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Single pellet grasping following cervical spinal cord injury in adult rat using an automated full-time training robot

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Abstract

Task specific motor training is a common form of rehabilitation therapy in individuals with spinal cord injury (SCI). The single pellet grasping (SPG) task is a skilled forelimb motor task used to evaluate recovery of forelimb function in rodent models of SCI. The task requires animals to obtain food pellets located on a shelf beyond a slit at the front of an enclosure. Manually training and testing rats in the SPG task requires extensive time and often yields results with high outcome variability and small therapeutic windows (i.e., the difference between pre- and post-SCI success rates). Recent advances in automated SPG training using automated pellet presentation (APP) systems allow rats to train ad libitum 24 h a day, 7 days a week. APP trained rats have improved success rates, require less researcher time, and have lower outcome variability compared to manually trained rats. However, it is unclear whether APP trained rats can perform the SPG task using the APP system after SCI. Here we show that rats with cervical SCI can successfully perform the SPG task using the APP system. We found that SCI rats with APP training performed significantly more attempts, had slightly lower and less variable final score success rates, and larger therapeutic windows than SCI rats with manual training. These results demonstrate that APP training has clear advantages over manual training for evaluating reaching performance of SCI rats and represents a new tool for investigating rehabilitative motor training following CNS injury.

Keywords

Single pellet grasping; Spinal cord injury; Rehabilitation; Skilled motor task; Motor behavior; Automated animal training; Reaching; Grasping

1. Introduction

It is widely acknowledged that task specific motor training is a key factor in recovery of fine motor function after CNS injury or disease. Skilled reaching tasks are important research

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tools for studying motor recovery in animal models of nervous system injuries such as spinal cord injury [SCI; 1,2–7] and stroke [8–11]. There are a number of manually administered reaching tasks used to study forepaw function and deliver forepaw rehabilitation in rodent models of CNS injury or disease including the Montoya staircase test [12,13], the Whishaw tray task [14], the isometric pull task [15], and the single pellet grasping (SPG) task [16]. However, because these reaching tasks rely on individual researcher interaction with animals they are subject to high degrees of variability between experiments, researchers, and laboratories. Moreover, these tests can be time consuming and tedious to administer (e.g., SPG, Montoya, and isometric pull tasks), especially in animals with forelimb dysfunction, and/or provide limited insight to kinematics and mechanisms of recovery from injury or disease (e.g., well-grasping, Whishaw tray, and isometric pull tasks).

The SPG task is frequently used to evaluate motor function and reaching motions before and after cervical SCI. It has several advantages compared to other reaching tests for evaluating forelimb motor function after cervical SCI. For example, SPG training and testing can be limited to the forepaw affected by injury and detailed analyses of reaching and grasping motions are possible. Moreover, the SPG task is a complex motor task and the SPG motions are not regularly performed in the home cage. As a result, changes in SPG performance can be attributed to training and testing within the enclosure rather than self-training in the home cage, which has been proven a problem for locomotor training [17]. Yet despite its advantages manual administration of the SPG task requires extensive one-on-one researcher-to-rat training, which is time consuming and can be a source of variation between laboratories and from day to day within the same study. Also, manual training and testing of the SPG task before and after CNS injury or disease often results in small therapeutic windows (that is, the difference in SPG performance between pre-injury baseline and post-injury final scores), thus limiting the value to studies testing the effect of rehabilitative training or other treatments.

The role of rehabilitation training is growing in animal models partly because it is standard practice in the clinic, but also because there is mounting evidence drug and cell therapies are more effective when combined with rehabilitation therapy [2,18,19]. Given the limitations of current training methods there is a need for novel high-throughput and standardized training methods that accurately test the recovery of reaching and grasping function in animal models of CNS injury and disease.

We recently described an automated pellet presentation (APP) system to present pellets to rats 24 h a day, 7 days a week, which allowed APP trained rats to perform an automated version of the SPG task ad libitum [20]. Rats with APP training were successfully trained to perform the SPG task and employed similar grasping motions as manually trained rats. A key difference between the APP SPG task and the manual SPG task is that for the manual task pellets are presented from a small notch in a presentation shelf and can be scooped or dragged to the enclosure without grasping the pellet, whereas for the APP system there is a gap between the pellet presentation pedestal and the enclosure which precludes scooping or dragging pellets. Scooping and dragging is a common compensatory strategy used by rats with cervical SCI for obtaining pellets without grasping [21,22]. However, whether APP trained rats with SCI could perform the SPG task without access to compensatory scooping

or dragging and whether limiting compensation improves functional recovery remains unknown.

The purpose of the present study was to explore whether rehabilitative APP training would allow for a simplified and more standardized training of the SPG task, thus opening the door for systematic exploration of the effects of task specific rehabilitative motor training in rats with SCI. For this we tested whether rats could perform the SPG task using the APP systems following unilateral cervical SCIs that affected forelimb motor function. We found that rats with APP training were able to obtain pellets using the APP system after SCI, but with lower success and attempt rates than pre-injury baseline. Importantly, APP trained rats had larger therapeutic windows than manually trained rats, indicating that APP training will be a useful tool for identifying treatment effects that could not be detected using manual training approaches.

2. Materials and methods

Eighteen female Lewis rats (Charles River Laboratories, Wilmington, MA, USA) weighing 210–240 g were trained to perform the SPG task either manually ($n = 10$ rats) or using an APP system ($n = 8$ rats). All animals were individually marked on their tails and housed in groups of 2–5 and kept on a 12/12 h light/dark cycle. Both manual and APP trained rats were housed in standard static home-cages with a PVC tube and small cedar block ($\sim 3 \times 3 \times 3$ cm) for enrichment. Additionally, the static cages of the APP trained rats had a small hole with a tube connecting the home-cage to the APP task enclosure. All procedures were approved by the Health Sciences Animal Care and Use Committee of the University of Alberta.

2.1. Manual SPG training

Manual SPG training followed the same training protocol as previously described [21,22] and is consistent with similar training protocols for this task [1,2,5,7,16]. Briefly, upon arrival to the animal facility the rats had ad libitum access to rat chow and water. Several days prior to the start of SPG training the average food intake per rat per day was measured. On the day prior to each training session food was restricted to 95% of the average food intake, usually between 9 and 11 g of food per rat per day, otherwise rats were fed ad libitum. Rats were weighed daily and their weight was maintained at about 95% the weight of ad libitum fed animals (c.f., APP trained animals who were fed ad libitum; Fig. 1E) by adjusting the amount of home-cage food provided.

To begin each training session, a rat was placed at the back of a standard acrylic SPG training chamber (40 cm long, 12.5 cm wide, 45 cm tall) with a 1 cm wide and 10 cm tall vertical slit in the front wall. Since each rat has a preference to use either their right or left paw to perform grasping tasks, the manual SPG task enclosures had two fixed pellet presentation wells located about 0.5 cm left and 0.5 cm right relative to the center of the slit. In the first few training sessions banana flavored sugar pellets (45 mg, TestDiet, 5TUT sucrose tab, St. Louis USA) were placed on both wells of the pellet presentation shelf at the front of the chamber. Once the rat had approached the front of the chamber and had completed a grasp attempt, the trainer placed a sugar pellet at the back of the chamber to

encourage the rat to return to the back of the enclosure. The rat then returned to the back of the enclosure, another pellet was placed on the pellet presentation shelf, and the process was repeated for the entire session. Once the rat learned to shuttle, pellets were no longer placed at the back of the enclosure. Following completion of each training session the rat was returned to their home-cage. The preferred paw was determined by tracking which paw each rat used to obtain pellets. After a few training sessions, once paw preference was determined, pellets were presented in the left-well to rats with a right-paw preference, and vice versa.

All rats with manual training were trained to perform the SPG task for four weeks before SCI. One week following SCI, SPG training was continued for 6 weeks post-injury. For baseline and final scores in the reaching task the success rates of each animal were averaged for all training sessions in the final week before SCI and the final week of post-injury training respectively. With manual training SCI rats sometimes use compensatory strategies such as scooping or dragging the pellet to their mouth rather than grasping the pellet from the presentation shelf [21,23]. Since scooping is not possible with APP training, for this study scooped or dragged pellets observed during manual training were scored as a 'scoop' and counted as an attempt, but were not considered successful grasps.

2.2. Automated pellet presentation system

The same APP systems used for this study was used in a previous study [20]. Briefly, the APP systems consist of an APP robot integrated within a modified SPG task enclosure, which is connected to the home-cage. The APP system was designed so that the rats had unrestricted access to both their home-cage and the APP task enclosure (45 cm long, 10 cm wide, 30 cm tall) and the robot within the SPG enclosure 24 h a day, 7 days a week, except during cleaning times. From within their home-cage, the rats had ad libitum access to food (standard rat chow) and water. Additionally, the APP trained rats could also obtain banana flavored grain pellets (45 mg, TestDiet, 5TUM grain-based rodent tablet, St. Louis USA) from the APP system by successfully performing the SPG task (see below for details). In the center of the front wall a 1 cm wide and 10 cm tall vertical slit was cut, through which the rats had access to the pellet platform. All electronic robotic components were purchased from Phidgets Inc. (Calgary, Canada), and robot-controller software written in Java using NeatBeans IDE 7.3. Motors were controlled using a PhidgetAdvancedServo 8-Motor controller board connected to a controller computer via USB. Sensor activity was monitored and LEDs were controlled using a Phidget Interface Kit 8/8/8 w/6 Port Hub connected to a controller computer via USB.

The APP system included a custom made pellet retrieval arm with a 3 mm diameter loop at the distal tip, called the platform, and could hold a single food pellet. To retrieve a pellet the pellet arm motor was activated so the platform would pass through the pellet hopper and would continue to rotate until a pellet was detected on the pellet platform by a pellet sensor, at which point a stop signal was sent to the controller to stop the pellet arm motor. The pellet retrieval process was repeated if the pellet sensor indicated the pellet had been removed from the pellet platform. The location at which pellets were presented could be adjusted in 3D space (i.e., proximal-distal, medial-lateral, and up-down) relative to the slit opening by

adjusting the position of the pellet retrieval system along the aluminum frame. Since each rat has a preference to use either their right or left paw to perform grasping tasks, manual SPG training enclosures typically have two fixed pellet presentation wells located about 0.5 cm left and 0.5 cm right relative to the center of the slit. Pellets are presented in the left-well to rats with a right-paw preference, and pellets are presented in the right-well to rats with left-paw preference. With the APP system, presentation position was adjusted depending on the stage of training (see Section 2.3) and the paw preference. Pellets were typically presented at a distance of 1.3–2 cm from the slit, up to 0.5 cm left or right of the center of the slit, and 2 cm up from the level of the enclosure floor. Access beyond the slit was controlled by an adjustable barrier system.

Sensors to detect the presence of a rat were located in the front half of the enclosure. Two floor sensors were located between a fixed base floor and a thin acrylic floating floor above the sensors to indicate whether a rat was standing on the floating front floor. One proximity sensor was positioned in the middle of the enclosure to detect rats that were resting their forepaws on the floating floor (i.e., remained within the front half of the enclosure) without activating the floor sensors. Together, these sensors could effectively detect the presence of a rat in the front half of the enclosure.

2.3. SPG task training using the APP system

APP pre-injury training followed the same training protocol as previously described for uninjured rats [20]. Briefly, SPG training was divided into four stages; three pre-injury training stages (Stages 1–3) as described previously [20], followed by one post-injury stage (Stage 4; Fig. 1A). Stage 1 was designed to allow the rats to acclimatize to the pellet presentation system and to have easy access to the pellets. In Stage 1 the rat sensor system was deactivated and the pellets were presented very close to the slit opening (<15 mm) and aligned with the center of the slit. In this configuration the rats had easy access to the pellets and were not required to return to the back of the enclosure for subsequent pellet presentation. All rats that had at least one day with 30 attempts ($n = 8$ of 8) in Stage 1 were considered to have learned the location of the pellets and proceeded to Stage 2.

Given the closeness of the pellets in Stage 1 (<15 mm), a small number of rats ($n = 3$) preferentially obtained pellets by licking rather than grasping. Stage 2 was designed to promote forelimb grasping instead of licking and to train the rats to move to the back of the enclosure for each attempt. To train the rats to move to the back of the enclosure, the barrier would remain closed until there was no rat detected by the rat sensors in the front half of the enclosure. Since a rat forepaw can reach through the slit further than an extended tongue, the distance of the pellet from the slit was gradually increased by 0.5–1 mm every 3–7 days from <15 mm to as high as 20 mm, until the rats no longer attempted to obtain pellets by licking.

In Stage 3 rats were housed according to their preferred paw. Additionally, the APP system was setup so that the pellet presentation platform was located 14.5–15 mm from the slit opening and aligned with the left edge of the slit for right-pawed rats or the right edge of the slit for left-pawed rats. In this configuration rats did not relapse into obtaining pellets by licking. The APP system remained in this configuration for the remainder of training.

Stage 4 started 6–8 days after SCI and continued for 6 weeks post-injury for $n = 7$ animals. One animal performed an average of 4 attempts per 24 h cycle in the first 6 days of Stage 4 training and zero attempts for the following 14 days of Stage 4 training. This animal was therefore removed from the APP system after only 4 weeks of post-injury training.

2.4. Video recording

Reaching attempts were video monitored 24 h a day, 7 days a week using Blue Iris software (v. 3.5, Perspective Software) and Foscam light/dark digital cameras (FI9821W) equipped with an integrated infrared LED array for dark-cycle recording. The cameras automatically activated the LED array and switched input gain and intensity during the dark-cycle. Video acquisition was set at 30 fps with a resolution of 640×480 pixels.

2.5. Spinal cord injury

Following reaching training each rat received a dorsal lateral quadrant (DLQ) spinal transection injury on the side of their preferred paw as established during reaching training. Rats were anesthetized using Isoflurane (2.5% in 50:50 air:oxygen mixture) and the dorsal neck was shaved and disinfected with 10% chlorhexidine gluconate. Buprenorphine was injected s.c., immediately post-op (0.03 mg/kg) and again 8–12 h later (0.02 mg/kg). Body temperature was maintained with a heating blanket. The spinal cord between C4 and C5 was exposed with a small laminectomy of the caudal half of C4. The dura was resected from over the intended injury site and a custom-made blade was lowered 1 mm into the spinal cord at the midline and then moved laterally. The muscle layers were sutured, the skin closed with staples, and 4 ml of saline was injected s.c. to maintain hydration. Animals were kept on a heating blanket until fully awake. Post-operative pain was managed by s.c., injections of buprenorphine (0.05 mg/kg) every 12 h for 2 days.

2.6. Histology and lesion size quantification

Upon completion of post-injury training, rats were euthanized and perfused with saline followed by 4% formalin solution containing 5% sucrose. Spinal cords were dissected and post-fixed overnight then transferred to 30% sucrose in PBS for 3 days. Spinal cord cross-sections were cut at $25 \mu\text{m}$ on a cryostat. Tissue was dried for 30 min at 37°C , rehydrated in TBS, then placed in 0.5% cresyl violet for 4 min. Tissue was rinsed 3 times in dH_2O and then serially dehydrated in EtOH (50%, 75%, and 99%) for 2 min each. Finally, tissue sections were cleared in xylene for 2 min and cover-slipped with Permount (Fisher Scientific, Canada).

Lesion sizes were evaluated using light microscopy. Lesion sites were considered as any part of the tissue section with intense cresyl violet staining and/or where tissue integrity was compromised. Lesion sizes were calculated as a percentage of the total cross-sectional area of the tissue section using ImageJ (NIH, Bethesda Maryland, USA). Lesions were reconstructed on a schematic of a C4 spinal cord cross section based on a stereotaxic atlas [24]. Multiple tissue sections were evaluated for each animal and the tissue section with the greatest cross-sectional lesion area was used to calculate lesion size. Using ImageJ, the lesions were further analyzed to determine the extent of injury to specific spinal tracts such as the corticospinal tract (CST) and rubrospinal tract (RST) using reported projection sites

[24–26]. For this, a C4 spinal cross-section diagram with the CST and RST superimposed over the diagram (Fig. 5A) was used to estimate the locations of the CST and RST in the injured spinal cords (Fig. 5B,C). The percentage of the CST and RST that was cut on the intended injury side was categorized as either 0, 25, 50, 75, and 100% cut for each spinal tract. In some animals the DLQ injury extended slightly beyond the midline thus resulting in damage to the contralateral CST. In these cases only the CST ipsilateral to the intended DLQ site was considered for CST injury analysis.

2.7. Analysis of APP video data

Videos were analyzed using BlueIris software. Most attempts required ~10 s of video and video playback rates were set at 4× normal speed, so each attempt required ~2.5 s to analyze. The animal performing each attempt was determined based on tail markings and results were sorted by animal. An attempt was defined as a forward motion of the dominant forepaw beyond the threshold of the slit opening towards the pellet platform. Attempts were evaluated as *pass* or *fail*.

1. *Pass*. A pass was scored when the rat successfully grasped a pellet from the pellet platform using their preferred forepaw and brought it back through the slit into the task enclosure.
2. *Fail*. A fail was scored when the rat made a grasp attempt, but failed to bring the pellet back through the slit.

Performance rates for each animal were calculated by dividing the number of passes by the number of attempts. Unlike with manual training, scooping or dragging pellets into the enclosure is not possible with the APP system because there is a gap between the pellet platform and the slit so that any scooped or dragged pellets would fall out of reach. Baseline performance before injury was calculated as the average success rate in the final week of training before injury.

During some attempts, more than one rat was present in the enclosure at the same time. In cases where a rat made an attempt while another rat(s) was located at the back of the enclosure and did not obstruct the movements of the attempting rat to move towards the slit and reach for the pellet, the attempt was included in the study. On rare occasions another rat was located towards the front of the enclosure and obstructed access to the slit or the reaching movements of the rat reaching for a pellet. Since in these cases the reaching and grasping motions were compromised, the grasping motion was not considered an SPG attempt and was excluded.

For quantification purposes each APP training session was considered as one light-dark cycle starting at lights-on. Rats were weighed daily partway through the light-cycle. Home-cage food consumption was measured daily and the average home-cage food per rat was calculated. For overall food consumption per rat, the number of food pellets obtained by the rat was multiplied by the weight of the food pellets (45 mg/pellet) and added to the average home-cage food consumed per rat.

2.8. Statistical analysis

Statistical analysis was performed using non-parametric tests including the Mann–Whitney-*U* test, the Wilcoxon Signed–Rank test, and the two sample Kolmogorov–Smirnov test. All averages are presented as mean $\pm$ s.e.m. Significance was set as $p = 0.05$.

3. Results

3.1. APP system and manual training before and after SCI

Our primary goal was to explore whether full-time ad libitum APP training is effective after cervical SCI with the idea to simplify and standardize training and testing of the SPG task and thus allow for a systematic exploration of SPG training after SCI in rats. For this we tested whether rats with DLQ SCIs could perform the SPG task using a previously described APP system [20]. The results from the SCI rats with APP training ($n = 8$) were compared to those of SCI rats with manual training ($n = 10$) [22]. As a first step, rats were trained and tested in the SPG task using the APP system or using a manual training protocol as previously described [22]. Average baseline attempt rates for the final week of training before injury was higher for rats trained in the APP system (average = 194 ± 9 attempts/24 h) compared to rats with manual training (Fig. 1B; average = 47 ± 3 attempts/24 h; $p < 0.001$, Mann–Whitney test for two independent samples). There was, however, no significant difference in the baseline SPG success rates between rats with APP training (average = $48.8\% \pm 2.3$) and manually trained rats (Fig. 1C; average = $43.7\% \pm 3.0$; $p = 0.27$, Mann–Whitney test for two independent samples). One week following SCI, rats with APP training were re-introduced to the APP system for ad libitum full-time rehabilitative SPG training and rats with manual training were provided 10 min of rehabilitative SPG training 5 days per week ($n = 25$ training sessions/rat) until 6 weeks post-injury. Average daily post-injury food intake of rats with APP training was higher throughout the post-injury period compared to rats with manual training, likely because these rats were food restricted, which is typical of manual training, whereas rats with APP training were not (Fig. 1D). After SCI average body weight dropped by 13 ± 1 g for APP trained rats and 17 ± 2 g for manually trained rats, followed by steady gains for both groups consistent with normal rat growth (Fig. 1E). These results show that rats with APP training tolerated the APP system very well throughout the post-injury period.

Performance in the APP system was analyzed from digital video recordings of every attempt. Each attempt was evaluated as either a *pass* or *fail*. Examples of reaching by uninjured rats in the APP system have previously been shown [20]. Examples of *pass* and *fail* attempts of SCI rats are illustrated in Fig. 2 (Videos 1–2). The average amount of researcher time per rat per week to train and test SCI each rat using the APP system was calculated by multiplying the average time required to evaluate the outcome of each attempt ($n = 2.5$ s/trial) and multiplied by the average number of attempts per day ($n = 70.7$ trials/day) by the number of training days per week ($n = 7$) to yield an average of 21 min/rat/week. The average amount of research time per week for manually trained animals is fixed at 10 min/day multiplied by 5 days/week, to yield an average of 50 min/rat/week. Comparable to uninjured rats [20] post-injury training required less than half the researcher time per rat per week using the APP system relative to manual training.

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3.2. Comparison of APP and manual training in attempt rates post-injury

Rats with APP training quickly resumed SPG training once reintroduced to the APP system post injury. Attempt rates for rats with APP training rose quickly and peaked at 10 days post-injury and slowly declined to a plateau at 30 days post-injury (Fig. 3A). Similarly, rats with manual training reached plateau levels at 13 days post-injury, but attempt rates remained fairly constant thereafter (Fig. 3A). Pairwise comparison of baseline attempt and final score attempts rates showed that rats with APP training had lower attempts rates in the last week of training following SCI compared to baseline (Fig. 3B; final score average = 51 ± 2 attempts/24 h; $p = 0.012$, Wilcoxon signed-rank test for paired samples). Conversely, baseline attempt and final score attempts rates were not significantly different for rats with manual training (Fig. 3B; final score average = 37 ± 2 attempts/24 h; $p = 0.114$, Wilcoxon signed-rank test for paired samples). Despite the drop in number of attempts for rats with APP training, their average attempt rates were still higher than those of manually trained rats post-injury (Fig. 3C; $p = 0.05$, Mann–Whitney test for two independent samples). Moreover, similar to pre-injury APP training [20], most APP attempts were made during the dark cycle (Fig. 3D; $p = 0.006$, Wilcoxon signed-rank test for paired samples), whereas manual training was done exclusively during the light-cycle. Together these data suggest that standard manual training protocols do not provide adequate training time compared to APP training. Furthermore, comparison of the distributions of attempt rate final scores revealed that the attempt rates for rats with APP training were widely distributed ranging from 0 to 193 attempts per 24 h, whereas the attempt rates for rats with manual training were closely grouped between 23 and 55 attempts per training session (Fig. 3E; $p = 0.031$, two sample Kolmogorov–Smirnov test). These data show that although rats with APP training generally have higher attempt rates: (1) rats with manual training are highly motivated to perform the SPG task as indicated by the relatively high minimum attempt rate of 23, likely due to food restriction and the use of sugar pellets for training rather than grain-based pellets as used for APP training; and (2) overall attempt rates with manual training is limited by restricted training times as indicated by the tight grouping of overall final score attempt rates near the maximum, further suggesting that rats with manual training less than most rats with APP training.

3.3. Comparison of APP and manual training in SPG performance post-injury

At the start of post-injury training SPG success rates of rats with APP training followed a similar pattern as the attempt rates, with average daily SPG% increasing to peak levels at 7 days, two days after peak attempt rates, followed by a steady decline and to a plateau stage starting at about 27 days post-injury (Fig. 4A). Conversely, although the attempt rates for rats with manual training reached a plateau, SPG success rates slowly climbed throughout post-injury training with a short plateau period after 30 days post-injury (Fig. 4A). As a result of the steady increase in SPG success rate for manually trained rats there was no difference between baseline and final score results ($p = 0.093$, Wilcoxon signed-rank test for paired samples), whereas the SPG final score for rats with APP training was lower than baseline (Fig. 4B; $p = 0.036$, Wilcoxon signed-rank test for paired samples). Comparison of

the final score results of rats with APP training and rats with manual training showed that rats with manual training had a higher average final score SPG% (Fig. 4C) and higher average final score SPG% when normalized to baseline (Fig. 4D) compared to rats with APP training, but the differences were not significant ($p > 0.05$, Mann–Whitney test for two independent samples). Moreover, although most APP attempts were made during the dark cycle, SPG final score success rates were the same during the light and dark cycles (Fig. 4E; $p = 0.277$, Two Sample Kolmogorov–Smirnov test). Collectively, these results show that rats with APP training undergo a significant drop in success rates after SCI and have less variable final score success rates compared to rats with manual training.

3.4. SPG performance as a function of lesion severity and location

Possible explanations for why rats with APP training had lower final score SPG success compared to baseline, whereas rats with manual training did not, could be because rats with APP training had more severe injuries than rats with manual training or because the APP SPG task is more difficult than the manual SPG task. To test if rats with APP training had more severe SCIs, lesions areas were evaluated and measured as a percentage of the cross section area of the caudal C4 spinal cord (Fig. 5A–C). Surprisingly, rats with APP training had injuries that were less severe than rats with manual training, yet rats with APP training had worse performance than manually trained rats (Fig. 5D,E; $p = 0.008$, Mann–Whitney test for two independent samples). The two APP trained animals with improved SPG% after SCI had lesion sizes near the average lesion size of the other APP animals with lower final score SPG%. Correlation analysis revealed that final score SPG% was negatively correlated with lesion area with correlation coefficient values of -0.405 and -0.730 for rats with APP and manual training respectively, but was significant only for manual training (Fig. 5E; $p = 0.405$ for APP training, $p < 0.013$ for manual training, Spearman Rank Order Correlation). Since certain spinal tracts such as the corticospinal tract (CST) and rubrospinal tract (RST) are important for rat forelimb function [21], these results raise the possibility that rats with APP training sustained more damage to these key spinal pathways than rats with manual training. Comparison of the transected cross sectional areas of CST and RST for the affected forelimbs of rats with APP and manual training showed no difference in the transected CST areas (Fig. 5F; $p = 0.421$, Mann–Whitney test for two independent samples), but rats with manual training had more severe RST lesions than rats with APP training (Fig. 5G; $p = 0.041$, Mann–Whitney test for two independent samples). Finally, it is also possible that since surgeons tend to have a preferred side, lesion severity may be different for animals with right-side lesions and animals with left-side lesions. To test this, the lesion sizes and final score SPG% for APP and manually trained animals were tested. There were no significant differences between left- and right-paw preferred rats suggesting that there was no left-right bias in the lesions ($p > 0.05$, Mann–Whitney test for two independent samples; data not shown). Taken together, these results show that SCIs with modest severity can cause considerable reductions in SPG success rates for rats with APP training compared to rats with manual training and are consistent with the idea that the APP SPG task is more challenging than the manually trained SPG task.

3.5. SPG performance as a function of attempt rates

Following SCI, increasing the amount of manual rehabilitative training from 15 min/rat/day to 30 min/rat/day nearly doubles the SPG success rates (Fouad et al., unpublished results). However, unlike rats with manual training, the final score attempt and success rates of rats with APP training were lower than baseline. It is therefore possible that post-injury SPG success rates are not positively correlated with attempt rates as with manually trained rats. This was, however, not the case since correlation analysis revealed that final score SPG% was positively correlated with final score attempt rates with correlation-coefficient values of 0.74 and 0.83 for rats with APP and manual training respectively (Fig. 6A,B; $p = 0.029$ for APP training, $p < 0.001$ for manual training, Spearman Rank Order Correlation). Visual inspection of the SPG final score distributions in Fig. 5A and B (also seen in Fig. 4B) suggests that SPG final scores were evenly distributed across the final score range, but rats with manual training seemed to be grouped into two categories: 'high-SPG achievers' and 'low-SPG achievers'. Distribution analysis of SPG final scores revealed that the final scores of rats with APP training were normally distributed ($p = 0.35$, Shapiro–Wilk test for normality) within a single group with a range of 0–40% (Fig. 6C). Conversely, the final scores of rats with manual training did not follow a normal distribution ($p = 0.011$, Shapiro–Wilk test for normality), but rather formed two distinct groups; one with low-SPG final scores from 0 to 10% and the other with high-SPG final scores from 31 to 60% (Fig. 6D). This polarization of SPG success rates can lead to difficulties in performing statistical analysis and identifying treatment effects of manually trained rats [21], whereas the normal distribution of APP SPG final scores lends itself to simplified analysis and interpretation of results.

Given that success rate is negatively correlated with lesion size for manually trained animals, it is possible that the high-SPG achievers had less severe lesions than low-SPG achievers. Comparison of the lesion sizes between the high- and low-SPG achiever groups showed that although the average lesion sizes of high-achievers ($28 \pm 6\%$ of spinal cord cross section) was less than low-achievers ($38 \pm 5\%$ of spinal cord cross section), there was considerable overlap in lesion sizes between the two groups and the differences were therefore not significant (c.f., Fig. 5E; $p > 0.05$, Mann–Whitney test for two independent samples). Moreover, although manually trained high-achievers had considerably higher SPG% final scores ($50.4 \pm 4.4\%$) than APP trained rats ($13.7 \pm 4.8\%$; $p = 0.003$, Mann–Whitney test for two independent samples), manually trained high-achievers also had higher average lesion sizes ($28 \pm 6\%$ of spinal cord cross section) than APP trained animals ($18 \pm 2\%$ of spinal cord cross section), but this difference was not significant ($p > 0.05$, Mann–Whitney test for two independent samples). Taken together, these data are consistent with the idea that the APP SPG task is more challenging than the manually trained SPG task.

The fact that the APP SPG final scores of rats with manual training diverge into high- and low-success rate groups, whereas APP final score success rates are normally distributed, could be linked to the way pellets are presented in the different SPG tasks. For instance, one key difference between rats with APP and manual training is that rats with manual training can obtain pellets by scooping them from the presentation shelf, whereas this is not possible for rats with APP training since pellets that are not properly grasped will drop from the

pedestal and out of reach of the animal. It is therefore possible that scooping could influence grouping of SPG success rates. Comparison of SPG final scores of rats with manual training to the number of scoop attempts showed that manually trained rats from the high-SPG group made more scoop attempts than animals from the low-SPG group (Fig. 6E; $p = 0.038$, Mann–Whitney test for two independent samples). However, these results could be because the high-SPG group had a higher attempt rate. To determine if this was the case, the scoop rates between the groups were compared as a percentage of overall attempts, and again the high-SPG group made more scoops than the low-SPG group (Fig. 6F; $p = 0.030$, Mann–Whitney test for two independent samples). Furthermore, most scoop attempts occurred in earlier training sessions (Fig. 6G), which coincides with a period of steady increase in SPG success rates (c.f. Fig. 4A). This period of increasing SPG success rates is followed by a sharp reduction in scoop attempts (Fig. 6G) and coincides with a period of plateaued SPG success rates (c.f. Fig. 4A). These data suggest that scooping could be an effective training strategy used by some manually trained rats to perform more successful grasp attempts later in training. Taken together, unlike manual training, APP SPG performance is not dependent on compensatory scooping strategies. This leads to more normalized results that are less variable over time, suggesting that rats with APP training would be highly useful for studying the effects of rehabilitation, drug, and cell therapies in rats with SCI.

4. Discussion

Forelimb reaching and grasping tasks such as the SPG task are often used for testing therapeutic interventions to promote functional recovery of fine motor control after CNS injuries such as SCI [1–7] and stroke [8–11]. However, previous studies have highlighted the fact that following CNS injury manual implementation of the SPG task is time consuming and can yield highly variable results with success rates ranging from zero to better than baseline, which can lead to small treatment effect windows, and thus difficulties in identifying potentially useful treatment strategies [21]. Here we used an automated SPG task training technique, previously described for the training of uninjured rats [20], for task-specific reaching and grasping rehabilitation therapy after SCI. We showed that rats can be trained to perform the SPG task after SCI using the APP system, which increased treatment effect windows and reduced variability compared to manual training. Moreover, rats with APP training had an improved range of training intensities and few success rates of zero compared to rats with manual training, which allows us to test a full range of rehab intensities. On top of these advantages, the reduced researcher time required to train rats with APP training and the fact that compensatory strategies like scooping can be avoided, makes the APP system the preferred training and/or testing paradigm in forelimb reaching and grasping.

4.1. Treatment effect windows

Treatment effect windows in reaching and grasping tasks after SCI are the difference in baseline success rate before injury and the final score success rates post-injury [3,4,19,21]. Ideally, for animals that receive only post-injury task training (i.e., control animals), success rates should be high before injury, drop to low levels immediately following injury, and then rise to intermediate levels that remain significantly lower than baseline levels (Fig. 7). The

rise in success rates with progressive training can be attributed to post-injury rehabilitation therapy and/or spontaneous daily use therapy (e.g., using the affected limb for tasks such as locomotion and grooming). Although an idealized task would not fully model the large variability in reaching and grasping motor skills of SCI individuals, and robust treatment effects can be observed even with small therapeutic windows, large therapeutic windows remain desirable for scientific and practical purposes. For instance, identification of cell and/or drug treatment strategies with small beneficial effects are important since modest improvements can be considerably amplified when combined with rehab therapy [2,19]. Large therapeutic windows facilitate the identification of treatment strategies (e.g., drug or cell therapy) with small therapeutic effects. Moreover, the rate at which these changes occur can be measured to optimize the timelines of treatments.

Consistent with other studies [3,4,27] we found only small changes between average baseline and final scores of rats with manual training, with the average final scores being $72\% \pm 14$ of baseline. This translates to a treatment effect window of 28%. Conversely, there was a decline in the SPG% for rats with APP training post-injury, with the average final scores at $39\% \pm 7$ of baseline. This translates to a treatment effect window of about 60% for rats with APP training. The large treatment effect windows observed for APP trained animals might be due to an inadequate number of APP trained animals ($n = 8$), whereby a larger sample would have yielded a high- and low-achieving group as observed with the manually trained animals. However, this seems unlikely given that the final score success rates of manually trained animals were highly polarized with half ($n = 5$) of the animals having final score success rates close to or higher than baseline with the other half ($n = 5$) at or near zero. Conversely, APP final score success rates were more normally distributed with only two APP animals having final score success rates near baseline and only one APP trained animal with a final score success rate of zero. Given the high attempt rates of APP trained animals it is possible that a study with a wide range of post-SCI APP training intensities would reveal a multi-modal relationship between training intensity and performance rates where progressively higher training intensities eventually leads to a performance plateau or reduced performance. Together, our results indicate that positive treatment effects would be easier to identify with the APP system and that SPG training and testing with the APP system yields results that more closely resemble an 'ideal' behavioral task than the manual SPG task.

4.2. SPG task difficulty and compensatory strategies

Our results show that manually trained rats achieve higher post-injury success rates, which suggests that manual training is more effective than APP training. One possible explanation for the significant drop in post-injury SPG success rates for rats with APP training is they had more severe lesions, since SPG success rates are inversely correlated with lesion severity [4,21]. Surprisingly, comparison of lesion cross-section areas showed that rats with APP training had less severe lesions than rats with manual training, suggesting that rats require more intact spinal circuitry to do the APP task than the manual task. Alternatively, the drop in APP success rates may be due to the concomitant drop in APP attempt rates after injury. However, rats with APP training had higher final score attempt rates than rats with manual

training. Taken together, these results suggest that the APP SPG task is more difficult than the manual SPG task.

The increased difficulty of the APP task could be due to a variety of reasons. For instance, in the APP system rats can easily displace a pellet from the pedestal with a slight touch and fall out of reach prior to grasping [20]. As a result, more precise grasping motions are required to obtain a pellet with the APP system. Conversely, in the manual enclosures pellets are presented within a small groove located on a shelf [16]. With this setup animals can displace the pellet from the groove without knocking it from the shelf, thus allowing more time and a greater margin of error for the grasping motion. Furthermore, the manual pellet presentation shelf can be used to guide the paw during the reaching/withdrawal motion or rest the paw/digits during grasping motions [16,28], especially after SCI [2,21], which is not possible with the APP system. Indeed, our results show that manually trained rats that scooped in the early stages of post-injury training made more attempts and had higher SPG final scores than manually trained rats that did not scoop. Scooping could therefore be a key training strategy to fine tune forelimb movements and achieve higher SPG success rates over time. Whether rats with APP training would employ the same strategy could be tested in future studies by adding a shelf that extends from the slit to the pellet presentation pedestal of the APP system, which would allow APP trained rats to scoop pellets with the high attempt rates possible with the APP system. Taken together, our data suggest that allowing and/or encouraging compensatory strategies may be a useful rehabilitation tool to encourage training and to promote functional recovery of fine motor control after SCI.

4.3. Attempt rates and food consumption

Two key differences between manual and APP training are access to food and access to training. Rats with APP training have unrestricted home-cage food and can train to grasp pellets ad libitum every day of the week, but rats with manual training have limited access to home-cage food and are restricted to short training sessions 5 times per week. Fixed manual training time limits the maximum number of attempts per session and thus reduces the effectiveness of rehabilitation therapy. Also, final score attempt rates were tightly distributed near the maximum observed attempt rates suggesting that overall attempt rates of manually trained rats were limited by training time.

Following injury average attempt rates for rats with APP training decreased significantly. SCI typically results in pronounced changes in muscle physiology such as muscle atrophy [29], changes in muscle blood flow caused by vascular atrophy [30], and fiber type conversion [29,31,32], resulting in neuromuscular fatigue [29,31]. Fatigue is often a major limiting factor in motor function and motivation with rehabilitation training for SCI individuals [33,34]. It is possible that post-injury fatigue of the affected forelimb could explain the drop in attempt rates for rats with APP training. If true, training with the APP system could be considered a more clinically relevant fine motor task since it closely mimics the training fatigue experienced by individuals with SCI. Conversely, reduced attempt rates of rats with APP training after injury could also be due to diminished motivation. A reduction in motivation of the rats with APP training could be dictated by the ad libitum training schedule. For instance, the motivation of rats with APP training is higher during the

dark cycle compared to light cycle, with over 92% of attempts made during the dark cycle, consistent with their nocturnal sleep-wake cycle. It is possible that overall activity of rats decreases post-SCI, which could reduce attempt rates, but this remains to be tested. Similar to uninjured animals [20], the increased dark-cycle attempt rates for post-injury rats with APP training did not result in increased dark-cycle SPG success rates. Instead, we found that final score attempt rates were a good indicator of final score SPG success rates for rats with APP training. Whether post-injury SPG success rates drop because of an inability to perform more attempts, or whether attempt rates drop because rats with APP training become frustrated with their poor SPG performance, or a combination of the two factors remains unknown. Motivation levels of rats with manual training are maintained with food restriction and by training with sugar pellets rather than the grain-based pellets used for APP training. Either of these strategies may increase the motivation of rats with APP training. For example, in early trials with the APP system sugar pellets were used instead of grain-based pellets, which resulted in almost continuous training such that attempt rates were very high (>500 attempts per rat per 24 h cycle; unpublished results). Presumably the use of sugar pellets and/or food restriction would also increase attempt rates post-injury. Collectively, our results suggest that standard manual training protocols likely do not provide adequate amounts of training to maximize both pre-injury baseline and post-injury final score success rates. The dramatic drop in attempt rates from baseline to post-injury for APP trained rats suggests that it is difficult for animals to maintain very high attempt rates after SCI. It is therefore possible that additional manual training could result in significantly more attempts before SCI followed by a considerable drop in post-injury attempt rates resulting in an improved therapeutic window, similar to APP trained rats. Whether manual training protocols with increased training frequency and session times improved functional outcomes and improved therapeutic windows for SCI rats remains to be directly tested.

4.4. Significance for ad libitum training and translational studies

It is well established that repeated task rehabilitation therapy is one of the most effective treatments to improve fine motor function of the trained task following SCI. As robotic devices for rehabilitation therapy become more sophisticated and affordable, high-intensity repeated task rehabilitation will become more and more prevalent as part of rehabilitation in the clinic and at home. Though numerous animal studies have utilized various fine motor tasks to study the effects of rehabilitation therapy after SCI [e.g.,2,3,19], these studies limited the rehabilitation time of the tested animals to short weekday training sessions. These approaches are not appropriate models for testing full time ad libitum rehabilitation therapy, as is now possible with home based therapies. Therefore, an upper limit for high-intensity rehabilitation therapy have yet to be identified in either animal models or individuals with SCI, and the cellular mechanisms underlying this limit remain unknown. It is important to understand the relationships between training intensity and functional outcomes using animal models since these studies will provide valuable insight regarding the cellular mechanisms underlying functional improvements as well as identifying potential risk factors associated with full time ad libitum training. For instance, previous studies using task-specific rehabilitation therapy has shown that intensive training of the rehabilitation task can adversely affect performance in other motor tasks [2,3].

Collectively, APP SPG training opens the door to studies with maximal training rates, will allow researcher to more easily identify the limitations of rehabilitation therapy, and can help to facilitate the study of the mechanisms underlying the benefits of rehabilitation therapy alone and in combination with other drug and cell therapies.

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Abbreviations

SPG	single pellet grasping
APP	automated pellet presentation
SCI	spinal cord injury

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HIGHLIGHTS

- The single pellet grasping (SPG) task is used to study skilled forelimb movement.
- The SPG task is time-consuming and can yield results with high variability.
- Automated pellet presentation (APP) systems can train and test rats in the SPG task.
- We test whether rats with spinal cord injury can be trained using the APP system.
- Automation yields a bigger therapeutic windows and less variable results.

Fig. 1.

The APP system for automated continuous SPG training before and after SCI. (A) Time-line showing Stages 1–4 of training and setup of the APP system at each stage. (B,C) Bar graphs showing the baseline average number of attempts (B) and baseline average SPG% (C) per 24 h period for rats with APP training and rats with manual training. (D) Plots showing the average daily food consumed per animal immediately before SCI (arrow) and post-injury for rats with APP training (black circles) and rats with manual training (grey squares). * p 0.05.

Fig. 2.

Trial evaluation with APP system. (A–B) Image sequences from analysis videos showing examples of a *pass* (A) and *fail* (B). Magenta circles in the first frames show pellet on pedestal before both the *pass* and *fail* attempts, and the circles in the third and fourth frame of B show the displaced pellet before it fell into the hopper following a failed attempt. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Attempt rates before and after injury. (A) Bar graphs and plots showing the average baseline attempt rates (bar graphs) and average daily post-injury attempt rates (plots) for rats with APP training and rats with manual training. (B) Plots showing baseline and final score attempt rates of individual rats with APP training and rats with manual training. (C) Bar graphs showing the average final score attempt rates for rats with APP training and rats with manual training. (D) Bar graphs showing the average number of attempts per light and dark cycle. (E) Plots showing the cumulative percentage of final score attempt rates for rats with APP training and rats with manual training. * $p < 0.05$, ** $p < 0.01$.

Fig. 4.

SPG success rates before and after injury. (A) Bar graphs and plots showing the average baseline SPG% (bar graphs) and average daily post-injury SPG% (plots) for rats with APP training and rats with manual training. (B) Plots showing baseline and final score SPG% of individual rats with APP training and rats with manual training. (C) Bar graphs showing the average final score SPG% for rats with APP training and rats with manual training. (D) Bar graphs showing the average final score SPG% normalized to baseline for rats with APP training and rats with manual training. (E) Bar graphs showing the average SPG% during the light and dark cycle. * $p < 0.05$.

Fig. 5.

Lesion reconstruction. (A) Diagram of C4 cross section highlighting the intended location of the DLQ transections (pink), the corticospinal tracts (blue), and rubrospinal tracts (red). (B–C) Representative cresyl violet stained cervical (C4) cross sections demonstrating DLQ lesions from a rat with APP training (B) and a rat with manual training (C). Cross section and grey matter outlines shown with black lines, lesion site highlighted in green, estimated locations of CST highlighted in blue, and estimated locations of RST highlighted in red. Extent of CST and RST lesions in B were estimated as 100% and 50% respectively. Extent of CST and RST lesions in C were estimated as 100% and 100% respectively. (D) Bar graph showing the average lesion area at the lesion site as a percentage of total cross section area. (E) Scatterplots with linear-regression lines showing the average final score SPG% in relation to lesion area of rats with APP training and rats with manual training. (F) Bar graph showing the average ipsilateral CST lesion area as a percentage of total ipsilateral CST cross section area. (G) Bar graphs showing average ipsilateral RST cross section lesion area as a percentage of total ipsilateral cross section area. * $p < 0.05$, ** $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

SPG performance and compensatory strategies. (A–B) Regression plots showing final score SPG% as a function of the average number of attempts per day in the last week of training for rats with APP training (A) and rats with manual training (B). (C–D) Bar graphs showing the percent of post-injury training sessions relative to the average SPG% per training session over the last week of training for rats with APP training (C) and rats with manual training (D). (E) Bar graphs showing the average number of scoop attempts per rat with manual training in the high-achiever group and low-achiever group. (F) Bar graphs showing the average number of scoop attempts as a percentage of total attempts per rat with manual training in the high-achiever group and low-achiever group. (G) Line plot showing the number of scoop attempts from the high-achiever rats with manual training relative to post-injury time. * $p < 0.05$.

Fig. 7.

Schematic comparing performance of rats with APP training and rats with manual training to an idealized behavioral task. The average motor score and standard error of the mean are represented by black lines and the grey shaded areas, respectively, for both pre- and post-injury. The y-axis represents SPG% where up is a high score and down is a low score. The x-axis represents time before and after an SCI. In the ideal behavioral task there is a large drop in motor function after SCI, with a modest increase in motor function over time, thus resulting in a big therapeutic window. Our data suggest that rats with APP training have a considerable drop in SPG% after SCI followed by a modest increase in motor function over time resulting in a big therapeutic window. Conversely, rats with manual training have a similar drop in SPG% after SCI, but a more pronounced and highly variable recovery of motor function over time resulting in a comparatively small therapeutic window.