT. For 5-HT immunoreactivity, frozen sections mounted on slides were incubated with the primary antibody (Incstar, Stillwater, MN; diluted 1:1000) for 24 hr and then with biotinylated goat anti-rabbit IgG and with avidin–biotinylated horseradish peroxidase complex, as specified by the manufacturer (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized with 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 mM Tris buffer. Control sections prepared using preimmune serum showed no staining. Histological preparations were examined by two or more investigators who did not know the motor performance of the animal. 5-HT staining was used also in spinal rats to confirm the completeness of the transection; no 5-HT staining was seen caudal to the lesion in these rats.

Receptor binding autoradiography. Three control, four spinal, and four transplant rats were prepared for receptor binding autoradiography to determine whether serotonin receptors were modified by the lesions at 8 weeks postoperatively (postnatally). These animals were decapitated, the spinal cords were removed quickly, and blocks from thoracic and lumbar spinal cord were frozen. Serial 20 μm coronal sections rostral (T4–T7) and caudal (T12–L3) to the lesion site were collected and thaw-mounted onto chrom–alum-coated slides. The sections were stored at −70°C until used for receptor autoradiography. The block containing the lesion/transplant was sectioned horizontally, stained with cresyl violet, and examined for completeness of the lesion and survival of the transplant.

Corresponding sections from control, spinal, and transplant animals were thawed quickly using cool air from a hair dryer. The sections were incubated at room temperature for 15 min in 170 mM Tris buffer, pH 7.4, containing 20 nM spiperone to block dopamine D-1 and 5-HT_{2A} receptors, followed by a 2 hr incubation in 170 mM Tris buffer containing 20 nM spiperone and 3.0 nM [^{3}H]mesulergine, specific activity 76.0 Ci/mM (TRK845; Amersham, Arlington Heights, IL). Nonspecific binding was defined using 1.0 μM methysergide (Research Biochemicals). The incubation was followed by two 10 min washes in ice-cold buffer containing spiperone to eliminate excess ligand. After a brief dip in ice-cold water, the slides were dried quickly using a hair dryer and desiccated overnight under vacuum. The slides were placed in cassettes together with a set of tritium standards and exposed to ^{3}H-Hyperfilm (Amersham) for 45 d. The films were developed and analyzed using computerized densitometry and the NIH Image program. The distance between the central canal and the ventralmost extension of the ventral horn was measured, and the density of the reaction in the ventral half of the ventral horn was determined. Background binding was measured from an area of the film that contained no tissue. These values, in addition to those determined for nonspecific binding, were subtracted from the total binding measurements. The reported values therefore reflect specific binding. The densities obtained were converted to femtomoles per milligram of protein by comparison with commercially prepared (^{3}H) standards (Amersham), exposed to each film from which the optical density measurements were made.

RESULTS

Animals from all three groups (normal, spinal, and transplant) gained weight and appeared to be in good health for the duration of the experiment. The rate of weight gain, however, differed among the three groups. For example, in one set of rats weighed just before testing began, control rats weighed more than spinal and transplant rats (controls, 191 ± 8 gm, $n = 9$; transplant, 153 ± 4, $n = 20$; spinal, 127 ± 9, $n = 8$, $p < 0.05$), and transplant rats weighed more than the spinal animals ($p < 0.05$; one-way ANOVA followed by Newman–Keuls multiple range test for *post hoc* comparisons). There was no systematic difference in weight between those spinal or transplant rats that performed well and those that performed poorly on the treadmill.

All of the steps made by the control animals during locomotion on the treadmill were weight-supported and consecutive (linked to other steps). As we showed previously (Miya et al. 1997), the spinal and transplant groups displayed a wide range of locomotor function after completing training. Some spinal animals were able to use weight-supported steps, and some transplant animals failed to support their hindlimbs during stepping (Fig. 1). Nevertheless, animals with transplants were more likely than spinal animals to use weight-supported hindlimb stepping on the treadmill (Mann–Whitney U test; $p < 0.05$).

Actions of directly acting 5-$HT_{2a/2c}$ agonists

Effect of quipazine and DOI on total number of step cycles

Figure 2*A* shows the total number of step cycles (including both weight-supported and nonweight-supported step cycles) observed

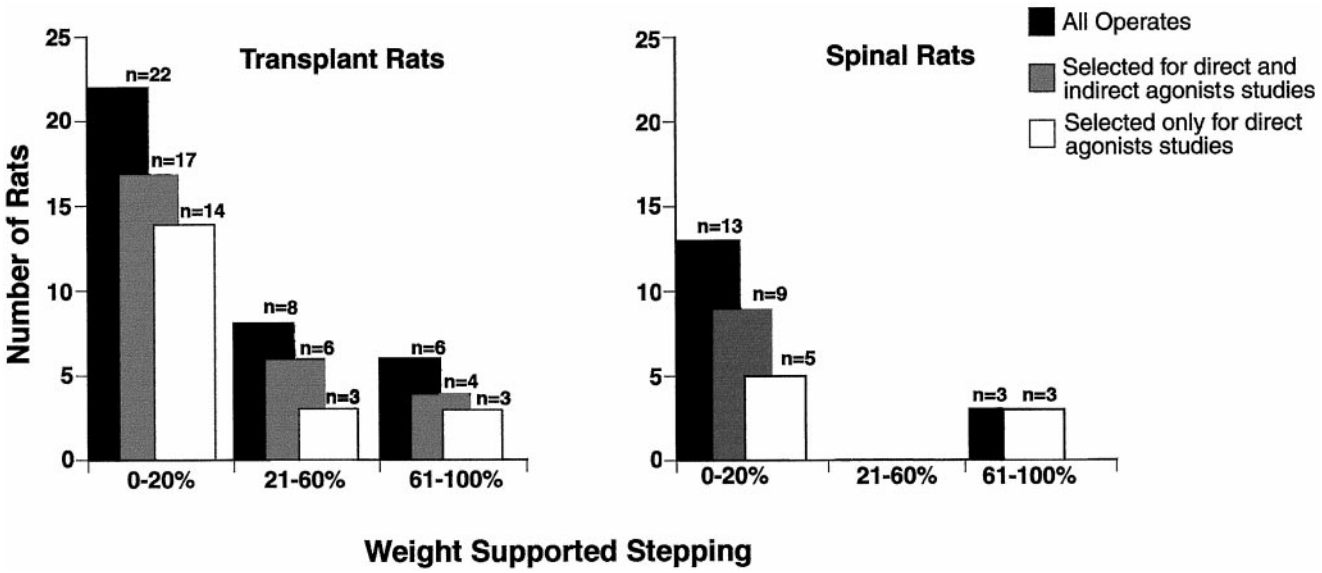

Figure 1. Histogram showing the numbers of transplant and spinal rats used in the behavioral study; rats are divided into groups with poor (0–20%), moderate (21–60%), and good (61–100%) baseline weight-supported stepping in treadmill locomotion. The *black bars* indicate all operated animals; the *gray bars* indicate all animals used for both direct and indirect agonist studies, and the *white bars* indicate those animals used for the quipazine–DOI study. The difference between the *gray* and *white bars* represents the animals used for the fenfluramine/sertraline study. The transplant rats as a group showed a greater percentage of weight-supported hindlimb steps during treadmill locomotion than did spinal rats (Mann–Whitney *U* test; $p < 0.05$). Both drug studies included transplant and spinal animals in the poor and moderate performing groups. The quipazine/DOI study also contained transplant and spinal animals in the best performing group; the sertraline/fenfluramine study did not contain spinal animals with moderate or high levels of locomotor performance.

in the 1 min analysis in the control, transplant, and spinal groups during baseline locomotor performance on the treadmill and after administration of several doses of quipazine or DOI. The total number of step cycles did not differ between control and transplant groups after saline injection (0 dose), although spinal animals in the quipazine but not the DOI study showed significantly fewer step cycles than controls ($p < 0.05$; Newman–Keuls multiple range test after ANOVA). Neither quipazine nor DOI changed the total number of step cycles from baseline in any group of animals (all p values > 0.10) although there was a tendency for an increase in number of step cycles in transplant rats given DOI.

In contrast to the absence of effect seen when considering total number of step cycles, both quipazine and DOI administration increased the percentage (Fig. 2*B*) and the number of step cycles (data not shown) that were weight-supported in a dose-dependent manner but only in rats that had received transplants. Spinal rats did not increase weight-supported stepping above baseline after either drug; control rats continued to make all of their steps with weight support regardless of drug or drug dose.

Actions of quipazine and DOI on the continuity of weight-supported stepping

The drug-induced increases in the number (and percentage) of weight-supported steps in the locomotion of transplant animals also improved the continuity of locomotion. As shown in Figure 2*C*, all doses of quipazine and DOI increased the number of linked weight-supported steps in transplant animals as compared with their baseline performance ($p < 0.05$). Neither drug altered the number of linked weight-supported steps in either controls or spinal rats. Importantly, although transplant rats had fewer linked weight-supported steps than control rats after saline injection ($p < 0.05$), the higher doses of quipazine and especially DOI increased both the number and percentage (data not shown) of linked weight-supported stepping to levels that did not differ from controls.

Pharmacological enhancement of function in transplant rats depends on baseline level of weight-supported stepping

As noted previously, spinal rats and transplant rats varied in their baseline level of locomotion. Thus, we examined the effects of quipazine (Fig. 3) and DOI (data not shown) on motor function after separating transplant and spinal rats, according to baseline weight-supported stepping, into poor (0–20%), moderate (21–60%), or good (>61%) groups. The rats with good weight-supported stepping did not improve significantly with either quipazine or DOI, which can be attributed to a "ceiling effect." Transplant rats in the moderate and poor function categories showed improved weight-supported stepping after DOI (data not shown) and quipazine administration. This was particularly striking in the case of five of the seven transplant animals that showed no baseline weight-supported stepping but improved to 20–80% after quipazine administration. In contrast, none of the spinal rats with poor baseline weight-supported stepping increased their weight-supported stepping after drug administration.

These results indicate that both quipazine and DOI improved the locomotor performance in rats that had received transplants. At the higher doses, both drugs induced hypermetria, identified as prolonged or exaggerated flexion, and/or tremors in the hindlimbs, but not the forelimbs, of some spinal and transplant rats. When severe, the tremors and hypermetria interfered with the animals' locomotion. Analyses of episodes of hypermetria during a 1 min period of treadmill locomotion for the transplant rats demonstrated that this effect was also dose-related, although far more pronounced after quipazine than DOI administration (Fig. 4). Control rats did not exhibit hypermetria or tremors at the doses used.

Kinematic analysis

We analyzed the kinematics of one control, three transplant rats, and one spinal rat before and after quipazine (0.3 mg/kg) administration. The transplant rats were representative of the good, moderate, and poor performing rats, and the spinal rat had the best weight-supported stepping in that group (Fig. 5). The primary focus was on the effect of quipazine administration on axial carriage and vertical excursions. Baseline locomotion, examined after saline administration, indicates that hindquarter elevation and the forward extension of the hindlimbs in the transplant rats was less than in the control rat and more variable. The kinematic figures show that quipazine has relatively little effect on posture or limb position in normal rats (Fig. 5*A*) and transplant rats with good baseline weight support (Fig. 5*B*) but that quipazine increases trunk elevation and hindlimb support in both moderate and poor weight-supporting rats (Fig. 5*C*,*D*). This is particularly evident in the case of the transplant rat with no baseline weight-supported hindlimb stepping who developed good hindlimb support after drug administration (see Fig. 7*D*). Quipazine administration in the spinal rat with good hindlimb weight support (Fig. 5*E*) increased hindquarter elevation accompanied by an increase in number of weight-supported steps that did not reach significance for the group. If kinematics provides a more sensitive method of analysis, this observation would suggest that spinal rats respond to quipazine, consistent with the results of Barbeau and Rossignol (1990). Nevertheless, the response is markedly less than for transplant rats, and no effect was seen in spinal animals with poor locomotor performance.

Step cycle duration

We compared step cycle duration in control rats and in subsets of spinal and transplant rats with baseline weight-supported stepping. The proportion of the step cycle spent in swing and stance did not differ among the groups, although the mean duration of weight-supported step cycles differed among the three groups (Fig. 6). Those steps made by spinal rats that were weight-supported were of shorter duration than those made by transplant rats, and both groups had shorter weight-supported step cycles than control rats ($p < 0.05$; Fisher's least significant difference). This is consistent with a beneficial but partial improvement in locomotion by rats with transplants. No significant effect of drug administration on step cycle duration was seen, although this may have reflected in part the variation imposed in some steps made by transplant and spinal rats by the hypermetria and tremors observed at the higher doses (Fig. 4).

Actions of indirectly acting 5-HT agonists

Neither the selective serotonin reuptake inhibitor sertraline nor the releaser/reuptake inhibitor D-fenfluramine modified the weight-supported stepping in any group at any dose (Fig. 7). This suggests that endogenously available serotonin does not contribute to the enhancement of motor function provided by transplants or by directly acting agonists.

Serotonin (5-HT_{2c}) receptor binding in ventral horn

Spinal transection increased the density of binding of 5-HT_{2c} receptors in the lumbar ventral horn at 8 weeks postoperatively (postnatally) (Table 1). There was no difference in binding between spinal and transplant animals. Receptor densities were not different in thoracic ventral horn rostral to the lesion site among control, spinal, and transplant animals.

Anatomical analysis of transplants

The spinal transections were complete in all animals. Spinal gray matter rostral and caudal to the lesion appeared healthy at a distance of one to two segments from the lesion/transplant site in each animal. In spinal animals, there was no immunocytochemical evidence of 5-HT caudal to the transection. In transplant animals, the size of transplants, the integration with the host, and the extent of 5-HT innervation varied considerably, as has been reported before (Miya et al. 1997), and showed no consistent relationship to baseline performance or to responsiveness to drug action. In no case in this series of animals did serotonergic axons extend to lumbar levels. Figure 8*A* shows an example of a well integrated transplant, stained with cresyl violet, from a rat that performed >61% baseline weight-supported steps. This animal had serotonergic staining into the transplant and caudally into the host (Fig. 8*B*). In contrast, another animal also had a well integrated transplant with substantial immunostaining for 5-HT within and caudal to the transplant (Fig. 8*C*), but showed no baseline weight-supported steps, although the animal improved to >61% after quipazine administration.

DISCUSSION

Our results demonstrate that administration of directly acting 5-HT_2 agonists acutely enhances motor function in rats that received fetal spinal cord transplants after spinal transections at birth. We have shown previously (Miya et al., 1997) that although transplant rats used more weight-supported steps than spinal rats during locomotion on a battery of tasks, their performance was impaired compared with controls. Administration of quipazine and DOI to transplant rats increased the frequency of weight-supported step cycles and the linking of those cycles into consecutive steps. Thus, these drugs improved significantly the motor competence of rats that had benefited only partially from the transplants. In contrast, serotonergic agents did not enhance significantly the performance of spinal rats. These data implicate a specific subclass of neurotransmitter receptor as a target for therapeutic agents in treating spinal cord injury. These results also provide new perspectives on the relative contribution of endogenous transmitters, receptors, and transplant-related factors in motor recovery.

Serotonergic agonists interact with spinal locomotor generators

Hindlimb locomotor function is expressed through the activity of motoneurons but is organized at the spinal level by groups of interneurons that make up the central pattern generator (CPG) for locomotion. Both the CPG and motoneurons are normally regulated by descending projections, including serotonergic axons (Cazalets et al., 1995a,b, 1996; Kiehn and Kjaerulff, 1996; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kremer and Lev-Tov, 1997; Lee and Heckman, 1998) and by afferent input (Sillar et al., 1997; Pearson and Ramirez. 1997); the midthoracic spinal transection removes descending influences on the CPG and motor neurons. The CPG remains functional, however, as evidenced by the similar patterns of stepping movements elicited by treadmill stimulation in both spinal and transplant rats (see also Stelzner et al., 1975; Weber and Stelzner, 1977). Transplantation increases the frequency and duration of step cycles and the likelihood of hindquarter weight support during locomotion and also enables cortical reorganization (Giszter et al., 1998). Transplants also appear to be permissive for the action of directly acting 5-HT agonists to improve motor function. The beneficial

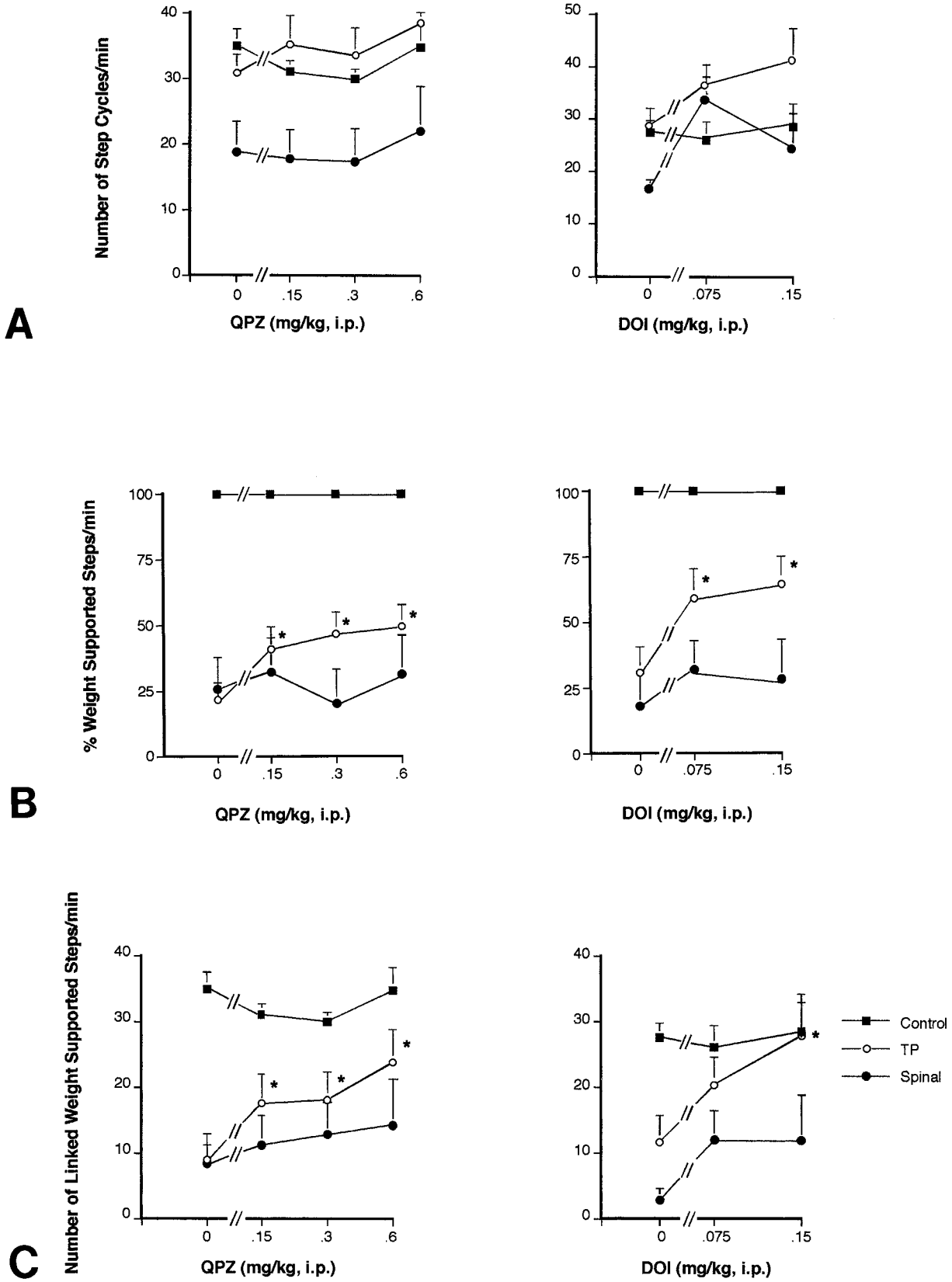

Figure 2. Effect of directly acting serotonergic agonists on stepping. *A*, Total number of step cycles (weight-supported + nonweight-supported) during a 1 min period of treadmill locomotion exhibited by control, transplant, and spinal rats. Baseline function is shown after saline injection (*0*). Spinal animals in the quipazine study showed fewer step cycles than controls. Increasing doses of quipazine and DOI had no effect on the number of step cycles in any group. B, Effects of quipazine or DOI on percentage of weight-supported steps made by control, spinal, and transplant rats. Control animals showed 100% weight-supported steps at all doses of either drug. Spinal animals showed no significant improvement over baseline (*Figure legend continues*)

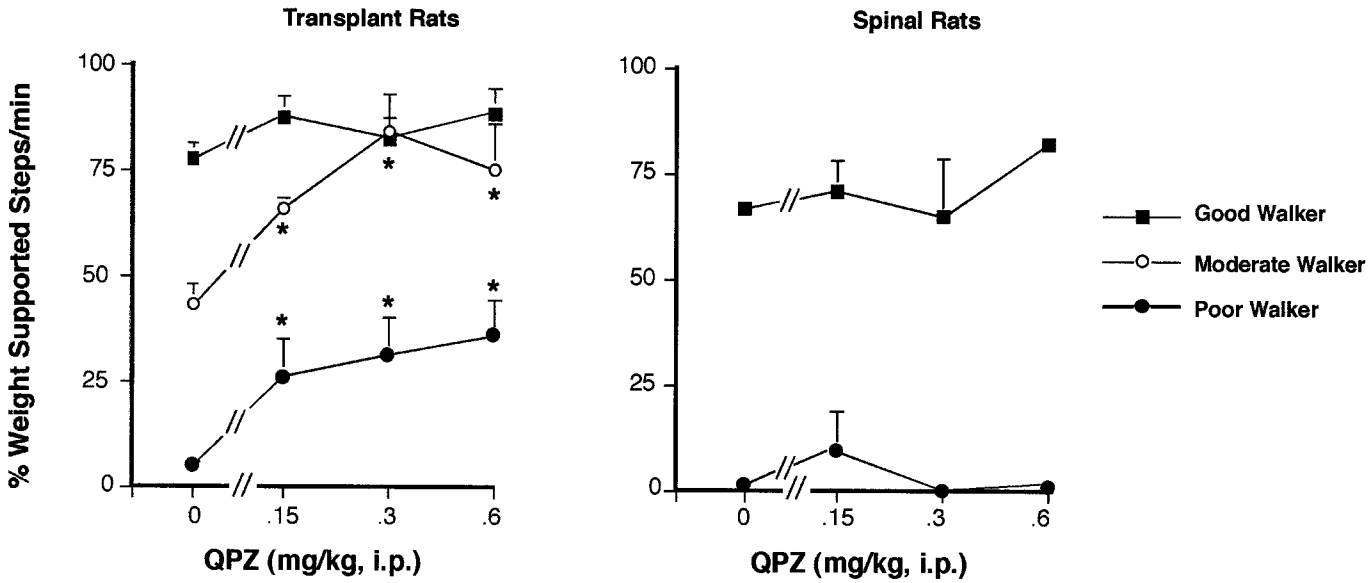

Figure 3. The effect of quipazine on locomotion by transplant rats depends on their baseline weight support. Transplant animals with >61% baseline weight-supported locomotion showed no improvement, but those with poor and moderate baseline locomotion improved significantly at higher does of quipazine. Spinal rats did not show a significant improvement with quipazine administration.

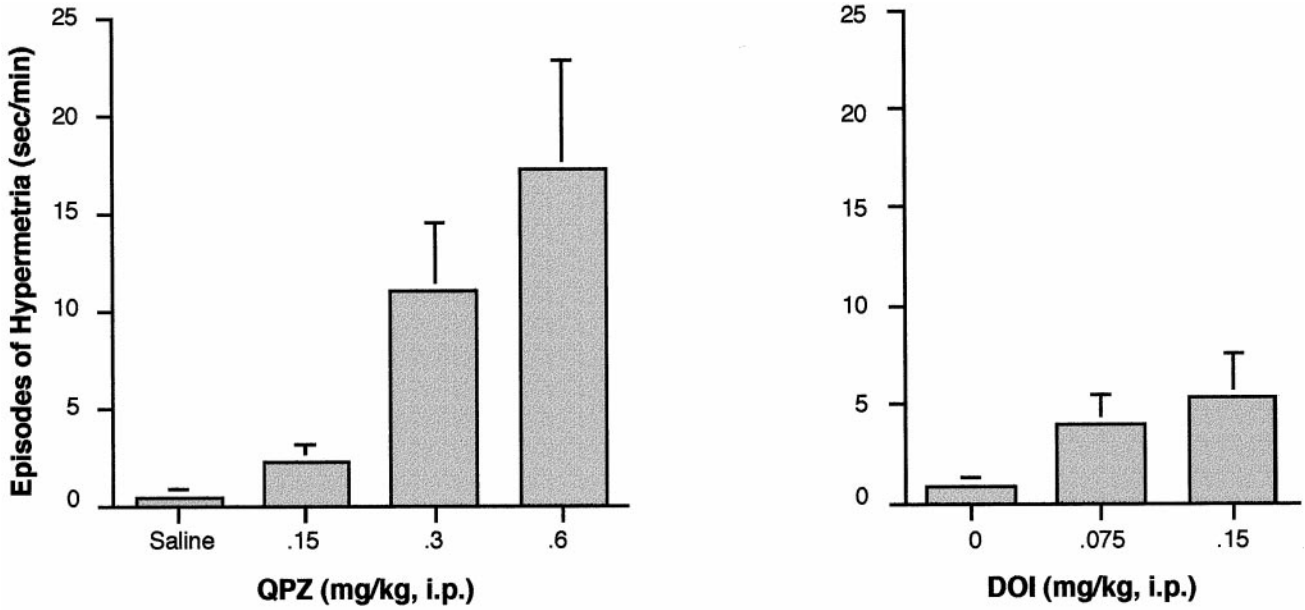

Figure 4. Frequency of hypermetria in transplant rats given quipazine or DOI. Episodes of hypermetria per minute of treadmill locomotion were measured in transplant rats during baseline locomotion and at increasing doses of quipazine and DOI. There was little hypermetria during treadmill locomotion after saline injection, but there were dose-related increased periods of hypermetria after agonist injections. Quipazine administration at higher doses produced significantly more pronounced hypermetria than DOI ($p < 0.05$).

effects of transplants and serotonergic drugs may operate via independent mechanisms. Nonetheless, our observation that 5-HT_2 agonists restored virtually to normal the frequency of weight-supported stepping by transplant rats with moderate baselines demonstrated an important interaction between the drugs and the remodeled spinal circuitry produced by the graft. Serotonergic drugs produced significant weight support in some transplant rats but not in spinal rats without baseline weight support.

←

levels at any dose of either drug. Transplant animals showed a significant improvement in weight-supported stepping at the higher dose of each drug. *C*, Effects of quipazine or DOI on linked weight-supported step cycles made by control, spinal, and transplant rats. Only weight-supported steps that are followed by another weight-supported step are counted. Increasing doses had no effect on linked weight-supported steps in control or spinal groups. Transplant animals showed a significant dose-related increase in the number of linked weight-supported steps with either quipazine or DOI administration.

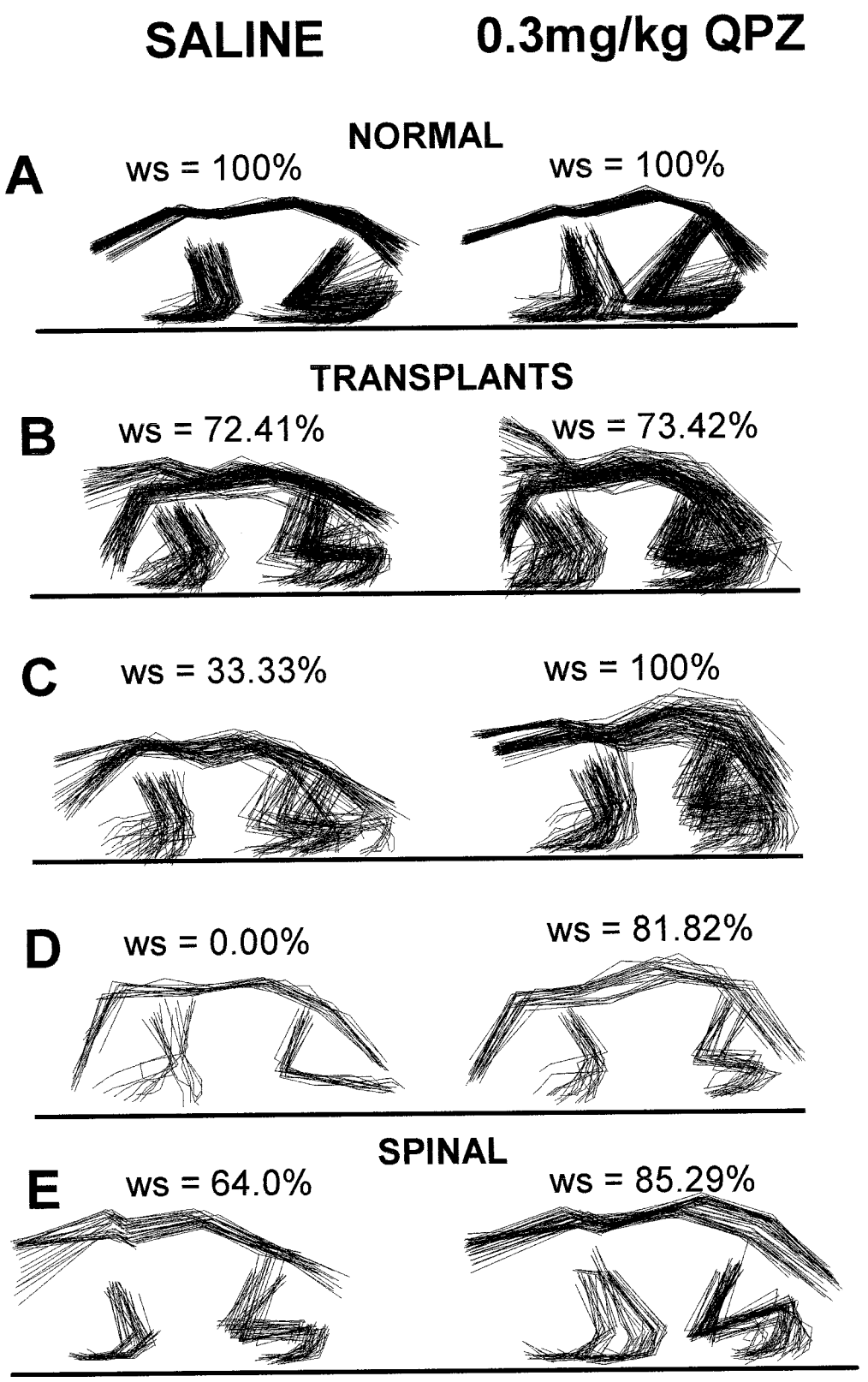

Figure 5. Kinematic analysis of a normal rat (*A*), transplant rats that showed good baseline weight support (*B*), moderate baseline weight support (*C*), and poor baseline weight support (*D*), and the best performing spinal rat (*E*) before and after quipazine administration. The most notable effects are seen in the moderate and poorly performing transplant rats in which hindquarter elevation, hindlimb support, and weight-supported stepping are increased after quipazine administration.

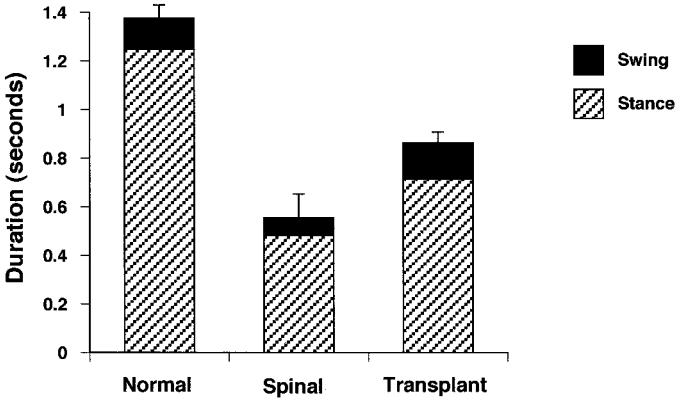

Figure 6. Histograms showing the duration of step cycles and amount of time in swing and stance for control rats, and a subset of spinal and transplant animals that developed weight-supported stepping. Operated animals had shorter step cycles, but there was little difference in the percentage of time in stance and swing among the groups. There was also no effect of quipazine on duration of step cycle or percentage of time in stance and swing.

These data established an important synergy such that stimulation of specific receptors can reveal transplant-mediated function that may not otherwise be recognized (see also Chau et al., 1998).

5-HT receptor subtypes in spinal cord

5-HT_{2A} and 5-HT_{2C} subtypes have been localized autoradiographically and immunocytochemically in the region of the interneurons and α-motoneurons of the ventral horn (Marlier et al., 1991; Thor et al., 1993; Sharma et al., 1997). Because drugs that stimulate 5-HT_2 subtypes, including quipazine and DOI, increase α-motoneuron excitability (Jackson and White, 1990; Yamazaki et al., 1992), we suggest that the therapeutic actions of these agents in the present study were mediated by one or more 5-HT_2 subtypes. The behavioral effects of quipazine and DOI differed; quipazine produced more episodes of hypermetria than DOI. Quipazine, unlike DOI, antagonizes 5-HT_3 receptors (Hayashi et al., 1993) and 5-HT_{1B} autoreceptors on the terminals of serotonergic neurons in the normal rat spinal cord (Monroe and Smith, 1985). Thus, some of the effects of quipazine, such as the exaggerated hypermetria, may be caused by multiple interactions with these receptors in addition to the 5-HT_2 subtypes. Neither quipazine nor DOI enhanced weight-supported stepping in spinal rats, despite similar upregulation of binding sites. Thus, activating 5-HT_2 receptors appears to be necessary but not sufficient for optimal recovery after transections of the cord.

Table 1. ^{3}H-Mesulergine (5-HT_{2C}) binding in ventral horn of spinal cord rostral (thoracic) and caudal (lumbar) to the lesion/transplant 8 wks postnatal

		fmol/mg protein			
Animal group	*n*	Thoracic	% control	Lumbar	% control
Control	3	50.9 ± 5.4		49.5 ± 6.5	
Spinal	4	53.7 ± 4.2	105	67.7 ± 5.7*	135.4
Transplant	4	50.6 ± 4.1	99	76.4 ± 5.1*	154.3

*$p < 0.05$.

Contribution of the transplant

The specific elements provided by the graft that contribute to improved motor responses are incompletely understood. Transplants placed into neonatal hosts do stimulate regeneration or permit elongation of late developing descending axons into and through the transplant (Bregman, 1987; Howland et al., 1995; Miya et al., 1997; Deiner and Bregman, 1998b) and thus provide a connection between rostral and caudal regions that does not exist in spinal animals. Given the distributed nature of the interneurons contributing to the CPG, even limited functional regeneration of axons into the host could engage the CPG and facilitate locomotion. Although we examined only 5-HT axons, the extent of growth of these axons in our model was quite variable and often slight, and did not reach caudal lumbar levels. In the present study the 5-HT reuptake inhibitor/releaser D-fenfluramine and the 5-HT reuptake inhibitor sertraline failed to enhance locomotor function in either transplant or spinal rats at doses that produced motor actions (Simansky and Vaidya, 1990) and increased extracellular 5-HT in intact rats (Rutter and Auerbach,

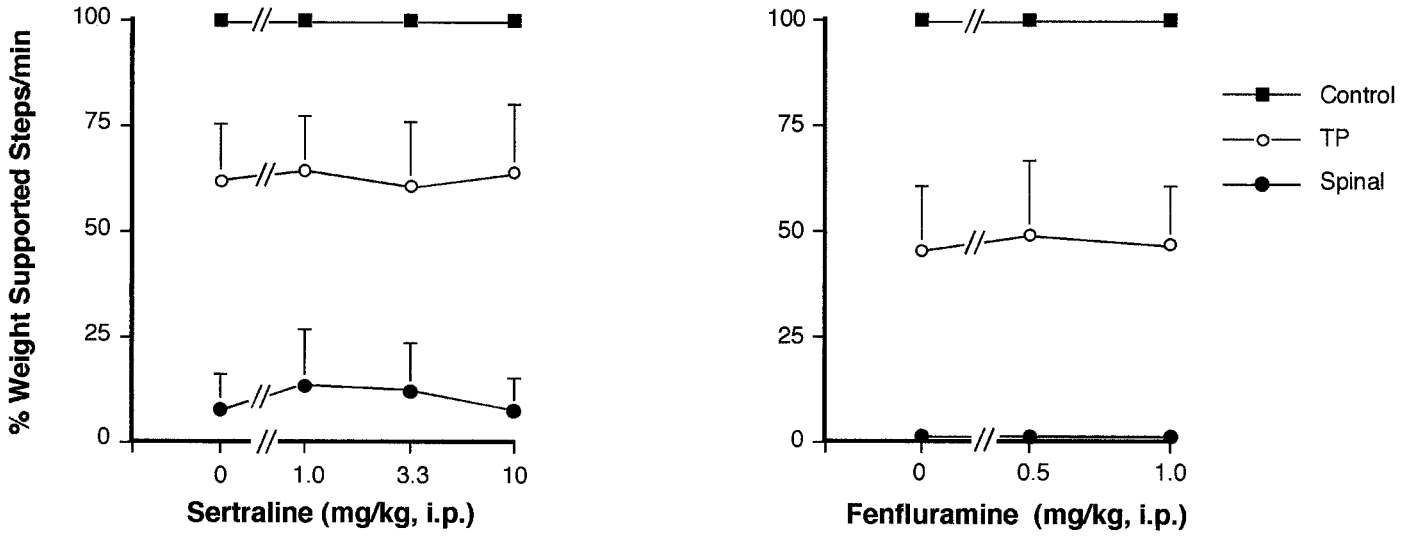

Figure 7. Effects of indirectly acting 5-HT agonists on weight-supported treadmill locomotion. Neither sertraline nor fenfluramine had an effect on weight-supported stepping in any group.

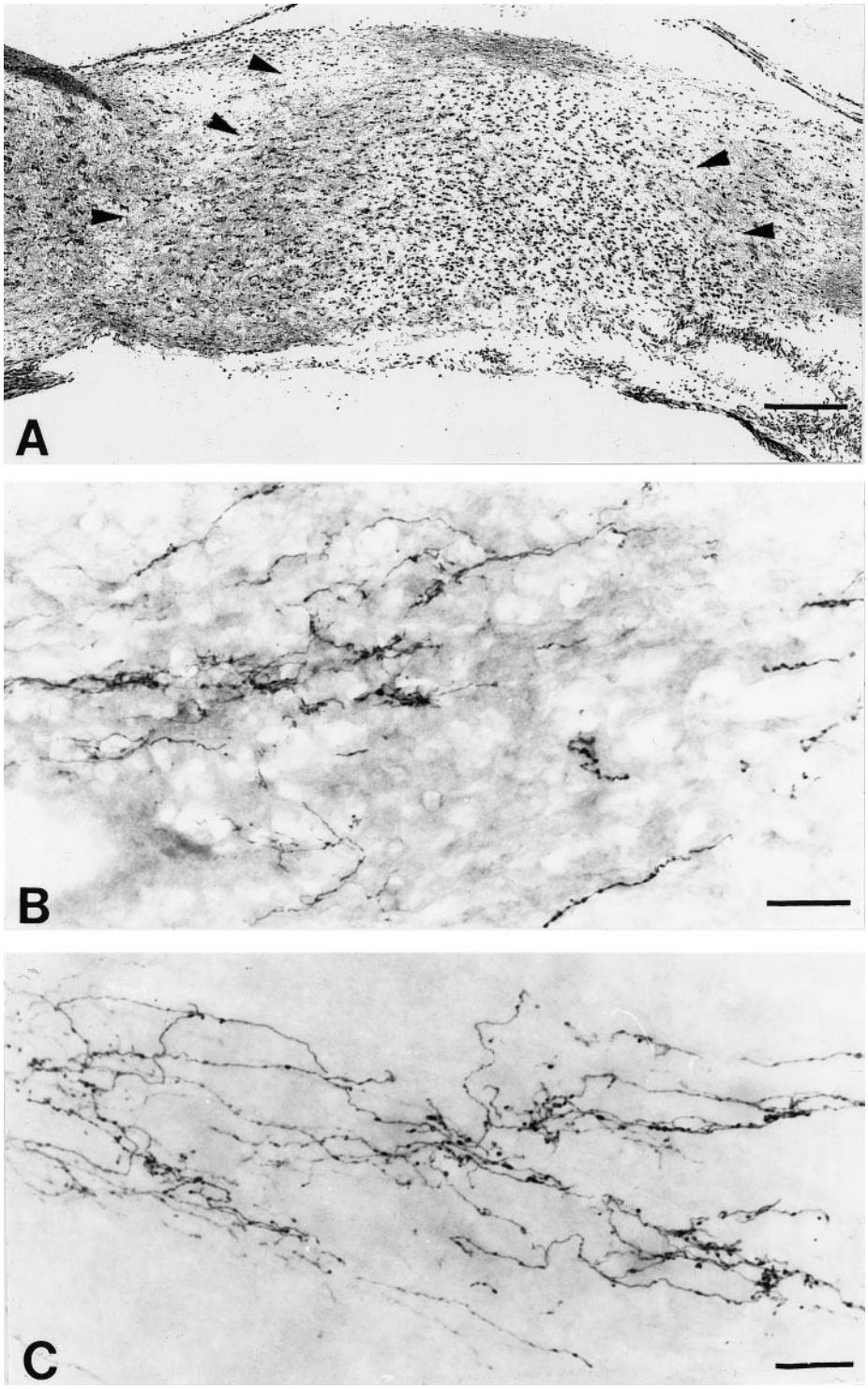

Figure 8. *A*, Sagittal section showing a transplant well integrated with the host. *Arrowheads* indicate transplant–host boundary; cresyl violet stain. Note absence of organized fiber bundles through the transplanted tissue. This transplant animal showed good baseline weight support (40×). Scale bar, 150 μm. *B*, Serotonergic axons in host caudal to transplant in animal shown in *A* (180×). Scale bar, 25 μm. *C*, Serotonergic axons in host caudal to transplant in animal which showed no baseline weight support. This animal showed improved weight-supported locomotion after quipazine administration (180×). Scale bar, 25 μm.

1993). Our data thus argue against a role for endogenous spinal 5-HT in mediating the recovery produced by our grafts in which axonal regeneration was minimal at best in the terminal fields of interest. In contrast, Feraboli-Lohnherr et al. (1997) reported that the 5-HT reuptake inhibitor zimelidine enhanced the effects mediated by embryonic serotonergic cells transplanted into lumbar levels of rats spinalized as adults. Thus, when adequate serotonergic neurites and endogenous transmitter exist, indirect agonists can potentiate the therapeutic effects of grafts in locomotion. Other recent studies (Bregman et al., 1995; Xu et al., 1995; Grill et al., 1997; Kobayashi et al., 1997; Ye and Houle, 1997; Liu et al., 1999) indicate that grafts supplemented by molecules that provide a more permissive environment will increase the amount of regeneration by axotomized neurons. Improving host regeneration could be expected to increase the size of endogenous pools of transmitter and to reveal a therapeutic effect of drugs that promote release of transmitter by the regenerated axons.

Transplants also rescue axotomized neurons that would otherwise undergo retrograde death (Bregman and Reier, 1986;

Deiner and Bregman, 1994; Himes et al., 1994; Mori et al., 1997; Shibayama et al., 1998). Grafting fetal tissue into the site of the transection may therefore rescue neurons relevant to locomotion, such as interneurons that contribute to the CPG or that modulate the excitability of motor neurons that are used in weight-supported locomotion. In addition, the presence of spinal cord transplants may also modify the development of function of sensorimotor cortex (Giszter et al., 1998). Thus, transplants placed into spinal lesions can modify existing circuitry or the circuitry that develops as a result of lesions. This reorganized circuitry may contribute to function, which we show can be further enhanced by the action of serotonergic agents on supersensitive target neurons. The lack of effectiveness of indirectly acting agonists suggests that serotonergic actions at the spinal level are critical to the improved function in transplant rats.

Drug effects in spinal animals

Barbeau and Rossignol (1990) demonstrated that quipazine increased activity of hindlimb muscles and consequent weight support in cats transected as adults, and McEwen et al. (1997) reported that a large dose (4 mg/kg) of quipazine increased hindlimb air stepping in neonatal rats with spinal transection, tested just 24 hr after the lesion. In contrast, we found that smaller doses of quipazine improved function and sometimes produced exaggerated hypermetria in transplant rats without significantly enhancing locomotion in most spinal rats, when tested 2 months postoperatively. Thus, the postoperative interval may dictate the response to quipazine after complete transection. The difference in efficacy of quipazine between spinal cats and our rats suggests differences in segmental reorganization after complete transection, depending on the species and age at which the animal is spinalized. In support of this, preliminary studies of spinal and transplant rats operated as adults showed increases in hindlimb movement in both spinal and transplant rats after quipazine administration, although the transplant rats showed a greater drug effect (Stackhouse et al., 1997).

In summary, our results demonstrate that acute administration of serotonergic agents improves transplant-mediated locomotion. This improvement is functional, as indicated by the increased number of linked weight-supported steps and is clinically relevant, as indicated by its dose dependence and its more pronounced effect on transplant recipients with poorer baseline function. Spinal rats, treated similarly, did not improve over baseline. The enhanced function appears to be attributable to an interaction between the spinal circuitry remodeled as a result of the fetal tissue and increased sensitivity of spinal neurons made supersensitive to 5-HT agents by the lesion.

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