Compensatory Plasticity Restores Locomotion Following Chronic Removal of Descending Projections

by

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Abstract

Homeostatic plasticity is an important attribute of neurons and their networks, enabling functional recovery after perturbation. Furthermore, the directed nature of this plasticity may hold a key to the restoration of locomotion after spinal cord injury. Here, we studied the recovery of crawling in the leech, Hirudo verbana, after descending cephalic fibers were surgically separated from crawl central pattern generators shown previously to be regulated by dopamine. We observed that immediately following nerve cord transection leeches were unable to crawl, but remarkably, after a day to weeks, animals began to show elements of crawling and intersegmental coordination. Over a similar time course, excessive swimming due to the loss of descending inhibition returned to control levels. Additionally, removal of the brain did not prevent crawl recovery, indicating that connectivity of severed descending neurons was not essential. Following crawl recovery, a subset of animals received a second transection immediately below the anterior-most ganglion remaining. Similar to their initial transection, a loss of crawling with subsequent recovery was observed. These data, in recovered individuals, support the idea that compensatory plasticity directly below the site of injury is essential for the initiation and coordination of crawling. We maintain that the leech provides a valuable model to understand the neural mechanisms underlying locomotor recovery after injury because of its experimental accessibility, segmental organization and dependence on higher-order control involved in the initiation, modulation and coordination of locomotor behavior.
**Introduction**

Neural circuits are remarkably resilient. Over a permissible range of perturbations, they can become reconfigured to maintain seemingly normal activity (Marder 2011; Turrigiano 2012; 2011). This ability is largely attributed to cellular- and network-level homeostatic processes that can retune a perturbed system and restore its functional state (Marder and Goaillard 2006; Pozo and Goda 2010; Turrigiano 2012). The idea of manipulating state-preserving homeostatic plasticity for restoring locomotor function after spinal cord injury appears promising, especially when repair or regrowth of neuronal connections across sites of injury is not possible (Edgerton et al. 2004; Rossignol and Frigon 2011). While such strategies may hold keys to significant biomedical advances for locomotor recovery, the cellular mechanisms underlying these and most other homeostatic or compensatory processes are just beginning to be understood (Husch et al. 2012; Pozo and Goda 2010; Sakurai and Katz 2009).

We propose that the leech serves as a valuable and highly tractable system in which to investigate homeostatic mechanisms contributing to functional locomotor recovery. Invertebrate preparations have long been pivotal in illuminating the cellular bases of rhythmic-pattern generation, its modulation, and stability (Marder et al. 2005; Marder and Calabrese 1996; Selverston 2010). For almost half a century, the medicinal leech, in particular, has served as a model organism to illustrate how neural circuits are organized and modulated to produce locomotor behaviors (Briggman and Kristan 2008; Brodfuehrer et al. 1995; Kristan et al. 2005; Mullins et al. 2011; Puhl and Mesce 2008; 2010). Similar to the functions of vertebrate spinal projections (Grillner 2006a; b; Grillner and Wallen 2002; Kiehn 2011), higher-order fibers descending from the leech’s
cephalic ganglion (i.e., brain) have been known to be vital for the initiation, modulation and coordination of crawling across iterated locomotor pattern generators (Puhl et al. 2012). Thus, in this report, we examined whether medicinal leeches (Hirudo verbana) could recover their ability to crawl after their nerve cords were fully transected, while noting whether or not regrowth of descending axons may have contributed to locomotor recovery. By observing leeches for days to months following such transections, we determined whether they could recover their ability to crawl in a coordinated manner, despite the loss of previously essential control elements for that coordination.

In mammals, compensatory plasticity is not always beneficial if it becomes undirected during severe supra-spinal injury (Beauparlant et al. 2013); thus discovering therapeutic strategies and how they work to steer beneficial features of plasticity hold promise for promoting adaptive locomotor recovery, especially those related to neuromodulatory intervention (Guertin 2014; Musienko et al. 2011; Sharples et al. 2014).

In the leech, we found that the crawl pattern-generating networks distal to the site of injury were able to achieve adaptive solutions for their reconfiguration and intersegmental coordination, independent of descending fibers from the brain becoming reconnected. Understanding how the leech nervous system correctly reconfigures itself post injury, while making occasional misdirected mistakes, will likely provide valuable insights about the cellular substrates of locomotor plasticity across animal species, including humans.
Materials and Methods

Animals and Surgical Procedures

Adult medicinal leeches (hermaphrodites), *Hirudo verbana* (Carena 1820; Siddall et al. 2007) were obtained from Niagara Medical Leeches (Westbury NY, USA) and stored at room temperature in a glass terrarium filled with water from a natural well at the University of Minnesota. After surgery, leeches were housed in individual containers (17 cm wide x 8 cm tall) with a thin layer of aquarium gravel covering the bottom and well water at 4-5 cm in depth.

Leeches chosen for behavioral assays weighed 2.45 +/- 0.48 g. Briefly, the leech CNS consists of: 1) a compound cephalic ganglion comprising the supra-and sub-esophageal (SEG) ganglion (i.e., the brain); 2) the 21 segmentally distributed ganglia (M1-M21) and the connectives that link them together (i.e., the ventral nerve cord); 3) the compound terminal ganglion ('tail brain'). Prior to surgery, leeches were anesthetized on ice until they were no longer responsive to tactile stimuli. Once anesthetized, they were pinned onto a dissecting tray lined with bees’ wax ventral side up. One thin pin was placed through the leech’s mouth, avoiding damage to the brain, and the other one placed through the caudal sucker. A small vertical incision spanning 2-3 annuli was made to access the nerve cord for complete transection. The incision was closed using one or two stitches made with 7-0 or smaller suture thread. A post-mortem examination was conducted in all animals to confirm that the nerve cord (connectives) had remained fully transected (Fig. 1A far right). Herein, we present data from individuals with several different types of manipulations, defined as follows: 1) **Transections**- A small incision was made 5 annuli below the mouth, which permitted a
complete transection of the nerve cord between the brain and first ganglion (M1) (see Fig. 1A) or between M1 and the second ganglion (M2). No differences in behavior were observed between these individuals. Three individuals were tested at 40 days and another four tested at 48-50 days following transection; these individuals were combined into a single grouping in the figures labeled as being tested at 40-50 days following transection; 2) M1 removal- M1 was surgically extirpated from the leech (see Fig. 6A); 3) SEG removal- The subesophageal ganglion was removed by surgically detaching it from the supraesophageal ganglion and M1 (see Fig. 7A); 4) Second Transection- Leeches that showed a complete recovery of crawling behavior were anesthetized and transected again at 40-50 days following the first transection (between the brain and M1). The second transection was made between M1 and M2 (see Fig. 8A); 5) Midbody- This type of transection was made between M9 and M10 or M10 and M11, resulting in the removal of all descending inputs to the posterior half of the leech while preserving those to the anterior half of the animal (see Fig. 9A inset); 6) Sham-Operated Controls- These surgeries were performed as a control for all of the above conditions, whereby the animal received identical surgical manipulations (e.g., skin and muscles cut and nerve cord tugged), except the nerve cord was not severed.

Behavioral Arena and Video Acquisition

Individuals were tested before and at multiple points following transection. The order in which the tests were presented was randomly chosen. The behavioral arena was a cylindrical acrylic dish, 20.3 cm in diameter by 8 cm in height. It was illuminated with LED lighting to approximately 750 lux. Water within the arena ranged from 19-21°C.
Videos were filmed at 38 fps using a DCC1455M high resolution CMOS camera (Thorlabs, Newton NJ, US).

Behavior under the following conditions was examined: Deeper water - the arena was filled with well water to a depth of 4 cm. Under this condition a leech was able to swim or crawl. Shallow water - the arena was filled with 15 ml of well water, which was just enough to coat the bottom of the arena with a thin layer of water 1-2 mm high. Under this condition the leech was only able to crawl. Under either condition, the leech's behavior in the arena was recorded for the 6 minutes immediately following placement of the animal in the arena. Evoked crawling - To initiate crawling, a round no. 4 artist's paintbrush was used for two minutes. The surface of the leech was touched with the brush in an anterior-to-posterior direction to stimulate crawling behavior. In response to this stimulus, a crawl or crawl-like behavior was counted if the leech performed one or more crawl-like cycles (note: an elongation and contraction phase equals one cycle) (Fig. 1D).

Behavioral Descriptions and Analysis:
Swim and crawl locomotor states were easily separable by visual inspection, defined as follows: Swim - the whole body is flattened and moves with a series of quasi-sinusoidal dorso-ventral movements progressing caudally (Brodfuehrer et al. 1995; Stent et al. 1978). During swimming, unlike crawling, neither of the leech's suckers attaches to the substrate. Crawling and Crawl-like behavior - A defining feature of normal crawling is that during each of its phases—elongation and contraction— the individual body segments are coordinated (Puhl and Mesce 2010). During elongation, each segment sequentially elongates (lengthens) in a stereotypic anterior-to-posterior directed wave.
During contraction, individual segments contract (shorten) in the same posteriorly-directed manner. Often, there is a delay of a few seconds before the next cycle is initiated. Here, we use the term 'crawl-like' to expand the traditional definition of crawling to include some of the atypical locomotor activity we observed during the recovery process. To be counted as crawl-like behavior, animals had to exhibit a body elongation phase followed by a body contraction phase with no body flattening typically associated with swimming. However, the crawl-specific coordination during each elongation or contraction phase (i.e., the caudally-directed metachronal wave) was not a requirement. For example, a reversal in wave direction, a partial wave or an uncoordinated wave would not limit use of the 'crawl-like' designator. Using recorded videos, swim and crawl behaviors were quantified by measuring the duration of bouts of swimming or crawling (in deep water) or by counting the number of crawl cycles (in shallow water) within a 6 minute (min) test period.

_Crawl movement analysis:_ To understand the recovery process better, videos of representative crawl cycles were used to generate a series of frames depicting the kinematics of the elongation and contraction phases. These analyses were based on the fact that, during the elongation and contraction phases, the body narrows and widens respectively—a consequence of hydroskeletal dynamics. Representative crawl phases were divided into 8 images (Fig. 4). The first and last silhouettes indicate the leech’s position at the beginning and end of a crawl cycle. The intervening images were separated by intervals of 15% of the total cycle duration (e.g., about 0.35 s for the pre lesion series). These images (for example, Fig. 4A i) were then analyzed in ImageJ by applying a threshold filter, which outlined the leech in black against the white
background (Fig. 4A ii). In those videos with some glare, the silhouettes contained a peppering of open spaces after thresholding (Fig. 4A ii). Such areas were subsequently filled in by using the line-and-fill tools in ImageJ so as not to cause errors during automated thickness (width) colorization. This process allowed for the local thickness plugin to be run. A reference of a 50 pixel-wide line was placed into the image such that all images could be calibrated to the same scale for coloration (Fig. 4A iii). The local thickness algorithm colorized images according to the largest circle that fit into a particular area, i.e., the leech’s silhouette. The local thickness algorithm was run and the image colorized for individual images such that blue-purplish colors represented less wide (narrower) areas of the leech and orange-yellow-white areas represented wider regions of the leech’s body—again, elements that correspond to the elongation and contraction phases of crawling (Fig. 4B).

Coordination analysis: To assess acquisition of the posteriorly-directed coordination of the crawl elongation phase, a given leech was visually divided into four approximately equal sections. When a body quarter was active during elongation, it would appear thinner due to the consequence of circular muscle contractions. For these analyses, up to 25 crawl elongations were measured for each individual in a 6 min window in shallow water, with the activity and order of body quarters in close succession counted. Only the elongation phases were used for analysis because they typically occupied a greater proportion of a given crawl cycle and active body movements were readily observed, assuring a more accurate measure of active body quarters and their coordination. For such analyses we used individuals that were available for all trials (n = 8 for transections; n = 4 for sham-operated controls).
Statistics and graphing methods for behavioral analyses

Unless otherwise noted, the statistical test used was the Skillings-Mack test (SM) that, when significant, was followed by a post-hoc Dunn's comparison; results are reported from this Dunn's comparison where applicable (Skillings and Mack 1981). For swim bout durations, this test required that we take the mean of each individual's swim bout duration and look at its change over time. The statistical test used for data presented in Fig. 1 was the Kolmogorov-Smirnov (K-S) test (Justel et al., 1997), a two sample analysis to compare non-parametric means with small sample sizes. Box Plots—Because many of the data sets included non-normal data and relatively small sample sizes, we used the Tukey box and whisker method of plotting data. In this method, the box surrounds the 25-75 percentiles. A line is present at 50% (the median). The whiskers extend to 1.5 times the interquartile ratio (Tukey 1977). For illustration purposes only, within these plots, the arithmetic mean is marked with a + sign. Outliers are marked by diamonds. Unless otherwise noted, all figures with measures of crawl or swim cycle number, cycle duration, or duration of uninterrupted locomotor cycles (bouts) incorporated the above mentioned box plots.

Results

The time course and process of crawl recovery after injury

The brain (cephalic ganglion) exerts a hierarchical control over behavior in the form of descending axons whose firing rate biases the animal toward (or against) the expression of swimming or crawling (Brodfuehrer and Friesen 1986; Esch et al. 2002; Mesce et al. 2008; Mullins et al. 2012; Puhl et al. 2012). While disconnection of the
brain has been shown to promote swimming and result in a loss of crawl coordination (Puhl et al. 2012; Puhl and Mesce 2010), these studies have only examined leeches with acute lesions (i.e., within hours of surgery). Here, we examined the recovery of normal function for weeks to months following transection of the nerve cord above M1 or M2 (n = 14), and in sham controls where the nerve cord was not severed (n = 7).

Consistent with prior studies, transection of the leech nerve cord between the brain and M1 (Fig. 1A) caused a 10 fold increase in the bout duration of swimming (Fig. 1B) (n = 14, p<0.0001, KS test). This change in swim expression must have been due to disconnection of the cephalic ganglion because no change in swim bout duration was observed one day after a sham surgery (n = 7). Interestingly, the increase in swimming following transection was not permanent; 40-50 days after surgery, transected leeches exhibited swim bouts of only 17.61 +/- 15.76 s/bout (n = 8), which was similar to sham-operated control levels and pre-test baseline (ns, p>0.9, KS test). In contrast, one day after surgery, transected animals showed a significant decrease in the number of crawl-like cycles, with only one transected individual of 14 exhibiting a single spontaneous crawl cycle (Fig. 1C, p<0.0001, KS test). In fact, during this time we could not evoke crawl-like behavior in any of the 14 individuals tested (Fig. 1D). This dramatic change in locomotor behavior, however, was once again temporary; 40-50 days after surgery (Fig. 1C), the mean number of spontaneous crawl cycles shown by transected leeches had increased to 39.38 +/- 16.42 cycles (n = 8), which was similar to corresponding sham-operated control levels (28.50 +/- 17.08 cycles) and not significantly different than pre-transection values 23.29 +/- 12.39 cycles (n=14; p>0.08, KS test). Sham-operated
individuals never showed a significant change in crawling (p>0.5, KS test) or swimming behavior (p>0.9, KS test).

Having determined that leeches show an initial deficit in crawling after transection, but exhibit the behavior readily at a later date, we examined the time course of recovery. First we wanted to examine when leeches begin to exhibit crawl cycles. These data are indicated in Figure 2A, where each leech is represented by a single row and each day following surgery is represented by a column. Prior to transection all leeches (n= 14) exhibited crawl behavior; however, following transection only a single individual exhibited one crawl cycle before Day 4. In contrast, all sham-operated individuals (n = 7) exhibited crawling the day following their surgery. In transected animals, by 40-50 days following surgery, all 8 leeches tested were able to exhibit multiple crawl cycles (Fig. 2A). To understand better the time course of crawl recovery, the specific number of crawl cycles exhibited was quantified when leeches were placed in either shallow water (Fig. 2B) or deeper water conditions (Fig. 3B). In shallow water, transected leeches exhibited a significant decrease in the number of crawl cycles performed following surgery (Fig. 2B, p<0.0001, SM test), and continued to exhibit lower numbers of crawl cycles until 13 days following transection (p<0.001, Dunn’s test). By day 13, the number of crawl cycles observed was no longer significantly different from pre-test values (p=.24, Dunn’s test). It should be noted, however, that as time progressed more individuals exhibited more crawl cycles. Long term, leeches were fully able to recover crawling, exhibiting 39.4 +/- 16.4 crawl cycles during a 6 min trial (compared to their pre-transection value of 23.4 +/- 12 crawl cycles). In contrast, individuals that received a sham surgery did not exhibit a significant decrease in the number of crawl cycles (p>0.28, SM test).
As noted in Figure 1B, swimming activity levels in transected animals also returned to control levels after 40 days of surgery. To examine this phenomenon more closely, animals were monitored daily over a period of 10 days (Fig. 3A). Before transection, swimming was exhibited in short bouts (12.71 +/- 11.34 s). Immediately following transection a dramatic increase in swim bout length was noted (212.4 +/-150.9 s, p<0.0001, Dunn’s test), something not observed in the sham-operated individuals (Fig. 3A gray, p>0.88, SM test). During the first 10 days following transection, a significant increase in swim bout duration persisted (p<0.05, Dunn’s test) with a declining trend, but by 40-50 days these bouts were no different from pre-test values (ns, p>0.32 Dunn’s test). Concurrent with the increase in swimming behavior, we saw a significant reduction in crawl bout duration for 10 days following transection (p<0.0001 for all, Dunn’s test, Fig 3B white), which subsided 40-50 days following transection (p>0.17, Dunn’s test). No significant changes in crawling were ever observed in sham-operated individuals, following surgery (Fig. 3B gray, p>0.95, SM test).

While leeches regained an ability to generate crawl-like cycles with two phases (i.e., elongation-contraction) after transection, we wanted to determine if within each phase the segments were able to regain their caudally-directed metachronal coordination. Thus, we analyzed movements of the leech by characterizing its body form during these two phases (Fig. 4). By colorizing videos of leeches crawling we were able to visualize the progression of the anterior-to-posterior wave of elongation (blue-red = narrower body) and contraction (white-orange = wider body) and thus examine the form of crawling during its recovery (see Methods section for details, Fig. 4A). In sham-operated individuals and pre-transection trials, there was a fluid anterior-to-posterior...
progression of coordinated segmental activity during both phases that propelled the
leech forward a considerable distance (Fig. 4B). Here, the notable curvature in the
silhouettes was due to the tendency of leeches to crawl thigmotactically around the
inside edge of the dish. In sham-operated individuals, just one day following surgery,
normal crawling was apparent (Fig. 4B). Figures 4C and 4D show two different
transected leeches as crawling was reacquired; note that metachronal body activity was
absent or weak at best, as depicted by a lack of blue-red color and its caudally-directed
invasion into the body (see Fig. 4C,D, Day 3 and 6 respectively). In fact, only a portion
of the leech’s body exhibited a crawl cycle (Fig. 4D, Day 6). Over time, however,
crawling did begin to regain its posterior-directed coordinated fluidity, and crawling
began to appear more normal. For example, on Day 12 (Fig. 4 C, 3rd-5th silhouette, left
to right) the purple-red color can be seen invading the leech in an anteriorly-directed
manner, reflecting the narrowing of the body segments as the circular muscles
contracted during the crawl elongation phase. In turn, the crawl contraction phase can
be appreciated (Fig. 4 C, Day 12, 6th-8th silhouette) by the caudally-directed wave of
orange-to-white color that progressively invades the leech body, reflecting the widening
(fattening) of each body segment as the longitudinal muscles contracted. By Day 40,
both leeches were showing crawl cycles that were similar in form and duration to pre-
lesion crawl cycles (Fig. 4C,D). It is noteworthy that individuals showed variability in
their recovery (for example, compare Figs. 4C and D on Days 10 and 9), which might
facilitate the future detection of factors that promote recovery across individuals. Lastly,
transected individuals initially exhibited long duration crawl cycles. For example, on the
first day that crawling behavior was expressed we saw an increase in crawl cycle
duration from the mean pretest value of 7+/-2.43 sec to 21.29+/- 13.16 sec/cycle (n = 9, p<0.0001, KS test). Sham-operated individuals (n = 6) did not show a change in their crawl cycle durations (5.95+/-2.57 sec and 6.44+/-2.95 sec between pretest and first day post-surgery trials, respectively, p=0.96, KS test). Long term (40-50 Days following transection), transected individuals (n=7) exhibited crawl durations of 5.83+/- 1 sec (p<0.09, KS test). Thus it takes time not only for the crawl oscillators to regain their coordination but also for the tempo of the rhythm to return.

To quantify attainment of the anterior-to-posterior progression of the crawl-specific wave further, we visually divided a given leech into 4 quarters and examined the activation of the quarters during the elongation portion of crawling (Fig. 5). This phase was selected, in part, because the metachrony was most apparent and thus more reliably measured. Within several days following transection, leeches typically utilized only one or two body quarters during crawl-like elongations (Fig. 5A). Curiously, these quarters were often the posterior-most two quarters (Fig. 5B). After Day 4, however, transected leeches began to utilize 3 or 4 quarters (Fig. 5A) and the anterior quarters were more likely to be the first activated (Fig. 5B). By Day 10, more than 80% of the elongation phases comprised all 4 quarters, and nearly all individuals began their forward crawl elongations with the first quadrant (Fig. 5B). To determine fully if bona fide crawl behavior had returned, we needed to examine how many of the quarters were activated in the correct sequence. For the first 4 days following transection, the vast majority of individuals exhibited activity in only one or two quarters. When two quarters were active they often had a sequential order (Fig. 5C). The exception was a 4-quadrant crawl-like elongation on Day 1 (Fig. 5), which the animal did not exhibit again.
until Day 8. Over time, as more quarters become active, they exhibited sequential
activation in the anterior-to-posterior direction. By Day 10, over 80% of the elongations
involved all four quarters activating sequentially (Fig. 5C). In a small percentage of
animals, elongations involving 2 or 3 quarters were sequentially activated but in the
reverse order (i.e., in the anterior direction) (Fig. 5C); rarely do non-sham control
leeches show backwards crawling.

**Testing for re-connectivity vs reorganization**

Visual inspection under a dissecting microscope did not reveal any evidence of axonal
reconnections being made across the transection site in animals in which crawling had
recovered (see Fig. 1A). To decrease the likelihood of axonal reconnections forming
across the lesion, we expanded our lesion size by removing the entire ganglion (M1)
below the brain (n = 6), as depicted in Figure 6A. Once again, we observed an
immediate and dramatic decrease in the number of leeches that spontaneously crawled
(p<0.001, SM test, Fig. 6B). By 8 days following surgery, all animals exhibited crawling
(Fig. 6B), and the increased number of crawl cycles (Fig. 6C) was no longer significantly
different from pre-surgery trials (p>0.09, Dunn’s test). For example, crawl cycles
recovered to 18.17 +/- 12.04 cycles by Day 10. Removal of M1 also initially increased
swim bout duration from 7.69 +/- 3.98 s/bout pre-surgery to 152 +/- 122 s one day post-
surgery (p<0.001, Dunn’s test; Fig. 6D). By 10 days after surgery, swim bout duration
had fallen to 24.92 +/- 45.65 s (n = 6). In transected animals, 8 days following surgery,
swim levels were no longer significantly different than pretest values (p>0.12, Dunn’s
test).
While we considered it unlikely that the observed recovery was due to reconnection of descending inputs across such a large lesion, as a further test, we removed the entire subesophageal ganglion (SEG) (Fig. 7A). Because the SEG contains the somata, neurites, and proximal axons of the descending brain cells, this surgery fully eliminated the possibility of reconnection across the lesion. Furthermore, removal of the SEG and M1 also tested for the possibility that these ganglia, specifically, were vital for locomotor recovery or provided an essential circulating (hormonal) factor. Similar to results from our prior tests, removal of the SEG caused a decrease in the number of leeches that exhibited spontaneous crawling in the shallow or deep water arenas, but this proportion recovered by 10 days after surgery (Fig. 7B). Similarly, the number of spontaneous crawl cycles in shallow water during a 6-minute window decreased from 8 +/- 4.8 cycles pre-surgery to only 0.16 +/- 0.4 cycles (one cycle of one individual) one day post-surgery (significant p<0.001, Dunn’s test), but returned to 5.3 +/- 5.5 cycles, which was similar to baseline by 10 days post-surgery (Fig. 7C). SEG removal further increased swim bout duration initially from 10.67 +/- 7.63 s/bout pre-surgery to 133.8 +/- 109.1 s one day post-surgery (Fig. 7D; n = 6, significant, p<0.001, Dunn’s test). By 30 days after surgery, swim bout duration had decreased to 42.63 +/- 28.84 s (n = 6), and at 115 days following surgery to 11.71 +/- 14.30 s, which was not significantly different from baseline (p>0.44, Dunn’s test).

**Potential neural mechanisms underlying crawl recovery**

Previous studies (Puhl and Mesce 2008; 2010) have shown that each of the 21 segmental ganglia houses its own crawl CPG. In the control state, these oscillators appear to need a command system for their activation and coordination. Thus
coordinated crawl activity in transected animals, directed from front to back, might need
to rely on the reorganization of neural networks in the rostral regions of the nerve cord.
We tested whether a putatively reorganized single ganglion, directly below the site of a
rostral transection, becomes necessary for the executive-like and coordinating functions
that are regained. We ran this experiment in leeches that had recovered normal
crawling behavior following a transection anterior to M1 (n = 7). Forty-fifty days
following their first transection, they received a second transection, which essentially
removed M1 (Fig. 8A). As in prior single-transection experiments, this manipulation
caused a dramatic decline in the proportion of leeches that exhibited spontaneous
crawling (Fig. 8B) and in the number of crawl cycles observed in 6 minutes (Fig. 8C,
p<0.0001, SM test). Consistent with the first transection, a temporary increase in swim
bout duration also occurred (Fig. 8D, p<0.001, SM test). Albeit slower than their initial
recovery, we still noted a second recovery of crawling behavior and of the suppression
of swim behavior to control levels (n = 7, day 76, p>0.32 and p>0.37 respectively,
Dunn’s test). In sham-operated controls, where the second transection did not cut the
nerve cord, 100% of leeches (n = 3) crawled immediately after the surgery and had
normal swim activity (N.S., p>0.5, SM test). The above results are compelling because
they bring into focus the potential site(s) of neural plasticity to include a single
experimentally tractable ganglion that has only a few hundred paired neurons.

Indeed, based on the previous experiment, it would appear that injury induces
changes in the ganglion immediately posterior to the transection site such that it
becomes the ‘lead ganglion’, possibly driving the caudally-directed coordination of
oscillator activation. Are these same changes possible in any ganglion immediately
posterior to a site of transection, or are only anterior ganglia specialized to allow for the initiation and coordination of crawling? To test this hypothesis, we transected 6 additional leeches in the middle of the nerve cord (between M9 and M11) (Fig. 9A, left panel). When we examined whether or not individuals exhibited crawl or crawl-like behavior, we observed that the body anterior to the transection site exhibited relatively normal and highly coordinated crawling as the full complement of cephalic inputs remained available (Figs. 9A,B; top panel in C). More importantly, however, we observed that after recovery the body region posterior to the middle-body transection was competent to initiate and properly coordinate crawling activity (Fig. 9 B,C middle panel). This crawling was independent from crawling exhibited by the front half of the leech (Fig. 9 C lower panel), thus supporting the idea that a middle-body lead ganglion had emerged.

In contrast to the crawl activity exhibited by the front half of the body, the body posterior to the transection site showed fewer spontaneous crawl cycles per 6-minute window (Fig. 9C, middle panel, p<0.01 for all time points). The body region anterior to the transection, however, showed an apparent decrease in swim bout duration (Fig. 9E, top panel), but this was found to be not significant (SM test, p>0.2). The back half of the body, however, swam with few interruptions (Fig. 9E, middle panel) (average bout duration 63.9 +/- 86.7 s, a significant increase from full body pre-test values p<0.005, SM test).

Leeches were also able to coordinate both halves of their body across the lesion (Fig. 9E bottom panel) and display full-body-coordinated swimming (Fig. 9D). Across-lesion coordinated swimming could be induced in all animals within a single day.
following transection and was not significantly higher or lower than in pre-test animals by 10 days following transection (p>0.33). Such across-lesion coordination, however, was not exhibited for crawling (Fig. 9C, bottom panel). In contrast, we observed that the two halves of the leech crawled independently, as if they were two separate animals generating their own crawl metachrony (Fig. 9B). By 115 days after surgery, 2 of the 6 leeches that showed some small degree of across-lesion coordination (Fig. 9C bottom panel) still did not exhibit the type of smooth, coordinated crawling observed in sham-operated controls.

A few additional details of the crawl-like activity that emerged after a mid-body transection are worth highlighting here. At Day 115, the average number of crawl cycles expressed by the body region caudal to the transection site was reduced compared to other M1 transections (compare Fig. 9C middle panel, with Fig. 2B). The number of crawl-like cycles, however, was greater at earlier time points during recovery (same comparison). We often observed the anterior half of the body crawling actively while pulling the passive posterior body along. After a bout of anterior-body-only crawling, the rear half would initiate a few crawl cycles, but these movements were rarely coordinated with those across the transection site (Fig. 9C bottom panel). These results are in sharp contrast with swimming, wherein the swim oscillators were able to be coupled by feedback from adjacent local proprioceptive inputs (Cang and Friesen 2002; Yu et al. 1999).
Discussion

Nature provides a clever strategy of compensatory or homeostatic plasticity to enable the functional recovery of neural circuits after injury (Nahar et al. 2012; van den Brand et al. 2012). Indeed, in the field of human spinal cord injury, the importance and manipulation of compensatory plasticity has been recently underscored as an inroad to ameliorate injury-related neuronal dysfunction (Rossignol and Frigon 2011). Research in rodents and humans, however, has shown that while compensatory plasticity is potentially beneficial, if left ‘undirected’ during severe supra-spinal injury, this same plasticity can play a detrimental role in the recovery of locomotor-related circuitry and sensorimotor function (Beauparlant et al. 2013). In the leech, such misdirected plasticity occasionally occurs when crawl coordination resumes in the reverse direction, leading to unproductive locomotion (Fig. 5). Thus determining how tractable small networks can achieve full and adaptive recovery after injury (or make mistakes) promises to provide valuable insights into general mechanisms of how circuits become retuned.

Restoration of locomotor activity:
The recovery of crawling following the chronic removal of descending inputs is, indeed, impressive as these inputs, including the cephalic neuron R3b-1, are known to be vital for the initiation, modulation and coordination of crawling movements (Esch et al. 2002; Mesce et al. 2008; Puhl et al. 2012; Puhl and Mesce 2010). It was anticipated that leeches would exhibit dysfunctional crawling after all neuronal connections between the brain and segmental crawl oscillators were removed via nerve cord transection (Fig. 1C,D). In invertebrates, the loss of descending inhibition has been associated with
changes in locomotor activity and coordination (Ridgel and Ritzmann 2005; Roeder 1937; Schaefer and Ritzmann 2001). What was unpredicted, however, was the leech’s ability to regain productive and coordinated crawling over the days-to-weeks after injury, a restoration that was not accompanied by any perceived reconnection of descending cephalic neurons (Fig. 1A).

We also predicted that leeches would express higher levels of spontaneous (i.e., unevoked) swimming after transection, as it was shown previously that the segmental swim circuits are under cephalic inhibition (Brodfuehrer and Burns 1995; Brodfuehrer and Friesen 1986; Cornford et al. 2006). Indeed, after transection, swim-bout durations were elevated in all leeches observed (Figs. 1B; 3A). Curiously, over time this elevated level of swim activity returned to control levels, and over a similar time course to crawl recovery (Fig. 6C,D). After a secondary transection, swim activity levels were again reset with a similar temporal match to a second round of crawl recovery (Fig. 8C,D).

After spinal cord injury in mammals, the loss of inhibitory projections also leads to maladaptive or spasmodic behaviors at first, which are similarly ameliorated when locomotor recovery ensues (Rossignol and Frigon 2011).

Regaining full recovery:

There are layers of complexity to crawling and its regulation; thus to gain insights into the mechanisms of crawl restoration, we needed to document which movements returned and when they appeared. In its basic form, leech crawling consists of a whole-body elongation (lengthening) followed by a whole-body contraction (shortening). During each of these phases, the segments exhibit a fluid caudally-directed metachrony (Cacciatore et al. 2000; Eisenhart et al. 2000; Puhl and Mesce 2010). Not until the
reports of Puhl and Mesce (2008; 2010), however, was it known that each and every segmental ganglion has a complete crawl CPG capable of directing its own segment’s elongation and contraction phases, and that specific interneurons in the brain (i.e., SEG) are required for across oscillator coordination (Puhl et al. 2012). Although we observed an infrequent and basic crawl-like mode of activity as early as 1-2 days post transection (Figs. 2A, 7B) as had others (Baader and Kristan 1995; Cacciatore et al. 2000), fluid and productive crawl-specific intersegmental coordination did not reliably occur until much later around Day 10 (Fig. 5C).

Reorganization of crawling: taking the lead

Knowing that descending projections were fully severed, how might the functions of such neurons be replaced? One mechanism could be the reconnection or regrowth of descending axons. Our experiments, however, were designed so as to make this possibility untenable. We not only made sequential and thus wider gaps across the transection sites, for example by removing M1, but we removed the entire SEG that housed the soma and integrative sites of R3b-1 and other projection neurons. Additionally, by removing the SEG, we excluded potential neuromodulatory influences specific to that portion of the brain. Still, in all such conditions, we observed the eventual recovery of coordinated crawling (Figs. 6, 7).

The most parsimonious explanation for our results is that the ganglion closest to the transection site acquires similar control features of the SEG. We call this idea the ‘lead ganglion hypothesis’. Inevitably, the lead ganglion must restore crawl activation and facilitate appropriate intersegmental phase relationships across the iterated crawl oscillators. If compensatory plasticity maximally involved this ganglion, then removal of
it would destroy all gains made for crawl restoration. Indeed, this is exactly what we observed when we performed a second transection after the initial recovery period (Fig. 8). Once again, leeches were able to recover coordinated crawling (Fig. 8C) and regain a suppression of swimming (Fig. 8D). Furthermore, our experiments hint at a non-brain related oscillator-coupling mechanism that may contribute to normal coupling. For example, it has been shown in untreated leeches that the crawl oscillators can initiate crawl-like bursting in neighboring segmental ganglia (Puhl and Mesce 2010). Although such intersegmental ‘drive’ cannot account for crawl-specific intersegmental phasing by itself, the addition and strengthening of descending inputs from a ‘lead ganglion’ may assist in the recovery of such coordination.

Finally, we studied whether a ganglion closest to the site of a nerve cord transection, but further away from the brain, could take the lead. To test this idea, we transected the nerve cord in the middle of the body (M9-11) to determine if caudally-directed crawl activity could be initiated and coordinated posterior to this transection. Similar to anterior transections, we observed a loss and eventual recovery of coordinated crawling by the posterior half of the body (Fig. 9 A,C). Crawling by the back half appeared to recover more quickly, which may have stemmed from increased sensory stimulation provided by the crawling front half. Although such effects may have had some restorative benefits, potential mechano- and/or proprioceptive activation generated anterior to the lesion were not sufficient to couple the crawl oscillators (Fig. 9C, bottom panel).

For swimming, in contrast, proprioceptive feedback is known to couple the swim oscillators when ganglia become physically disconnected through nerve cord
transections (Fig. 9E bottom) (Cang and Friesen 2002). This type of across-transection coupling was clearly not observed during crawling (Fig. 9B, C bottom panel), underscoring essential differences in oscillator-coupling mechanisms between the swim and crawl CPGs (Iwasaki et al. 2014; Kristan et al. 2005). We postulate that this inability for across-transection crawl coupling stems from the network reorganization that occurs in the ganglion nearest to the site of injury. Because the lead ganglion apparently directs its own propagating wave of crawl activity, it becomes refractory to anterior oscillator-induced proprioceptive or mechanosensory feedback. Future studies will test the role of proprioceptive inputs that likely become vital for the intersegmental coordination displayed after injury. The accelerated crawl recovery we observed below a midbody transection (Fig. 9) supports the idea that physical manipulation of the body can influence crawl CPG excitability, thus possibly facilitating crawl recovery.

Compensatory plasticity and locomotor recovery:

Crawling in leeches is a relatively slow and highly flexible locomotor rhythm with a number of parallels to walking in mammals and insects wherein ‘stepping’ can be paused and resumed mid-cycle without perturbation of the locomotor rhythm (Büschges 2005; Buschges and Borgmann 2013; Pearson 2000). Cephalic projections are vital for crawl performance, just as supraspinal and cortical projections are critical for stepping in primates, including humans (Courtine et al. 2009). It has been argued that neurons within rhythm-generating networks will self-tune to restore or maximize their synaptic inputs (Marder 2011). Included in this plasticity are changes in neuromodulatory state, especially changes in aminergic modulation or its sensitivity (Husch et al. 2012). As in vertebrate animals (Sharples et al. 2014), dopamine and
serotonin in the leech play pivotal roles in modulating locomotor CPGs (Mesce and Pierce-Shimomura 2010; Puhl and Mesce 2008; 2010; Willard 1981). Future studies will examine whether changes in aminergic projections and receptors provide a mechanism for recovery, and whether nerve cords isolated from the bodies of crawl-recovered animals, in the absence of sensory feedback, have the ability to generate crawl-specific coordinated fictive crawling. In addition, the roles of damaged ascending neurons, and not simply those cells descending from the brain, warrant future attention in our understanding of crawl recovery. Although perturbation of ascending neurons could play identified crawl recovery, it is important to note that the loss of ascending inputs from the terminal compound ganglion (Baader and Kristan 1995) and M21 (Mullins et al. 2011), known to promote crawling and swimming, did not prevent the front half of the leech from immediately crawling after delivery of a mid-body nerve cord transection (Fig. 9C).

On a final note, the relatively large variance in recovery rates obtained across individual leeches (e.g., see Figs. 2, 4) is likely telling about the solutions that each nervous system is competent to achieve and the specific physiological histories and internal states that either hamper or facilitate recovery outcomes (Marder and Taylor 2011; Sakurai et al. 2014). This richness in recovery capabilities will provide a valuable test bed for future explorations of what constitutes resiliency and how it can best be promoted.
Figure Legends

Figure 1. Acute and long-term changes in crawling and swimming following nerve cord transection. A, Photomicrograph (left) and diagram (middle) depicting the transection site, which separates the segmental ganglia (M1-M21) housing the crawl CPGs, from the descending inputs originating from the brain (i.e., the compound cephalic ganglion comprising the supra- and subesophageal ganglion, SEG). Representative post-surgery photo is shown at far right. B, Median duration (+/- 95% CI) of swim bouts in deep water (4 cm depth) during a 6 min observation window before and after transection. One day after surgery, swim bout duration significantly increased in transected leeches (white bars) (*, p<0.0001, Kolmogorov Smirnov (K-S) test), but not in sham-operated controls (gray bars). C, Number of unevoked crawl or crawl-like cycles in shallow water (6 min window). Cycle number was significantly lower 1 day post transection (*, p <0.0001, K-S test), but not in sham-operated controls p<0.5. D, Evoked crawl behavior within a 2 min window. One day following surgery, crawl-like behavior consisting of one or more cycles could not be evoked in any transected individuals (*, p<0.0001, X^2 test). Error bars represent standard deviation of the mean in C and D.

Figure 2. Daily changes in crawling following transection. A, Representation of crawl recovery in individuals (rows) over specific days post transection (columns). Locomotor activity containing 1 crawl-like cycle (light gray square) or 2 or more consecutive cycles (dark gray square) are reported. Note the abundance of dark gray squares for the sham-operated controls, and their continued rise in transected animals.
by Day 10. **B,** The number of crawl or crawl-like cycles occurring within a 6 min trial in shallow water. Note: in this figure and all subsequent ones, the + symbol marks the mean in the box and whisker plots. Boxes represent an interquartile range (from 25% to 75%) with the whiskers extending to 1.5 times the interquartile range (Tukey 1977). Diamonds represent outliers (see Methods). The repeated measures statistical test used in this and following figures was the SM test that, when significant, was followed by a post hoc Dunn’s comparison. By 13 days following transection, the decrease in crawling observed in transected individuals (white bars) was no longer significant compared to pretest values (ns, p>0.24, Dunn’s test). Activity in sham-operated controls (gray) remained indistinguishable from pre-test conditions (p>0.288, SM test).

Figure 3. Duration of swim and crawl bouts in deep water during a 6 minute trial.
Animals were placed in 4 cm of water, a depth in which they were permitted to swim (**A**) or crawl (**B**). Bouts of each behavior (i.e., the duration of 1 or more consecutive locomotor cycles) was measured in seconds over the course of a single 6 min trial. **A,** Compared to pre-test values, transected individuals showed a significant increase in swim-bout durations (p<0.001, SM test); at 40-50 days swimming returned to pre-test levels (ns, p>0.17, Dunn’s comparison). Sham-operated controls (gray) exhibited no significant change in swim bout duration following surgery (p>0.88, SM test). **B,** Crawl bout duration was decreased in transected individuals for the first 10 days (p<0.05, Dunn’s Comparison). By Day 40, these values ceased to be significantly different from pre-test values (ns, p>0.32). Sham-operated controls never showed a decrease in crawl-bout duration (p>0.95, SM test).
**Figure 4. Colorized video analysis of crawling in control leeches and those recovering from transection.** To analyze the progression and kinematics of crawl recovery, videos were obtained at varying time points post transection. **A,** Overview of colorization process: 8 frames were extracted from crawl videos at equal intervals starting and ending with the beginning and ending point of a crawl cycle. Upper panels: i, shows an extracted frame from a given video; ii, image with some glare analyzed with a threshold filter in ImageJ to render it black and white; small gaps are filled in with the ImageJ line and fill tools; iii, the 50 pixel-wide line added as a thickness (i.e., width) reference to calibrate the color scale; iv, the local thickness plugin run to colorize a given leech silhouette based on the color scale, shown at far left, which depicts various widths. For example, the blue-purple-red colors represent less wide (narrower) body regions of the leech as compared to the orange and white colors. **B,** Body silhouettes of a sham-operated control leech crawling before (top, pre-test) and 1 day after surgical manipulation with no transection (Day 1, bottom). Times at bottom right indicate crawl cycle duration. Looking at the silhouettes, from left to right (e.g., 3rd-5th ones over), the body became successively less wide and colors shifted from mostly white-orange to blue-red. Note that the blue-red colors invaded the body over time in the anterior-to-posterior direction. This wave of color reflected the metachronal wave of segmental circular muscle activity that produced crawl elongation. The notable curvature in crawl trajectories was a result of the animal’s tendency to use thigmotaxis to navigate along the inside edge of the circular arena. **C and D,** Crawl profiles of 2 different leeches before (pre-test) and many days after receiving a similar nerve cord transection (M1).
Although the time course of recovery differed across the two animals, by Day 40 the crawl form in both animals was indistinguishable from pre-test activity. For example, the silhouette colorization patterns of both animals were similar to those of the pre-test animals (for both elongation and contraction phases of crawling) whereby the blue-red color invaded the body caudally during the elongation phase and the orange-like color invaded the body (also caudally directed) during the contraction phase.

**Figure 5. Quantification of body coordination during the crawl elongation phase.**

To assess the time course of the return of coordinated crawling, leeches were visually divided into four sections, and the sequential activity of those quarters was recorded using video analysis (see Methods). In shallow water, up to 25 crawl elongations exhibited during a 6 min window were analyzed for 8 transected leeches and 4 sham-operated controls. The total number of elongations/6 min period for all animals recorded was: Pre-test (130); Day 40+ (177); Sham-operated control Day 1 (71); Days 1-10 (12, 8, 8, 6, 15, 30, 15, 26, 63, 53). A, Percent of quarters active during a given elongation phase over the days to weeks of recovery. Legend at right color codes the number of active quarters. B, First quarter of the leech body to elongate during a given elongation phase over the days to weeks of recovery. Legend at right depicts the 4 quarters of the leech from rostral to caudal region (A-D). Prior to surgery, the elongation phase began in the head region (quarter A, see inset at right). Following surgery, elongations typically began with activity localized in the rear quarters (e.g., quarters C and D). By Day 10, 100% of all forward elongations (over 80% of all elongations) regained their rostral initiation. C, Number of sequentially activated quarters observed during the elongation phase of
crawling over the days to weeks of recovery. Legend at right color-codes the number of sequentially activated quarters progressing in either the anterior or posterior direction. During the first 4 days of recovery, a caudally-directed sequence of more than 2 adjacent body regions was minimal, with only 1 transected individual exhibiting a four-quarter crawl-like elongation during this time frame (on Day 1). Activity was frequently seen in a single quarter or, in a few instances, across nonadjacent sections with no specific sequence (collectively, white with gray diagonal lines). After Day 5, a few partial waves were directed in the reverse direction (white and light gray), whereby the animal crawled backwards. Over time, however, the leech regained its original caudally-directed sequence (e.g., A, B, C, D) (black), thus exhibiting a fluid, productive anterior-to-posterior wave of normal crawl-specific movements.

Figure 6. Changes in crawling and swimming following complete removal of the first segmental ganglion (M1). Although axonal regrowth across transections was not evident 98 days after crawling had returned (Fig. 1A), the potential for axonal re-connectivity was further challenged by removing M1 in 6 animals. A, Diagram depicting M1 removal. B, Representation of the course of individual recovery where rows represent individual leeches and columns represent the days post transection. C, Number of crawl or crawl-like cycles occurring within a 6 min trial. After Day 6, the decrease in crawl behavior was no longer significant (ns, p>0.09, Dunn’s test). D, Bouts of swimming measured over the course of a single trial for all 6 individuals; plotted as described in Methods and Figure 2. Eight days following transection the increase in swimming behavior was no longer significant (ns, p>0.12).
Figure 7. Changes in crawling and swimming following complete removal of the subesophageal ganglion (SEG). To rule out axonal reconnection between the proximal and distal severed regions of all cephalic descending axons, we removed the entire SEG in 6 individuals and determined whether crawling could be re-established. A, Diagram depicting SEG removal. B, Representation of the course of recovery for individual animals as described in Fig. 6B. C, Number of crawl or crawl-like cycles occurring within a 6 min trial. C. After Day 10, the decrease in number of crawl cycles was no longer significant (ns, p>0.18, Dunn’s test). D, After Day 30, duration of swim bouts was no longer significantly longer compared to pre-test durations (ns, p>0.09).

Figure 8. Leeches repeat the recovery process following removal of the ‘lead’ ganglion. To ascertain whether or not the ganglion closest to the transection site had become vital for crawl recovery, we tested whether its removal would result in a second loss of crawling behavior with a second round of recovery. A, Diagram depicting the sites of the first and subsequent transections. B, Representation of the course of recovery for each animal as previously described in Fig. 6B. C, Number of crawl or crawl-like cycles occurring within a 6 min trial. At 72 days following the second transection, the number of crawl cycles was no longer significantly different than pre-test values (ns, p>0.32, SM test). D, Bouts of swimming measured over the course of a single trial for the same 6 individuals tested for crawling. At 8 and 72 days following a second transection, swim bout durations were no longer elevated compared to pre-test values (ns, p>0.08 and p>0.37 respectively, SM test).
Figure 9. Midbody nerve cord transections test possible anatomical constraints on the lead ganglion hypothesis and establish if across transection oscillator coupling is possible. To test whether posteriorly located ganglia were capable of setting up their own crawl wave along the body, nerve cords were transected below M9-10. A, Diagram depicting the midbody transection (left) and representation of the course of recovery for all 6 individuals measured. The blue and pink shadings over the diagram highlight behavioral activity controlled by a partial nerve cord attached to the brain and one that is not, respectively. B, Video image prepared, as in Fig. 4, of a leech crawling at 115 days after transection below M9-10. Note that in the 5th through 7th frames (silhouettes) the anterior half of the leech is entering its crawl elongation phase, while the posterior body half is in a crawl-contraction phase; indicative of 2 independent crawl cycles. C, Number of crawl or crawl-like cycles occurring within a 6 min trial. The blue and pink shading highlights the anterior and posterior body regions; purple denotes the potential for cross-transection activity. Note that no cross-transection body coordination ever returns for crawling (bottom panel). D, Video images of coordinated (sinusoidal) swimming (top clip), and cross-transection uncoordinated swimming (bottom clip). For uncoordinated swimming, the sinusoidal swim wave was not distributed along the entire body; often with the anterior body in a contracted state. E, Bouts of swimming measured over the course of a single 6 min trial for all 6 individuals observed. Swim suppression of the posterior body returned to control levels by Day 10. Cross-transection swim coordination, measured as a whole-body sinusoidal waveform, was not prevented. Note that the time scales on the y axes have been kept the same for purposes of comparison, thus insets (swim, top and bottom) are provided to showcase
smaller values.

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Figure 1

A

B

Swim behavior

Average swim bout duration (s) per 6 minute trial

Before transection 1 day after transection 40-50 days after transection

C

Unevoked crawl behavior

Average number of crawl-like cycles per 6 minute trial

Before transection 1 day after transection 40-50 days after transection

Shams Transections

D

Evoked crawl-like behavior

Probability of induced crawl-like activity

Before transection 1 day after transection 40-50 days after transection

SEG M1 M2 M3 Brain Ganglia
Figure 2
Figure 3

A. Deeper water swim behavior

B. Deeper water crawl behavior
Figure 4
Figure 5
Figure 6

A

B

Crawl behavior

M1 removal
Days after transection

pre 1 4 8 10 30 115

No crawl
Crawl
Only 1 cycle

C

D

Number of crawl-like cycles in 6 minutes

Swim bout duration (s)

Days after transection

Days after transection

Swim behavior
Figure 7
**Crawl Behavior**

**Number of crawl-like cycles in 6 minutes**

Days after second transection

**Swim Behavior**

**Swim bout duration (s)**

Days after second transection

Figure 8