The role of serotonin in the control of locomotor movements and strategies for restoring locomotion after spinal cord injury

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In this review we will discuss different ways for re-establishing serotonergic activity that can enhance recovery of coordinated plantar stepping after spinal cord injury in adult rats. It is well known that serotoninergic neurons located in the medulla are able to initiate locomotor activity. This effect is exerted by actions on motoneurons and on neurons of the locomotor CPG (Central Pattern Generator). Motoneuron and interneuron excitability is increased, and putative CPG interneurons display oscillatory behaviour in response to serotonin receptor activation. The medullary serotonergic nuclei play multiple roles in the control of locomotion, and they terminate on specific target neurons with different types of serotonergic receptors in the spinal cord. Activation of these serotonergic receptors can restore locomotor movements after spinal cord injury. Specifically, using defined serotonergic agonists the 5-HT1 receptors can be stimulated to control CPG activation as well as motoneuron output, while 5-HT receptors to control activity of the locomotor CPG. These results are consistent with the roles for these receptors during locomotion in intact rodents and in rodent brainstem–spinal cord in vitro preparations. The other possibility to encourage the remaining spinal cord circuitry below the total transection to control recovery of plantar hindlimb stepping is restoration of serotonergeric innervation by intraspinal grafting of embryonic 5-HT neurons. Our data show that grafting of different populations of 5-HT neurons dissected from embryonic brainstem provides differential control over multiple components of the spinal locomotor circuitry through specific serotonin receptors. Moreover, we demonstrated that the best effect of motor recovery is obtained after grafting of neurons destined to form the B1, B2 and B3 descending 5-HT systems. Using only one of the subpopulations for intraspinal grafting, for example, B3 or the lateral group of 5-HT neurons, induces only partial recovery of plantar stepping with a clear lack of proper interlimb coordination. This confirms the hypothesis that transplantation of 5-HT neurons from specific embryonic sources is necessary to obtain optimal recovery of locomotor hindlimb movement.

Key words: locomotion, spinal cord injury, 5-HT, intraspinal grafting

ABBREVIATIONS:

5-HT – 5-hydroxytryptamine; serotonin
5HT1a, 5-HT2a, 5-HT3c, 5-HT7 – serotonergic receptors
8-OHDPAT – (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide
B1, B2, B3 – caudal populations of serotonergic cells
ChR2 – channelrhodopsin2
CPG – Central Pattern Generator
DREADDS – designer receptors exclusively activated by designer drugs
EMG – electromyography
IML – intermediolateral cell column
Lhx3, Chx10 – transcription factors
NMDA – N-Methyl-D-aspartic acid
Nkx2.2, Pet-1 – transcription factors
PPR – the parapyramidal region
Sol – soleus muscle
SCI – spinal cord injury
S2 – sacral 2 spinal cord segment
T12 – thoracic 12 spinal cord segment
TA – tibialis anterior muscle
Vglut2 – vesicular glutamate transporter 2
YFP – yellow fluorescent protein

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INTRODUCTION

The effects of spinal cord injury on the control of locomotion can be attributed in large part to the loss of specific descending neural pathways that normally serve to initiate and control locomotion. Chief among these in rodents is the descending serotonergic (5-hydroxytryptamine, 5-HT) pathway, that originates in cells of the medulla and terminates at all levels of the spinal cord. The importance of serotonergic descending neurons for the control of locomotion has been reviewed (Gimenez y Ribotta et al. 2000, Schmidt and Jordan 2000, Jordan et al. 2008, Boulenguez and Vinay 2009, Jordan and Sławińska 2011, Pearlstein et al. 2011). Restoration of serotonergic control after spinal cord injury has been a goal of numerous studies. And despite the fact that serotonergic fibers have a greater propensity for regeneration than other descending pathways (Hawthorne et al. 2011), strategies to improve 5-HT neuron regeneration have not led to very extensive recovery. A combination of techniques for activating the locomotor CPG have been developed, including epidural and intraspinal stimulation (Mushahwar et al. 2002, Courtine et al. 2009, Tator et al. 2012) as well as application of drugs that serve to replace the actions of transmitters normally released from descending locomotor pathways to control the CPG (Orsal et al. 2002), and training (Barbeau et al. 1999, Edgerton et al. 2008, Rossignol and Frigon 2011). Here we will review the effects of systemic drug application that mimics the action of serotonin and the effects of grafting of 5-HT neurons from specific embryonic sources that are necessary to obtain optimal recovery of well-coordinated locomotor hindlimb movements in paraplegic rats.

PLANTAR STEPPING AFTER SPINAL CORD INJURY

Stepping movements are well known to occur after transection of the spinal cord in several species, but in rats spinalized as adults, locomotor activity is difficult to elicit without some form of exteroceptive stimulation, such as mechanical stimulation of the tail (see Fig. 1). This effect appears to result from the sensory activation of neurons of the spinal central pattern generator (CPG) for locomotion, but after complete transection it falls short of producing well-developed plantar stepping, because the rhythmic output is uncoordinated (Jordan and Sławińska 2011). Tail stimulation has been recognized as a means to promote locomotor function in adult rats with a spinal cord transection (Meisel and Rakerd 1982), and it has been used in our studies on recovery of treadmill locomotion (Sławińska et al. 2000, 2012b, Majczyński et al. 2005). It has also been used to allow treadmill locomotor training in adult spinal rats (Macias et al. 2009) and mice (Leblond et al. 2003). Tail stimulation and electrical stimulation of the cauda equina have been known for some time to be effective means of eliciting fictive locomotion in isolated rodent spinal cord preparations (Smith et al. 1988, Lev-Tov et al. 2000, Whelan et al. 2000, Delvolve et al. 2001, Norreel et al. 2003, Gordon and Whelan 2006). Tail nerve electrical stimulation is now a standard procedure to induce body weight-supported stepping in adult rats with contusion injury (Zhang et al. 2010). After total transection, however, there is apparent “paralysis” of the limb, but rhythmic muscle bursts of activity that are not coordinated persist, resulting often in ineffective or little actual movement, without placement of the plantar surface of the paw on the substrate, “dragging” of the limb (see Fig. 1E) on the dorsal surface of the paw, and little weight support. According to Kaegi and coworkers (2001), “Using EMG recordings … after a SCI in adult rats, we gained detailed insight into various changes occurring in the stepping pattern, with many of them undetectable for behavioural tests or kinematic analysis.” (p. 249). The advantage of EMG recording is demonstrated when an apparently paralyzed limb displays well-developed rhythmic activity, but the “paralysis” is actually a failure of coordinated activity among the various muscles of the hindlimb to allow proper placement of the paw on the plantar surface (Majczyński et al. 2007, Liu et al. 2009). After various interventions, this uncoordinated activity can be transformed into muscle synergies that can give rise to effective placement of the paw on the plantar surface, with weight support throughout the stance phase of the locomotor cycle.

Our analysis of locomotor activity under various conditions in spinal rats (e.g., drug-induced locomotion, locomotion after intraspinal transplants) led us to suggest a means of defining plantar walking based largely upon EMG recordings, as illustrated in Figure 1. Here we compare typical data from a spinal rat two months after spinal cord injury during locomotor activity on a treadmill induced by tail stimulation (Fig.
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1, left side) with normal treadmill locomotion in an intact rat (Fig. 1A–D, right side) and a rat that received a graft to restore serotonin control of the CPG (Fig. 1E, right side). Figure 1E2 presents a photo of a walking spinal rat that received an intraspinal graft of serotonergic neurons, which recovered the plantar stepping pattern resembling natural walking of intact rats. The EMG activity recorded from the ankle extensor muscles (soleus, Sol) and the ankle flexor muscles (tibialis anterior, TA) of the left (l) and right (r) hindlimbs during an episode of treadmill locomotion is shown in Figure 1A. The disorganized activity seen in an untreated spinal animal is evident, and the pattern observed in an intact rat is clearly different. The placement of the plantar surface of the paw on the treadmill is a key feature of recovery toward the normal condi-

Fig. 1. Criteria for plantar stepping based upon EMG analysis. Tests were conducted with the experimenter manually placing the animal in the horizontal posture on the treadmill at speeds of 5 and 10 cm/s. Hindlimb movement was induced by tail pinching. In the left panels (A1–E1) are illustrated typical results obtained in spinal rats without any intervention. In the panels on the right (A2–E2) are results from intact (A2–D2) or repaired (E2) animals. The criteria that we use for detecting plantar walking are stated. (A) EMG – electromyography; (B) Linear envelops of rectified and integrated EMG; (C) Burst duration/cycle duration analysis (D) Inter- and intralimb coordination, 0 – onset of r TA; (E) Single frame from treadmill locomotion trials of experimental rats (left – spinal; right – grafted)
tion (Fig. 1E), and this is termed “plantar stepping” (Sławińska et al. 2012b, 2013). Using EMG recordings and some means of detecting weight support, it has been possible to establish criteria for plantar stepping, as illustrated in Figure 1. Placement of the paw on the plantar surface and some degree of weight support is necessary, and this is achieved by muscle synergies produced by activation of the locomotor CPG to produce the rhythm and the recruitment of coordinating interneurons. Sustained ankle extensor muscle (soleus) activity throughout the stance phase of locomotion is a necessary feature (Fig. 1A–B), resulting in the soleus muscle burst duration being strongly correlated with the step cycle duration (Fig. 1C), with an alternating brief period of ankle flexor muscle activity (TA) of consistent duration regardless of step cycle durations. Consistent intra- and interlimb coordination is necessary to allow proper placement of the paw for plantar stepping. In our experiments the trials are conducted with the experimenter manually placing the animal in the horizontal position on the treadmill, which allows any weight support applied by the animal to be readily detected. Other studies have employed force plates (Muir and Webb 2000), lever arms (de Leon et al. 2002), and height of the pelvis (Fouad et al. 2010) to detect weight support.

**DRUG-INDUCED LOCOMOTION**

There are a number of descending pathways that are effective in producing and/or controlling locomotor activity, and these have been reviewed elsewhere (Majczyński and Sławińska 2007, Jordan et al. 2008, Rossignol and Frigon 2011). We have focused on the capability of serotonin receptor activation to elicit locomotion, based on our finding that brainstem stimulation in a particular part of the medulla (the serotonergic parapyramidal region – PPR) elicits well-coordinated fictive locomotion (Liu and Jordan 2005). This was the first demonstration of a specific descending pathway with this capability, and it remains the only study defining a discrete population of locomotor command neurons. Other workers have recently demonstrated, using optogenetics, the capacity for glutamatergic neurons in the brainstem of a vesicular glutamate transporter 2–channelrhodopsin2–yellow fluorescent protein (Vglut2-Chr2-YFP) mouse to elicit locomotion (Hagglund et al. 2010). A discrete population of glutamatergic reticulospinal neurons defined by the transcription factors Lhx3 and/or Chx10 has been shown to possess many of the properties expected of a glutamatergic command pathway for locomotion (Bretzner and Brownstone 2013). Other descending pathways using a variety of transmitters possess this capacity, including dopamine and norepinephrine, but the most effective means of eliciting locomotion in rats with exogenous drugs has been with 5-HT (Schmidt and Jordan 2000). A combination of agonists and other treatments has been used to elicit locomotion (Antri et al. 2002, 2003, Landry et al. 2006, Courtine et al. 2009). In the most recent of these, 5-HT acting at 5-HT2 receptors has been implicated (Courtine et al. 2009, Fouad et al. 2010). We have focused on defining the 5-HT receptors important in the production of locomotion, and our results lead to the suggestion that the major receptors involved are 5-HT2A and 5-HT7.

In our investigations systemic administration of agonists of 5-HT receptors (5-HT2 - quipazine and 5-HT7/1A - 8-OHDPAT) facilitated locomotor hindlimb movements in the paraplegic rats placed in horizontal posture above the moving belt of the treadmill (Sławińska et al. 2012b). These results confirm the observation by Antri and coauthors (2005) that these two drugs in combination facilitate locomotor recovery in spinal rats. An important observation that emerged from our results was that although both quipazine and 8-OHDPAT improved locomotor-like activity, with coordinated plantar stepping resulting with either drug, interlimb coordination was significantly better after 8-OHDPAT than after quipazine. We propose that the activation of 5-HT2 receptors facilitates the rhythm generator neurons for locomotion, and activates the coordinating neurons (Schmidt and Jordan 2000, Liu and Jordan 2005, Pearlstein et al. 2011), while activation of 5-HT7 receptors directly influences rhythm generating neurons of the CPG and increases the excitability of motoneurons and output elements of the CPG. Combined treatment has been used by others (Landry et al. 2006, Courtine et al. 2009, Musienko et al. 2011) including the use of these two drugs in combination with epidural stimulation (equivalent to the tail stimulation used in our experiments and caudae equine stimulation *in vitro*) and locomotor training. In our experiments using only these two drugs, neither training nor any form of exteroceptive stimulation was necessary for plantar stepping after the combined drug treatment (Sławińska et al. 2012b).
It is well known that the action of quipazine could be mediated by 5-HT$_{2A}$, 5-HT$_{2B}$, or 5-HT$_{2C}$ receptors. The predominance of 5-HT$_{2A}$ receptors in control of locomotor movement after quipazine application was demonstrated in adult rats (Ung et al. 2008). The role of 5-HT$_{2A}$ receptors in the production of locomotion by stimulation of brainstem serotonergic neurons have been also implicated in neonatal rats (Liu and Jordan 2005). On the other hand, Murray with colleagues (Murray et al. 2010) claimed that for recovery of locomotor function after staggered hemisection the constitutively active 5-HT$_{2C}$ receptors are responsible. In their study 5-HT$_{2C}$ receptors were upregulated in the spinal cord below the level of injury. However, below a total spinal cord transection, in contrast to significant upregulation of 5-HT$_{2A}$ receptor mRNA, there were no changes in 5-HT$_{2C}$ receptor mRNA expression detected (Navarrett et al. 2012). The upregulation of 5-HT$_{2A}$ receptors after complete spinal cord injury was described in motoneurons (Jordan et al. 2010, Kong et al. 2010, 2011, Navarrett et al. 2012). We also have preliminary evidence using intrathecal drug application of serotonergic antagonists that in intact adult rats 5-HT$_{2A}$ receptors but not 5-HT$_{2C}$ receptors are involved in locomotor control (Sławińska et al. 2012a). Thus, except for the staggered hemisection preparation, the consensus seems to be that quipazine acts through 5-HT$_{2A}$ receptors.

Regarding the effectiveness of 8-OHDPAT for inducing locomotor movement the importance of both 5-HT$_{1A}$ receptors, and 5-HT$_{7}$ receptors, was also implicated since locomotion could be blocked by antagonists of either of them and application of 8-OHDPAT could facilitate locomotion in 5-HT$_{7}$ receptor knockout mice or in the presence of a selective 5-HT$_{7}$ receptor antagonist (Landry et al. 2006). On the other hand, there is growing evidence showing that 5-HT$_{1A}$ receptors control the activity of motoneurons (reviewed in Perrier et al. 2013) as well as locomotor interneurons (Noga et al. 2009). Thus, it is possible that the actions of 8-OHDPAT that do not require 5-HT$_{7}$ receptors are exerted on motoneurons rather than on locomotor CPG neurons. It was demonstrated that the decrease caused by 5-HT in NMDA-induced periodicity in fictive locomotion of the neonatal rat spinal cord in vitro was attenuated by pharmacological agonists of the 5-HT$_{7}$ receptor family (Beato and Nistri 1998). Moreover, it was shown that locomotor activity evoked by brainstem stimulation was depressed by effects of serotonin exerted on 5-HT$_{1A}$ receptors, while in the absence of these receptors the effect of increasing available 5-HT resulting in initiation of locomotion was mediated by 5-HT$_{7}$ receptors (Dunbar et al. 2010). So, since the effects of 5-HT$_{1A}$ receptor activation are normally a decrease in neuronal activity, while those mediated by 5-HT$_{7}$ are excitatory (Beato and Nistri 1998, Schmidt and Jordan 2000, Hochman et al. 2001, Perrier et al. 2013, Noga et al. 2009), it is possible that the effects of 8-OHDPAT that do not require 5-HT$_{7}$ receptors are exerted on motoneurons. So, since the effects of 8-OHDPAT that do not require 5-HT$_{7}$ receptors are exerted on motoneurons.

Fig. 2. Grafs of 5-HT neurons of different populations (B1–B3, B3 and lateral) are differentially effective for improving hindlimb locomotor movements of spinal rats. (A) Immunolabeling of a horizontal section of a brainstem showing 5-HT positive populations of neurons in an E14 embryo from which the tissue was dissected for intraspinal grafting of spinalized rats. (B1, C1, D1) Bilaterally well-coordinated EMG patterns in the soleus (Sol) and tibialis anterior (TA) muscles are seen in a spinal rat grafted with B1–B3 neurons (B1, top panel) vs. less coordinated EMG activity in a spinalized rat grafted with lateral or B3 only 5-HT populations (C1, D1). The same raw EMG records rectified, filtered, and normalized to the step cycle (onset of activity in the right TA considered the onset of the cycle), show left-right and flexor-extensor coordination in a rat grafted with B1–B3 neurons (B1, lower panel) vs. a rat grafted with lateral or B3 only 5-HT populations (C1 and D1, lower panels). Regression lines showing the relationship between step cycle durations and burst durations for the left and right TA and Sol muscles in three groups of grafted rats are illustrated (B2, C2, D2). Polar plots of the relationships between the onset of r TA burst activity and either the contralateral TA showing interlimb coordination and (B4, C4, D4) or the ipsilateral right Sol showing intralimb coordination. The 0 position on the polar plots corresponds to the onset of activity in the right TA muscle, and the positions of the filled circles (black) indicate the times of onset of activity in the left TA (B3, C3, D3). The polar plots in B4, C4, D4 show intralimb coordination, with the times of onset of ipsilateral Sol activity plotted in relation to the times of onset of the ipsilateral right TA. E1 provides bar diagrams showing the mean slopes (±SD) for the regressions in three groups of grafted rats (B3, C3, D3). E2 presents bar diagrams showing the means (±SD) of the angles of the left-right TA (l-r) and of the right Sol–right TA (r S-T) relationships (relative timing of the onsets for the two muscles) represented in each polar plot in three groups of grafted rats animals. Bar diagrams in E3 show the means (±SD) of r values between the times of onset of activity in both left-right and flexor-extensor EMG comparisons in three groups of grafted rats. Statistical significance (Student’s t-test) comparing the animals grafted with various populations of 5-HT neurons is indicated by red stars: * P<0.05; ** P<0.005. The comparisons include rats with the B1–B3 transplants (n=7), rats grafted with the lateral group of 5-HT neurons (n=5) or rats grafted with only the B3 population (n=4).
Heckman et al. 2009, Dunbar et al. 2010), we have focused on the latter as the most likely mediator of 5-HT activation of the locomotor CPG.

In general, therefore, interpretation of the effectiveness of these two drugs, quipazine and 8-OHDPAT, requires consideration of both the type of preparation and the various sites of action of the drugs that are likely to be involved in enhancing locomotor hindlimb movement.

We have argued that the problem in spinal rats that impairs locomotion is the loss of some descending system that controls coordination (Jordan and Ślawińska 2011). This is based on the observation that without descending serotonin control, there is rhythmic activity in the hindlimbs, but it is not sufficient to produce coordinated plantar stepping. The rhythm generator is functional, but its output is disturbed such that co-contractions of antagonists occur, and interlimb coordination is impaired. Addition of 5-HT or its agonists appears to restore coordinated muscle activity (Pearlstein et al. 2005, Liu et al. 2009, Jordan and Ślawińska 2011). We have shown that spinal locomotion improved by quipazine is disrupted by strychnine, which confirms a role of glycinergic interneurons in reciprocal inhibition and interlimb coordination (Pearlstein et al. 2005, Boulenguez and Vinay 2009, Jordan and Ślawińska 2011). Other factors controlling these same cells can be expected to emerge.

LIMITATIONS OF DRUG-INDUCED LOCOMOTION

Even though the serotonergic agonists are effective in eliciting coordinated hindlimb locomotor movements, in our experience it is difficult to obtain a sustained effect using this approach. Thus, the use of these drugs to elicit well-coordinated plantar stepping indicates that activation of this system can be effective, but systemic administration is unlikely to be a solution for restoring functional stepping. Some of the limitations of the systemic drug application approach are discussed below.

As pointed out below, serotonin can interfere with the natural sensory feedback processes that promote coordinated locomotion. It is also the case that these drugs can have other unwanted side-effects. Tachyphylaxis, defined as the rapid appearance of a progressive decrease in the response to a given dose after repetitive administration of a pharmacologically or physiologically active substance, is an obvious drawback to the repeated use of drugs to elicit locomotion (definition provided by Stedman's Medical dictionary 2000). If this occurs, increasingly large doses are required to achieve the desired effect. This may be due to alterations in the receptors over prolonged treatment or produced by the injury. For example, work in Edmonton has provided evidence that certain 5-HT receptors become constitutively active after injury (Murray et al. 2010). Some such effects are likely to occur with repeated doses of 5-HT agonists, making the efficacy of this approach limited.

Side effects of serotonin treatment can also occur, and the most dramatic of these is the Serotonin Syndrome, which can include: agitation or restlessness, confusion, rapid heart rate and high blood pressure, dilated pupils, loss of muscle coordination or twitching muscles, heavy sweating, diarrhea, headache, shivering, goose bumps (Volpi-Abadie et al. 2013). Severe serotonin syndrome can be a life-threatening condition.

As the effects of the Serotonin Syndrome symptoms suggest, there are peripheral effects of systemic administration of 5-HT and its agonists. These include effects on the heart, respiratory system, gut, vessels and the immune system. The effects of systemic actions of 5-HT and its agonists are therefore widespread, and the possible effects of sustained applications of drugs to induce locomotion are unknown. It seems likely that the efficacy of this approach may be limited by such side effects.

LIMITATIONS OF COMBINATORIAL APPROACH

Recent efforts to restore locomotion after spinal cord injury have incorporated a number of different approaches in combination, including systemic drug application, and at least one has resulted in potent protection for the procedures followed (Guertin et al. 2011). An example is the use of systemic drug application in combination with electrical stimulation of the surface of the spinal cord below the level of the lesion, and locomotor training with the animals held in a jacket over a treadmill or walkway in the upright posture, with only the hindlimbs available to support the animal’s weight. Pharmacological enhancement of locomotor recovery after SCI has been coupled with the benefits of training and epidural stimulation (Fong
et al. 2009). Courtine and coworkers (2009) reported the use of specific combinations of pharmacological and electrical stimulation interventions, together with locomotor training, could induce full weight-bearing locomotion in the upright posture. Their pharmacological treatment consisted of a combination of 5-HT\textsubscript{2} and 5-HT\textsubscript{1A/7} agonists. Subsequently this same group has expanded the pharmacological treatment to include pharmacological modulation of serotonergic, dopaminergic and noradrenergic receptors to facilitate specific features of locomotion (Musienko et al. 2011). They describe facilitation of consistent locomotor movements with 5-HT\textsubscript{1A/7} agonists, and they improved weight bearing abilities with 5-HT\textsubscript{2A/C} agonists. The results of these combinatorial approaches can be dramatic, but there are drawbacks that need to be pointed out.

First, the system of neurons responsible for coordination can also be activated from the plantar surface of the paw (Sławińska et al. 2012b). In this case placing the animal in the upright posture and allowing the paws to be artificially placed so that cutaneous receptors detect the presence of weight support induces well-coordinated stepping similar to that provided by 5-HT agonists or transplants. This effect can be blocked by anaesthesia of the foot. A balance exists between the 5-HT effect and the afferent feedback effect however, because in some cases the combination of the two leads to disordered coordination (Sławińska et al. 2012b). Recognition of this effect of paw afferent feedback should be taken into account when interpreting the data that has arisen from experimental paradigms where rats are placed in an upright posture to detect their recovery from spinal cord injury. This posture alone clearly facilitates locomotor activity in the absence of any other intervention (Sławińska et al. 2012c).

The ability of epidural electrical stimulation of the spinal cord to elicit locomotion is a promising intervention that is now being tested clinically (Harkema et al. 2011). It was first demonstrated in spinal cord injured patients (Dimitrijevic et al. 1998). It was subsequently shown to be effective in the cat spinal cord, and various animals models have allowed the search for the most effective sites and modes of delivery to persist. In rats and mice the effects are also encouraging, as recently reviewed (Gerasimenko et al. 2008, Boulenguez and Vinay 2009). Afferent feedback is important for epidural stimulation evoked locomotion, because contact of the plantar surface of the foot with the treadmill and partial weight support is necessary (Ichiyama et al. 2005). After T12–S2 unilateral deafferentation (Lavrov et al. 2008), epidural stimulation is less effective for eliciting stepping on the deafferented side. Recent experiments have confirmed the efficacy of epidural stimulation in humans (Harkema et al. 2011), and have demonstrated that the procedure can influence other functions besides locomotion. The epidural stimulation approach is likely to continue to be developed for restoring function after spinal cord injury, but its combination with repeated systemic drug applications to achieve well-coordinated locomotion has the limitations described above. It is possible that some form of electrical stimulation can be used in combination with cell replacement therapy to some advantage, however.

RESTORATION OF LOCOMOTOR FUNCTION BY INTRASPINAL GRAFTING OF SEROTONINERGIC EMBRYONIC CELLS

Various methods for local intraspinal application of monoaminergic drugs are being explored, as pointed out above. One promising approach is the grafting of embryonic cells destined to release monoamines taken from the brainstem into the spinal cord below the site of the lesion. It has been demonstrated that these embryonic cells can survive and release monoamines in the host environment. The results of first homologous fetal brain grafts were published almost 40 years ago (Nygren et al. 1977). The development of the transplantation approach has been reviewed in detail (Vrbová and Sławińska 2012). The improved hindlimb locomotor function restoration brought about by grafted serotonin-releasing cells was shown for the first time to be mediated in part by 5-HT\textsubscript{2} receptors, since cyproheptadine (5-HT\textsubscript{2} antagonist) IP application disrupted the hindlimb movement restored by the graft (Majczyński et al. 2005).

In our recent research we have extended these findings by demonstrating the effectiveness of grafting of different populations of serotonergic cells in adult rats. There are three recognized populations of 5-HT neurons (B1, B2, B3) that send their axons into different parts of spinal cord grey matter. The axons of neurons located in raphe obscurus and raphe pallidus (B1 and B2) terminate in the ventral horn. Raphe magnus neurons (B3) send axons to the dorsal horn and to the ventral horn as well. The precise termination of neu-
rons located in the parapyramidal region (PPR), classified as a part of B3, are unknown. However, there is information that shows the more laterally placed 5-HT neurons are derived in part from more caudal descending 5-HT groups (Tork 1990). Moreover the sources of serotonergic fibers present in laminae VII and X in the spinal cord are not yet known. The molecular genetics of the brainstem 5-HT system is now emerging (Cordes 2005, Jensen et al. 2008, Bang et al. 2012). We presume that different cell population can play different roles in control of locomotor movements, and we explored this possibility in our recent publication (Sławińska et al. 2013), where we used all three populations of 5-HT neurons (B1, B2 and B3).

In our recent paper (Sławińska et al. 2013) we demonstrated that for better recovery of hindlimb locomotor movements in paraplegic rats simultaneous supplementation of various populations of 5-HT neurons (B1, B2, B3) that innervate different parts of the spinal cord network is required. We have now extended this research to include a comparison of B1–B3 grafts with grafts of neurons from a more lateral region of the fetal brainstem and from the B3 region only (Fig. 2A). The effects of these grafts on locomotion are illustrated in Figure 2 (panels C and D). These effects were compared with those obtained from transplants of the B1, B2 and B3 regions (Fig. 2B). The results show that the lateral transplants give rise to plantar stepping with extensor muscle activity related to the step cycle duration. This is clear in the regression lines comparing burst duration to cycle duration (Fig. 2). However, coordination is impaired compared to the B1–B3 transplanted animals, as illustrated in the polar plots in Figure 2B for the B1–B3 grafted animals and Figure 2C for the animals with grafts taken from the lateral group of 5-HT neurons in the fetal brainstem. Figure 2D shows the locomotor activity produced in animals with grafts from the B3 region only. The quality of plantar walking in these animals was significantly less than in the other animals. Significant differences were observed between the three groups of animals when intra- and interlimb coordination was compared (Fig. 2E). We conclude from these observations that the lateral group of neurons is capable of restoring some features of plantar walking, but the restoration of intra- and interlimb coordination is incomplete. The poor locomotion observed in the B3 grafted animals in comparison to other groups of grafted rats may be associated with the absence of 5-HT innervation of the area around the central canal and lamina VII. Comparisons of the walking produced by each of these grafts with control spinal animals that received no grafts reveal that all the grafted animals that received a piece of lateral brainstem or the B1–B3 serotonergic populations were significantly better than control spinal animals. Improvement of locomotion in those that received only B3 serotonergic cells was not statistically significant (to see control rats see Sławińska et al. 2013).

Figure 3 shows examples of the distribution of 5-HT fibers found in the B1–B3 and lateral graft animals. It was a consistent observation that the B1–B3 grafts provided robust input to the area around the central canal, as well as cells in lamina VII and the dorsal horn in the area near the graft (Fig. 3A). Furthermore, there was often substantial innervation of motoneurons in lamina IX of the lumbar spinal cord (Fig. 3B). The animals that received the grafts from the lateral region of the fetal brainstem only showed consistent innervation of the intermediolateral cell column (IML), the central canal, and some innervation of motoneurons in the area of the spinal cord nearest the transplant (Fig. 3C). The innervation was consistently more sparse in the lateral graft cases than in the B1–B3 cases. In more caudal areas of the spinal cord, there was innervation of the central canal region and some sparse innervation of lamina VII, but little innervation of the caudal motor nuclei (Fig. 3D). The B3 grafted animals illustrated in Figure 2D did not reveal any 5-HT innervation in the central canal area (data not shown).

The innervation provided by the grafted cells appears to be guided to some degree by the target cells they were originally intended to innervate. This is consistent with the observations of Rajaofetra and colleagues (1992), who showed discrete innervation patterns for different groups of 5-HT neurons derived from different parts of the fetal brainstem. One issue that arises from these observations is whether merely the restoration of some 5-HT in the lesioned spinal cord is sufficient to facilitate plantar stepping, rather than reinervation of specific spinal neurons, so that any 5-HT neurons can produce the effect. We argue that the innervation of certain groups of neurons is necessary, however, because recovery of some aspects of plantar walking is associated in all cases with innervation of the area around the central canal, a region where many neurons active during locomotion are
located (Dai et al. 2005). Neurons in this area of the cat spinal cord that are active during locomotion receive 5-HT input and are also rich in 5-HT$_{2A}$ and 5-HT$_7$ receptors (Noga et al. 2009). Locomotor cells in this region have been shown to be excited by 5-HT (Dai et al. 2009). It is noteworthy that left-right coordination is consistently improved by the grafts, because commissural neurons have been shown to be excited by 5-HT as well (Carlin et al. 2006, Zhong et al. 2006). In our experiments, interlimb coordination is impaired in animals with sparse or no 5-HT innervation of the area around the central canal. Furthermore, plantar stepping is poorly developed in the B3 transplanted animals that had no 5-HT innervation of the central canal area. Thus, there is an abundance of evidence implicating neurons of this area in the production of locomotor activity in both cat and rat preparations. The neurons responsible for this effect have not yet been identified.

In our recent investigations we made an effort to determine the relative contributions of 5-HT$_2$ and 5-HT$_7$ receptors to transplant-induced locomotor hindlimb movement recovery (Sławińska et al. 2013). The crucial role of 5-HT$_2$ receptors was confirmed using systemic application of cyproheptadine, an antagonist at 5-HT$_{2A/2B/2C}$ receptors, which altered left-right coordination and the cycle duration of hindlimb locomotor movement in grafted spinal rats. Moreover, cyproheptadine application shortened soleus burst duration and decreased its peak amplitude. Thus, our results showed that blockage of 5-HT$_2$ receptors interfered with the ability of the graft-derived intraspinal serotonin to activate components of the CPG as well the motoneurons. In addition we established that blockade of 5-HT$_7$ receptors using SB269970 (an antagonist of 5-HT$_7$ receptors) impairs inter- and intralimb coordination in locomotor hindlimb movements of grafted spinal rats. It is important to note that

Fig. 3. Immunolabeling of transverse spinal cord sections showing 5-HT-positive neurons and fibers in rats grafted with different populations of 5-HT neurons. (A) shows the 5-HT positive neurons present in a large graft occupying the central region of the gray matter and numerous 5-HT positive fibers within and emerging from the graft of the B1–B3 serotonergic populations. (B) shows example of 5-HT fibers in close proximity to 5-HT$_{2A}$ receptor positive motoneurons in the ventral horn at the L3 spinal cord level of spinal grafted rat with the B1–B3 serotonergic populations. (C) and (D) show 5-HT-positive neurons and fibers present in the ventral horn, around the central canal and in the intermediolateral zone emerging from a graft of the lateral population of serotonergic brainstem neurons. We found that in the lateral and B3 only (data not shown) grafted spinal rats there are fewer 5-HT positive fibers in the ventral horn and intermediolateral zone compared with B1–B3 grafted rats.
in contrast to cyproheptadine, SB269970 did not alter muscle activity (burst duration or amplitude). Thus, our results show that blockade of 5-HT\textsubscript{7} receptors alters activity of interneurons responsible for generating the reciprocal pattern of flexor and extensor motoneuron activity, as well as neurons responsible for coordinating activity in the two hindlimbs during hindlimb plantar stepping of grafted rats but does not influence motoneuron excitability directly. Our results allow us to conclude that both receptors, 5-HT\textsubscript{2} and 5-HT\textsubscript{7}, mediate the improved locomotor movements in spinal rats after grafting, but through actions on different populations of spinal locomotor neurons. We are persuaded that 5-HT\textsubscript{2} receptors control CPG activation as well as motoneuron output, while 5-HT\textsubscript{7} receptors contribute primarily to activity of the locomotor CPG and associated interneurons involved in intra- and interlimb coordination.

**FUTURE PROSPECTS FOR CELL REPLACEMENT THERAPY**

One promising idea for the cell replacement strategy is the use of defined populations of neurons for replacement therapy. There is evidence showing that grafted embryonically defined motoneurons leads to significant functional improvement following ventral root avulsion. It has been shown that in adult rats the missing motoneurons can be replaced by embryonic grafted neurons and their axons can be guided to denervated muscles via a reimplanted ventral root so the grafted motoneurons contribute to the innervation and functional recovery of the denervated muscles (Nógrádi and Vrbová 1996, Gal et al. 2012). As we described above the transplantation of discrete populations of embryonic serotonergic cells into the spinal cord below the total transection of adult rats give rise to improvement of plantar stepping (Sławińska et al. 2000, Majczyński et al. 2005, Sławińska et al. 2013). Other sources of defined cell populations are now being developed, particularly the use of stem cells directed to a particular lineage. As reviewed by Rossi and Keirstead (2009), transplantation of cells with an oligodendroglial lineage (Totoiu et al. 2004, Keirstead et al. 2005), Schwann cells (Kohama et al. 2001), olfactory ensheathing cells (Imaizumi et al. 1998), and various stem cell types (Brustle et al. 1999, Keirstead et al. 2005, Nistor et al. 2005) remyelinates axons in animal models with demyelination. Restoration of motor neuron circuitry occurs following transplantation of motor neuron-primed human neural stem cells (Gao et al. 2005, 2007) or mouse embryonic stem cell derived motoneurons (Deshpande et al. 2006) into models of motoneuron disease. Cholinergic processes exit the ventral root and travel through the sciatic nerve to form physiologically active neuromuscular junctions. This raises the question of whether cell replacement therapy using stem cells might be developed for replacement of serotonergic neurons. Recently a new method allowing induction of serotonergic neurons from embryonic stem and pluripotent stem cells has been described (Shimada et al. 2012). It was found that noggin, a known antagonist of bone morphogenetic protein, induced embryonic stem cells to express genes involved in serotonergic differentiation, such as Nkx2.2, Pet-1, Sonic hedgehog, tryptophan hydroxylase 2, and serotonin transporter. These cells could also demonstrate high potassium-induced release of serotonin. Thus, this method might provide an option for serotonergic differentiation of pluripotent stem cells and can open some new therapeutic perspectives for developing a strategy aiming to replace missing serotonergic innervation in various pathological situations including spinal cord injury.

**CLINICAL IMPLICATIONS**

The results of experiments on animals can be used as a basis for development of clinical approaches for restoring locomotion. Locomotor training is now being subjected to clinical trials. An early report consolidating data from a number of centers has been published (Dobkin et al. 2007), but the human locomotor training approach has only produced anecdotal evidence in favour of a beneficial effect, in contrast to the animals studies. Nevertheless, these approaches are still being applied clinically. For example, a recent case report (Harkema et al. 2011) has incorporated epidural stimulation of the lumbar spinal cord and demonstrated effects on voluntary movement, standing, and assisted stepping after motor complete paraplegia. It can be expected that other combinations of approaches that have had positive reports in animals will continue to be applied in humans, including systemic and/or epidural drug applications.

Could interventions such as replacement of serotonergic neurons be a viable clinical approach for functional recovery after spinal cord injury? Pearse and
Bunge (2006) have reviewed the problems associated with designing cell- and gene-based regeneration strategies to repair the injured spinal cord. The use of Schwann cells to promote regeneration (Williams and Bunge 2012) is now in the clinical trial stage, and U.S. Food and Drug Administration approval has been awarded. This is a promising first step in the development of cell replacement therapies for spinal cord injury. In the case of replacing serotonergic neurons, the positive preclinical data is all from fetal cell transplants, although 5-HT secreting cell lines injected intrathecally have been attempted (Eaton et al. 2011). This approach did not result in significant improvement in locomotor control, however. As the data reported here suggest, it will be necessary to develop ways to create not only 5-HT neurons, but those destined to become specific descending 5-HT neurons, including neurons from the B1, B2 and B3 groups. Further refinement of the tools to recognize and guide the development of these specific neurons is necessary. Furthermore, methods to control the transplanted cells have yet to be developed, and a means of providing a supply of appropriately differentiated 5-HT neurons for transplantation into the spinal cord has not been developed. The cells also must be in the right stage of development, as is the case with the E14 embryonic cells used in our experiments, otherwise they will not be able to grow and find appropriate targets. This is an area of research with considerable potential, however, because even without any means to increase the activity of the command neurons, they provide facilitation of locomotion. It will be advantageous to develop methods to allow for voluntary control of the activity of these transplanted cells as well. The transplanted cells have specific receptors that allow them to be selectively activated after transplantation using intrathecally applied drugs that have minimal effects on the spinal cord below the lesion but can activate the transplanted neurons. Less specific activation tools include epidural and intraspinal stimulation. The potential for these approaches to selectively activate the transplanted neurons is not yet known. The electrophysiological and pharmacological tools that are standard ways of activating spinal cord neurons need to be developed in the laboratory. It is now possible to selectively activate genetically defined transplanted neurons. One such approach is optogenetics, were identified 5-HT neurons can be engineered to express ChR2 so that a light stimulus can activate the transplanted cells. Alternatively, it is possible to use systemic application of a designer drug to activate “designer receptors exclusively activate designer drugs” (DREADDS) (Dong et al. 2010).

**CONCLUSION**

In conclusion, we argue that replacement of 5-HT neurons responsible for the initiation and control of locomotion is a worthy research goal in the spinal cord injury field. What is left to do is to further define the 5-HT neurons suitable for this approach, ones that can survive and innervate the appropriate targets, particularly the area around the central canal and the motoneuron area. The cells must be at a stage of development equivalent to the E14 neurons used in successful fetal cell transplants, and they must be destined to develop into the cells of the B1–B3 groups that are likely to provide maximal functional recovery. Finally, methods of controlling the activity of these cells need to be developed.

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