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Running Title:

Intraspinal stimulation caudal to spinal cord transections in rats. Testing the propriospinal hypothesis.

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Abstract

Many laboratories have reported the successful regeneration of neurons across damaged portions of the spinal cord. Associated improvements in hindlimb locomotor movements have been attributed to the formation of functional neuronal connections with the locomotor central pattern generator (CPG). But regenerating axons generally extend no more than 10mm caudal to the lesion sites, terminating about 20mm short of the lumbar segments thought to contain the CPG. It has therefore tacitly been assumed that the locomotor improvements were due to activation of propriospinal neurons relaying excitation to the CPG. Here we report a test of this assumption, which we call the propriospinal hypothesis. Intraspinal microstimulation (ISMS) was used to activate the putative propriospinal relay neurons. Approximately 2-3 weeks after complete spinal cord transection at T8-T9 in rats, an array of 6 Pt-Ir microwires was chronically implanted in the intermediate and ventral grey matter of T10-T12 segments. ISMS pulse trains with amplitudes of 0.8-0.9 times threshold for activating axial muscles were delivered during open field locomotor tests (BBB). ISMS significantly increased BBB scores over control tests, but did not produce limb coordination and weight-bearing sufficient for locomotion. These results support the main assumption of the propriospinal hypothesis, namely that neuronal activity elicited in thoracic spinal segments caudal to a complete spinal cord transection may propagate caudally and activate the locomotor CPG.

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Introduction

Many recovery strategies are currently under development to restore spinal cord function after injury. Unlike peripheral nerves, damaged neurons in the central nervous system (CNS) fail to regenerate, apparently because of the formation of impenetrable scar tissue (Fawcett and Asher 1999) and the presence of growth inhibitory molecules (Caroni and Schwab 1988; Schwab and Thoenen 1985). Aguayo and colleagues pioneered the use of peripheral nerve grafts to achieve long distance axon regeneration in the CNS (Aguayo et al. 1981; Bray et al. 1987). Numerous types of graft and molecular strategies to promote regeneration have since been studied, particularly in rats. In some cases neuronal regeneration across complete spinal cord transections was associated with significant functional improvements, including the restoration of hindlimb weight-bearing and coordinated locomotion (Cheng et al. 1996; Coumans et al. 2001; Ramon-Cueto et al. 2000). These results are particularly crucial because completely spinalized rats do not recover weight-bearing locomotion spontaneously (Basso et al. 1996), though they may do so with extensive locomotor training (Edgerton et al. 2001). Another surprising feature of these results is that significant improvements were observed even though the extent of regeneration was quite limited. Typically the regenerated axons extended only one or two spinal segments below the lesions, which were usually in the mid- to lower thoracic segments (Bamber et al. 2001; Ramon-Cueto et al. 1998; Rapalino et al. 1998). In most cases this meant that the regenerating axons terminated about 20 mm short of the lumbar spinal cord containing the hindlimb locomotor pattern generator (Cazalets et al. 1998; Cazalets et al. 1995; Cowley and Schmidt 1997; Kjaerulff and Kiehn 1996; Kiehn and Kjaerulff 1998).

What is the mechanism of the functional recovery accompanying the regeneration of axons below such lesions? One plausible explanation that is tacitly accepted in the regeneration field is that regenerating axons make connections with propriospinal neurons (PSNs), which extend caudally

and activate hindlimb locomotor circuitry in the lumbar spinal cord (Figure 1A). We now propose the term "propriospinal hypothesis" to describe this explanation. Recently (Bareyre et al. 2004) found that injured corticospinal neurons sprouted and made functional connections with long PSNs projecting to the lumbar spinal cord. The propriospinal hypothesis would require that such connections are also made by transected axons growing through and beyond a complete spinal cord lesion (Figure 1A). Furthermore, the hypothesis would require that non-specific activation of these descending PSNs would activate elements of the CPG sufficiently to cause the observed locomotor improvements (Figure 1B). The aim of our study was to test this latter part of the propriospinal hypothesis by non-specifically activating PSNs with intraspinal microstimulation (ISMS) caudal to a complete spinal transection.

Materials and Methods

Animals. The experiments were performed on 11 adult Sprague-Dawley female rats weighing 200-250g. Animal treatment and surgical procedures were approved by the University of Alberta Health Sciences Animal Welfare Committee. They conformed to the guidelines of the Canadian Council on Animal Care.

Spinal cord transection. Animals were deeply anesthetized with isoflurane and underwent an aseptic laminectomy at the level of the T8 spinal segment. Complete spinal cord transections 2-3 mm wide were produced with the use of fine suction pipettes at the T8 segment until no neural tissue remained and the inside of the dura could be visualized over the entire circumference. The musculature and skin were suture closed, analgesic (Buprenorphine, 5 μg/kg every 8 hrs) was administered and animals were placed in a heated environment for postoperative recovery. The bladder was emptied manually 3-4 times daily for the next two weeks until reflex voiding was established.

Implantation of electrode array. Approximately 2-3 weeks after complete spinal cord transection, when bladder and bowel functions had recovered, the animals were anesthetized again and underwent a laminectomy of T9 and T10 vertebrae. An array of 6 platinum-iridium microwires (diameter - 25μm), insulated except for the beveled tip, arranged in two rows and spaced 2 mm apart was implanted to stimulate bilaterally intermediate and ventral grey matter one segment below the lesion (Figure 1B). The implant physically occupied just over one thoracic segment (Waibl 1973). The microwires were inserted manually into the dorsal surface of the spinal cord, about 1mm lateral to the midline, a 5mm length of photocopy transparency served as a guide to insertion. Though care was taken to insert the wires as vertically as possible into the spinal cord, histology in 4 animals revealed that the actual tip positions ranged from 0 to 2mm lateral to the midline. However, prior studies have shown that propriospinal neurons active during locomotion are located throughout this medio-lateral range, so we feel that the electrode positions achieved were appropriate for the purpose of hypothesis testing (Dai et al. 2005). The implantation and stabilization procedures were based on a technique developed for intraspinal microstimulation (ISMS) in cats (Mushahwar et al. 2003). The electrode leads were spot-glued to the dura with cyanoacrylate and covered by a thin piece of plastic film to prevent connective tissue from dislodging the implant. A silastic tube carrying the microwires was secured to the T11 spinous process with dental acrylic. The microwires led percutaneously to a connector, which was secured to the skin overlying the sacrum with sutures. Post-operative recovery and subsequent bladder and bowel management were as described above.

Behavioural testing. Following postoperative recovery, we filmed and rated locomotor performance before and during ISMS using the standard BBB open-field locomotor rating scale (Basso et al. 1995). The BBB test was chosen to allow comparisons between our results and those of many published regeneration studies. The bladder was manually emptied and motor thresholds of each of the implanted electrodes were determined 30 minutes prior to the experiment. Animals

were rated in two successive 4 minute sessions, a control session without ISMS and a test session with ISMS. The threshold of each microelectrode for eliciting contractions of trunk and abdominal muscles was individually determined by careful visual observation and palpation prior to open-field testing sessions. During the test session with ISMS, trains of stimulus pulses (biphasic, 200μsec, 50s-1) with amplitudes 0.8-0.9 times threshold (20-200μA) were delivered through each microelectrode in the ISMS array in an interleaved sequence, such that the action of each microelectrode was independent of the others. The testing was conducted for 1-3 weeks after implantation until the local motor thresholds exceeded 300μA. The change in the threshold could have been caused by microelectrode migration over the course of several weeks or it could have been due to electrode encapsulation, local tissue damage, or gradual failure of insulation at the connector.

Histology. At the end of the experiment animals were deeply anesthetized with sodium pentobarbital and perfused through the heart with a 3.7% formaldehyde solution. Thoracic and lumbar spinal columns were extracted and the positioning of the arrays within the T10-T11 spinal segments was confirmed using thoracic vertebrae as reference. We were only partly successful in histologically identifying microelectrode tip positions in the chronically implanted animals. The extensive growth of connective tissue in the area of the spinal cord injury extended to the site of implantation and provided a challenge to remove without dislocating the microwires. In 4 animals, the thoracic segments of the spinal cord with embedded microelectrodes were manually sectioned and the relative positions of the implanted microelectrode tips were established to confirm the accuracy of targeting during implantation.

Statistics. To compare changes in the in the BBB scores in response to the microstimulation, the difference in scores before and during stimulation is reported as mean ± standard deviation (s.d.). The critical significance level α was set at 0.05. A result was considered significant if the

achieved significance p-value was lower than α. Bootstrap analysis was used as an additional nonparametric test with the same α. Bootstrapping is a procedure for estimating the distribution of a data set by resampling with replacement from the original sample. The variation of the resulting difference between the scores was achieved by comparing the difference between randomly chosen data samples 10000 times. The confidence interval was then calculated as 2.5 and 97.5 percentiles of the resulting distribution. This method is effective for testing mediation for small samples of data without the requirement for the normality assumption to be met (Efron and Tibshirani 1993).

Results

To test the "propriospinal hypothesis" rats were implanted with ISMS arrays about one spinal segment below a T8 lesion, at least 10 mm rostral to the L1 spinal segment. Figure 2A shows the relative position of the tips of the implanted electrodes, which targeted intermediate and ventral grey matter. Figure 2B shows a bar plot of the average scores 10 days before and 10 days after the implantation and the individual animal scores (connected with a line). In spite of the small diameter (25μm) of the electrodes, the implantation procedure was associated with a significant 1.3±1.3 ($p=0.01$) point decrease in BBB scores during the period of 10 days after the implantation. The likely cause is mechanical damage of neuronal tissue by the penetrating microwires. Though post-mortem histological sections showed little obvious damage with the 25μm electrodes used, local damage was more evident in pilot studies with 30μm electrodes. Because recovery after complete spinal cord transection peaks within a week after injury (Basso et al. 1996), the single earliest sessions performed in 3 animals within a week after transection may have slightly decreased the mean score before microelectrode implantation. The decrease in BBB scores after the implantation provides indirect evidence that the implanted section of spinal cord contributed to the generation of spontaneous hindlimb movements.

BBB scores increased significantly in 8 out of 11animals during the test session with ISMS, even though animals were less prone to explore the test enclosure after the 4 min control test that immediately preceded each ISMS test. Figure 3A shows a sequence of frames demonstrating the hindlimb movements of the animal with the biggest response to ISMS. The sequence shows extensive coordinated movements around 3 joints when stimulation was applied, in contrast to the control trial when this animal only produced occasional single joint movements. On average, the scores of all animals significantly increased in response to ISMS by 1.5±2.04 ($p=0.02$). Notice that in Figure 3B, which shows individual scores in the same format as in Figure 2B, two animals had high scores in the control sessions. These same animals had developed a persistent urinary infection, which, as a source of irritation, might have contributed to the additional inputs from the sacrocaudal afferents to the pattern generating networks (Strauss and Lev-Tov 2003). It is well known for example that bouts of air-stepping can be initiated in spinalized rats by the procedure of bladder expression. To avoid biasing from these 2 animals and the animal with the highest response, the data from the remaining 9 animals were tested separately, but the difference remained significant, 1.2±1.45 ($p=0.03$). Because BBB scores of individual animal performance may not be normally distributed (see Figs. 7-8, Schucht et al. 2002), we used the Bootstrap test of significance, which does not rely on the assumption of a normal distribution (Efron and Tibshirani 1993). Figure 3C shows a resampled population of the differences of BBB scores between the control and test conditions. The median difference of 1.5 (solid grey line) remained significant for 95% confidence interval [0.4, 2.7] (dashed grey lines).

It is important to note that though the change in the motor performance was very small, it was comparable to the changes reported in numerous regeneration studies. Furthermore, a difference of 1.5 on the BBB scale represents an almost 50% increase on the baseline score for chronic spinal rats. This indicates that non-specific electrical activation of T10-T11 spinal segments can

modestly improve locomotor performance of rats with complete spinal transections assessed by the BBB open-field locomotor rating scale.

Discussion

This study demonstrates that tonic ISMS of gray matter immediately caudal to a complete spinal cord transection, and several segments rostral to the region assumed to contain the locomotor CPG improves locomotor performance in adult rats. The extent of the improvement scored according to the BBB open-field locomotor scale was comparable to that after long-distance axon regeneration below the site of a spinal cord injury (GrandPre et al. 2002; Hausmann et al. 2002; McDonald et al. 1999; Tuszynski et al. 2003; Verdu et al. 2003). Overall, these results support an important component of the propriospinal hypothesis, namely that nonspecific activation of descending PSNs may partly activate the locomotor CPG.

PSNs are located in ventral as well as in dorsal laminae of the spinal cord and in fact are likely to represent the majority of spinal neurons (Chung et al. 1984; Chung et al. 1987; Menetrey et al. 1985; Skinner et al. 1979). Midthoracic PSNs are likely to be involved in coordinating the activity of the cervical and lumbar enlargements and thus mediating forelimb-hindlimb coupling (Juvin et al. 2005). Our choice of intermediate and ventral areas as targets for stimulation was based on the evidence that sparing of the gray matter in these areas after spinal cord injury is more correlated to locomotor recovery than sparing of dorsal laminae (Schucht et al. 2002; You et al. 2003). However, this does not rule out the potential importance of dorsal propriospinal pathways, which may also be involved in the activation of the hindlimb locomotor CPG. This is supported by the evidence of locomotor recovery induced by epidural stimulation of the most caudal thoracic and lumbar segments below a spinal cord lesion (Gerasimenko et al. 2003; Ichiyama et al. 2005).

Three alternative possibilities should be mentioned. The first is that ISMS (or axons regenerating through grafts) could activate neurons that elicit contractions in local trunk muscles. These contractions could stretch hip muscles and evoke proprioceptive feedback to the locomotor CPG (Giszter et al. 1998). This in turn could improve performance in the open-field locomotor tests, without any direct activation of descending PSNs. Though we cannot eliminate this mechanism, it does not satisfactorily explain improvements we observed in foot and toe movements in the absence of improvements at the hip.

The second possibility is that we may have activated sensory axons, which have a low threshold to ISMS (Gaunt et al. 2006). Thus we may have antidromically activated terminal branches of group I and II afferents, which project rostrally and caudally from their entry points in the dorsal columns to provide excitatory input to motoneurons up to two segments away (Henneman and Mendell 1981). We doubt this for two reasons. First, all afferent projections descending from dorsal roots T8 and above were severed by the T8 transection. Second, the stimulated areas were well within the range of secondary spinal cord injury, where remaining axonal pathways go through a process of extensive demyelination and retraction (Beattie et al. 2000). It is therefore unlikely that we were antidromically stimulating afferents of more caudal thoracic or even lumbar segments, though again the possibility cannot be entirely ruled out.

The third possibility is that neuronal networks in the caudal part of the thoracic spinal cord, which have been shown to be capable of generating rhythmic activity in neonatal rats (Cowley and Schmidt 1997 19) are in fact part of the hindlimb locomotor pattern generator. ISMS of thoracic oscillators could conceivably generate rhythmical waves of excitation propagated caudally, as seen in lower vertebrates (Matsushima and Grillner 1992). Neonatal rats are capable of locomotion and rhythmical activity of axial trunk muscles is correlated with that of hindlimbs during gait at moderate speeds. However, as rats mature, the correlation disappears (Gramsbergen

et al. 1999) except in high-speed locomotion, where postural stabilization is needed and stretch reflexes are significant (Macpherson and Fung 1998; Zedka and Prochazka 1997; Zomlefer et al. 1984). Thus, it is unlikely that thoracic and lumbosacral spinal cord comprise a common locomotor pattern generator, which can be employed by the regenerating neurons.

The limited amount of functional improvement in our ISMS trials points to a possible limitation of recovery strategies based only on a non-specific activation of thoracic descending PSN systems. In our experiments, none of the animals before or during ISMS developed sufficient weight-bearing or intra- and inter- limb coordination necessary for locomotion. However, the spared spinal cord was capable of a high level of coordination and rhythmogenesis as was evident from long-lasting bouts of coordinated air-stepping observed after mechanical stimulation applied during bladder expression in the same animals. This observation is in agreement with the recent finding of pathways from sacrocaudal afferents to the lumbosacral locomotor pattern generator in neonatal rats (Strauss and Lev-Tov 2003). Also, it suggests the possibility that PSNs may increase locomotor performance not by the direct activation of lumbar locomotor CPG networks , but by a non-specific increase in the overall motoneuron excitability. It is possible that the efficacy of descending thoracic PSN inputs could be potentiated when combined with other recovery strategies, e.g. locomotor training, activation of the lumbosacral spinal cord with ISMS, epidural stimulation and/or pharmacological agents. Finally, these results point out the necessity to identify the specific descending propriospinal pathways that are involved in mediating the observed improvement. This future direction of research may have an important bearing on the success of studies promoting regeneration in the spinal cord.

To conclude, in this study we tested a key part of "the propriospinal hypothesis," namely that nonspecific activation of descending pathways below a complete midthoracic transection can improve locomotor performance. We found that tonic ISMS of the gray matter immediately

below the lesion produced small, but significant improvements of locomotor performance comparable to those observed in studies of long distance axon regeneration.

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Figure Legends

Figure 1. Testing the "propriospinal hypothesis". A. The “propriospinal hypothesis” attributes observed behavioral improvements of the axon regeneration through nerve grafts to nonspecific innervation of propriospinal neurons, which activate lumbosacral locomotor circuitry several segments below the lesion. B. We test if nonspecific activation of the spinal cord with the implanted ISMS array below the complete transection can improve the locomotor performance in rats.

Figure 2. Implantation of ISMS array in thoracic T10-T11 spinal segments. A. Location of the implanted electrode tips. Microelectrodes were implanted to target intermediate and ventral grey matter. B. Comparison of the open-field locomotor scores (BBB score) before and after the implantation of ISMS arrays. Averages of 10 days before and after the implantation are shown for individual animals. BBB scores are significantly lower after implantation (t-test $p=0.01$).

Figure 3. Effect of ISMS on the locomotor performance. A. Video sequence (15 frames/s) of evoked responses in a spinalized rat. Note the extensive range of movements and interlimb coordination in the hindlimbs. B. Comparison of BBB scores in a session without microstimulation of the spinal cord and in the following session with ISMS one segment below T8 lesion. BBB scores are significantly higher during the stimulation session (t-test $p=0.02$). C. Complimentary Bootstrap analysis of the differences in the BBB scores in sessions with and without ISMS shows that the difference is significant (95% confidence interval is indicated with dashed grey lines around the median, solid grey line).

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Figure 1 (yakovenko)

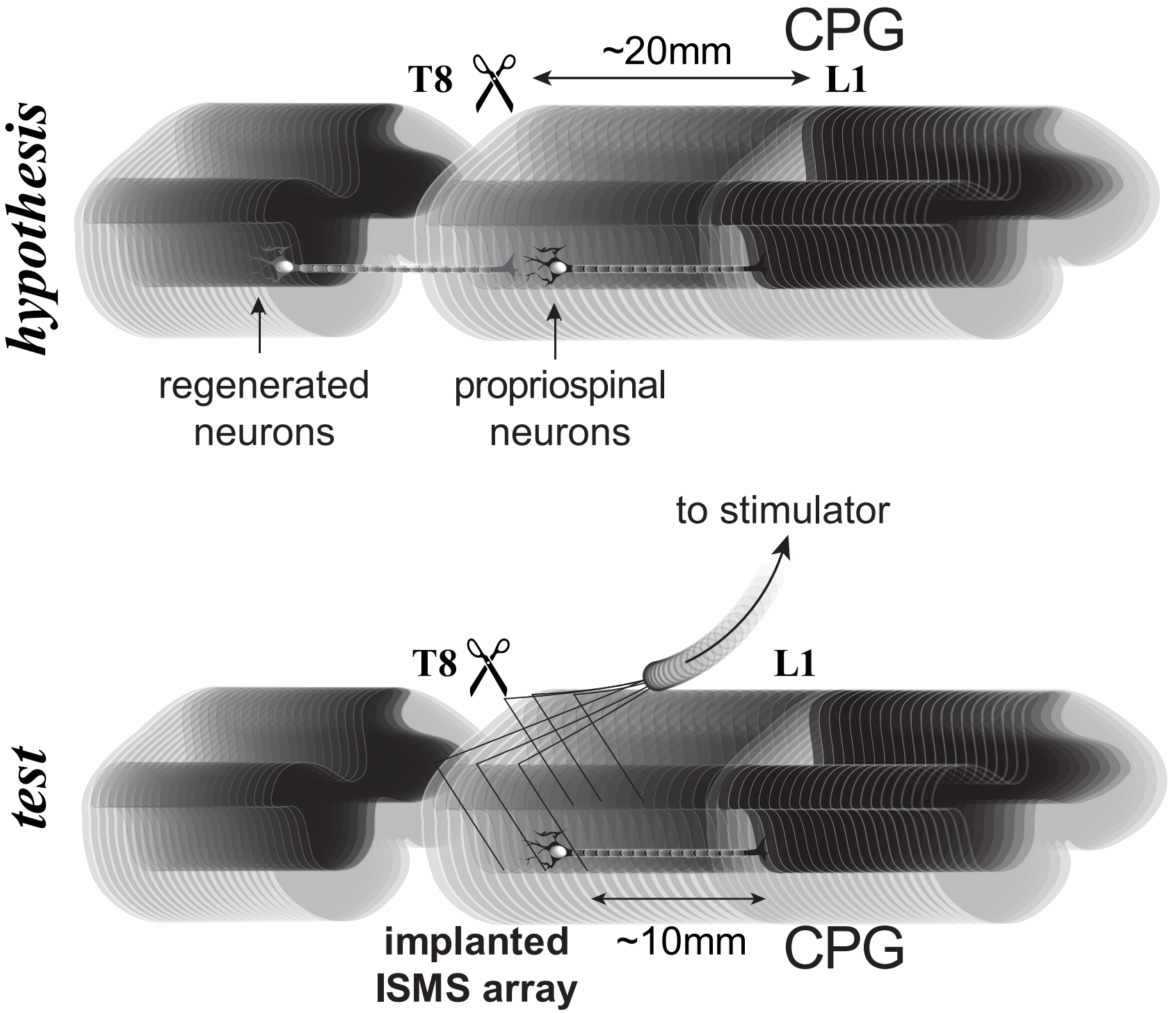


Figure 2 (yakovenko)

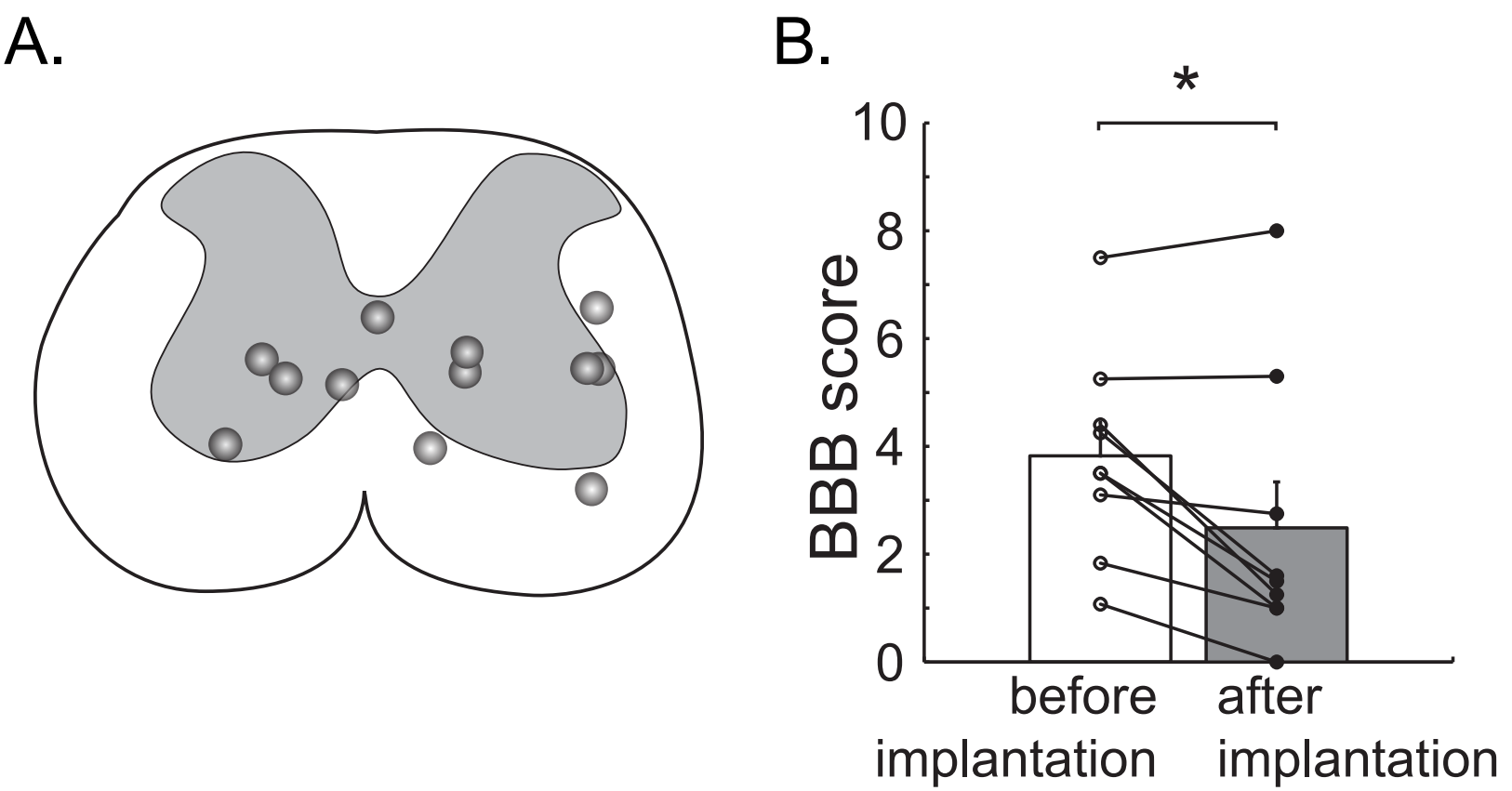
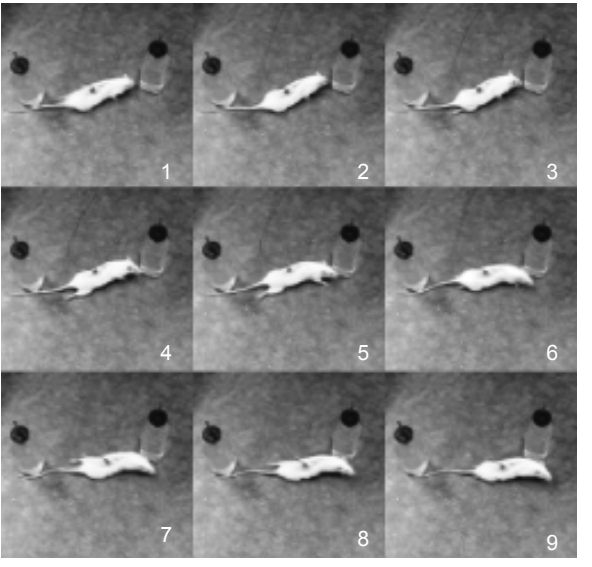
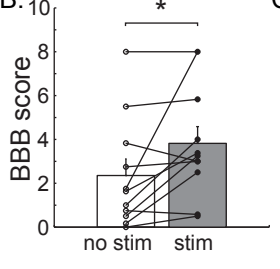


Figure 3 (yakovenko)

A.



B.



C.