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# Long-Term Motor Deficits after Controlled Cortical Impact in Rats Can Be Detected by Fine Motor Skill Tests but Not by Automated Gait Analysis

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## Abstract

Animal models with constant, long-lasting motor deficits together with the right tests to assess behavioral abnormalities are needed to study the effectiveness of potential therapies to restore motor functions. In the current study, controlled cortical impact (CCI) was applied in rats to induce damage to the forelimb area of the motor cortex and the dorsal striatum. Motor behavior was assessed before and after CCI, using fine motor skill tests such as the adhesive removal test, the cylinder test, and the Montoya staircase test as well as the automated gait analysis system CatWalk XT over a 6 week period. CCI caused a variety of unilateral motor deficits, which were characterized in detail by using the selected fine motor skill tests. In striking contrast to previous studies on CCI in mice, neither forelimb impairments, nor general changes in gait, were detected with the CatWalk XT. These data suggest that the adhesive removal test, the cylinder test, and the Montoya staircase test are the methods of choice to detect long-term unilateral motor deficits in rats after CCI, whereas the use of automated gait analysis systems might not be suitable to measure these behavioral deviations.

**Keywords:** adult brain injury; behavioral assessments; CCI; locomotor function; traumatic brain injury

## Introduction

**B**RAIN INJURIES CAUSED BY STROKE OR TRAUMA can result in long-lasting motor impairments, which severely affect the patients' quality of life and prevent them from performing daily activities independently. Current research approaches to treating cortical lesions may include electrical stimulation, stem cell transplantation, or neuroprotection.<sup>1–3</sup> To investigate whether these therapies are able to restore motor functions in animal models, suitable paradigms with a robust and long-lasting behavioral phenotype have to be used. Nonetheless, in the majority of animal models, it is difficult to detect chronically persistent motor deficits opposed to which are observed in the clinics. A chronic behavioral phenotype is essential for testing new treatments relevant to the patient population, because those treatments are usually administered chronically after the insult. To produce motor deficits in rodents, the motor cortex is among the main targets for lesion induction. In the present study, a controlled cortical impact (CCI) model was used in rats to create long-lasting motor deficits specific to the dominant or, in other words, preferred forelimb. CCI is a widely used method to model traumatic

brain injury (TBI) in rodents, in which an impactor tip strikes the exposed cortical surface at a predefined depth, velocity, and dwell time. Advantages of this model are the well-defined lesion and high reproducibility caused by a low mortality and low variability.<sup>4,5</sup> However, reliably detecting behavioral deficits that last >2–4 weeks after the lesion remains a well-known problem.<sup>6</sup>

Previously, Zörner and coworkers thoroughly profiled impairments in gross locomotion and coordinated limb movements after different kinds of central nervous system damage, among which is unilateral cortical stroke.<sup>7</sup> Our study aims at creating a comparable profile of forelimb impairment after a CCI to the forelimb area of the motor cortex, with a special focus on a variety of fine motor skills. Previous studies provide some indications as to which behavioral testing methods can reliably detect deficits after CCI; however, the overall picture remains inconclusive. We compared and validated four behavioral tests after CCI in rats. The behavioral tests used in this study all measure a distinct aspect of forelimb motor behavior: integration of sensorimotor cues and purposeful forelimb motion are measured by the adhesive removal test, exploration along a vertical surface is assessed by the cylinder test,

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and specific deviations in grasping small objects can be evaluated with the Montoya staircase test. These three tests have been used in rats to detect motor deficits, and have been described in previous publications on CCI.<sup>8–11</sup> To increase readability, we refer to the tests as “fine motor skill tests” in the remainder of the article, although the cylinder test also measures elements of balance and posture. In addition, we included the automated gait analysis system CatWalk XT, which has been used previously to show functional impairments after CCI in mice that were assessed up to 31 days post-surgery.<sup>12–14</sup> The CatWalk XT provides quantitative information about different aspects of gait with regard to all limbs in concert as well as each limb on its own.

We used a systematic approach to measure the presence and duration of forelimb impairment after CCI. Our data show which motor deficits are still detectable in rats 6 weeks after injury induced by a standard model of TBI, and which behavioral tests can be used to reliably measure those deficits.

## Methods

### Subjects

All animal experiments were conducted according to the European Union Directive 2010/63/EU for animal experiments, and were further approved by the local ethical committee for animal experiments at Maastricht University. Seventeen male Sprague–Dawley rats (Charles River, L’Arbresle, France), 4 months of age and weighing  $416 \pm 8$  g at the time of surgery, were housed in pairs under a reversed 12 h light/dark cycle. Only male rats were used, because the prevalence of TBI is highest in young adult men<sup>15</sup> and the immune system, which determines the extend of secondary damage after TBI, is substantially influenced by the estrogen cycle.<sup>16</sup> Animals received standard laboratory chow (Sniff, Soest, Germany) and acidified water (pH 2.3–2.7) *ad libitum*. Animal housing took place in individually ventilated cages (IVC) at a constant temperature of 22°C and a humidity of 40–60%.

### Surgical procedures

Surgical procedures were performed as previously described, with minor modifications.<sup>17</sup> Anesthesia was induced and maintained with 1.5–3% isoflurane, and rats were fixed in a stereotaxic frame (Stoelting, Wood Dale, IL). A craniotomy of  $3.5 \times 3.5$  mm was made, exposing the forelimb area of the motor cortex contralaterally to the preferred paw, as determined prior to surgery using the Montoya staircase test (see subsequent description), at the coordinates anteroposterior (AP) 0–3.5 mm anterior to bregma and medial lateral (ML) 0.5–4 mm lateral to bregma. The exact location of the forelimb area on the motor cortex was derived from a cortical mapping study and a rat brain atlas.<sup>18,19</sup> CCI was created by an electromagnetically driven impactor device (Leica Impact One, Leica Biosystems, Richmond, IL). Ten rats received a lesion using an impactor tip of 3 mm diameter with an impact depth of 5 mm and a velocity of 3 m/sec. Seven rats received only a craniotomy, using the same coordinates as the CCI rats to function as controls, taking the potentially damaging effect of the craniotomy itself into account. The order of CCI and control surgeries was randomly alternated throughout the day. After CCI or a craniotomy was performed, a bioabsorbable coagulant mesh was placed on the lesion to stop the bleeding. The skull was closed with a thin layer of bone wax covering the craniotomy, which was reinforced with dental cement (Paladur, Heraeus, Hanau, Germany). The dental cement was anchored by placing two miniature screws on top of the skull close to the craniotomy, and the incision was sutured. After the surgery, rats were left to recover for 2 weeks. The mortality rate among the 17 operated rats was zero and in the course of the experiment no rat had to be excluded for other reasons.

### Behavioral testing

Behavioral testing was performed at three time points: before surgery, and 2 and 6 weeks after surgery. All behavioral assessments took place during the dark phase of the reversed night–day cycle (between 7 a.m. and 7 p.m.), which was the active period for the rats.

Before the adhesive removal test was conducted, rats were first habituated to the testing environment, a Perspex cage measuring  $40 \times 40 \times 50$  cm, by placing them inside the cage for an increasing amount of time once daily during 1 week. On testing days, adhesive color coded stickers with a diameter of 8 mm (Avery, Holzkirchen, Germany) were placed on both forepaws covering the thenar and hypothenar muscles. Rats were placed in the testing cage and filmed from below (see Video S1; Logitech HD Pro C920, Lausanne, Switzerland; Debut Video Capture Software, NCH Software, Greenwood Village, CO) (see online supplementary material at <http://www.liebertpub.com/neu>).

A trial was finished when the rat had removed both stickers from its forepaws, and one testing session consisted of three consecutive trials. Based on the video footage, time was stopped when the rat noticed the sticker under each paw (*time to contact*, visible by shaking the paw or bringing it to the nose) and when the rat removed the sticker (*time to remove*, usually with the teeth; VCL media player, VideoLAN Organization, Paris, France). Values representing the *time to remove* the sticker were treated as independent latencies and did not include the values measuring *the time to contact* the sticker. Data are presented in seconds for both forelimbs separately, and by showing the *difference score*, which is calculated by subtracting the score of the unpreferred forelimb from the score of the preferred forelimb.

Asymmetry of paw use was assessed using the cylinder test as previously described.<sup>20</sup> In short, rats were placed in 37.5 cm high Perspex cylinders with a diameter of 19 cm, and recorded from above during 10 min (Video S2; EthoVision XT, Noldus, Wageningen, the Netherlands) (see online supplementary material at <http://www.liebertpub.com/neu>). The first 20 wall contacts with the forepaws were counted and used for analysis (VCL media player, VideoLAN Organization, Paris, France). Individual paw contacts with either the preferred and unpreferred paw were differentiated from each other, or there was one simultaneous contact with both forepaws. An individual paw contact was counted as such when the rat initiated a rear or when both paws had left the wall before the respective contact. In a case in which one paw remained on the wall and the second paw also made contact, this was counted as “both” paws making contact. When both paws remained on the cylinder wall, but crossed over irregularly, it was still counted as one contact (“both”), to keep a focus on the individual paws. After 20 paw contacts were counted, scores for the individual forepaws were calculated as a percentage of the total 20 paw contacts. To assess asymmetry between the preferred and the unpreferred paw after surgery, post-surgery scores were set relative to their corresponding baseline scores, followed by subtracting the score of the unpreferred paw from the score of the preferred paw.<sup>11</sup> A lower value therefore indicated a decreased usage of the preferred paw in leaning against the cylinder wall.

To be tested in the Montoya staircase test, rats were trained to crawl into translucent boxes, with a staircase located on the left and right side of a central, narrow platform. In the 1st week, rats were familiarized with the testing boxes by allowing them to freely enter and leave the box followed by closing the box for an increasing amount of time, gradually habituating the rats towards the testing duration of 15 min.

During 3 weeks, rats were trained twice daily to reach a stable grasping performance that was considered sufficient when >55% of the pellets were eaten with at least one forelimb. Training conditions were similar to testing conditions. All steps, except the upper two, were baited with three sucrose pellets (Test Diet, Richmond, USA), resulting in a total of 15 sucrose pellets per site. The pellets were mixed with food coloring powder (Crazy Colors, Warburg,

Germany) diluted in water, and each step was baited with pellets of a distinct color. The upper two steps were left empty, because the rats were able to retrieve the pellets with their tongue, which would prevent initiating the desired grasping behavior.

During the training phase and on the testing days, rats were food restricted to increase their motivation to retrieve the pellets. One test trial consisted of 15 min, and the average score from the four trials was calculated at baseline, and at 2 and 6 weeks after surgery. Individual test trials took place with a time interval between trials of at least 3 h, which in most cases resulted in one test trial per day for each rat. Furthermore, the fourth test trial was recorded using cameras placed next to the stairs on both sides to allow offline qualitative assessment of performance (Video S3; Logitech HD Pro C920, Lausanne, Switzerland; Canon LEGRIA HF R16, Canon, Canon Nederland N.V., 's-Hertogenbosch, the Netherlands; Debut Video Capture Software, NCH Software, Greenwood Village, CO) (see online supplementary material at <http://www.liebertpub.com/neu>).

After the rats finished the test, grasping performance was assessed threefold. First, the total number of eaten pellets, regardless of their color, was calculated for each side. Baseline values in the total number of pellets eaten were used to determine the preferred limb for each rat; that is, the side where more pellets were eaten. Second, based on pellet colors, the following parameters were assessed for each separate step: *pellets remaining* for pellets still lying on their original step, *pellets misplaced* for pellets that had been knocked off their original step onto a different (mostly lower) step, and *pellets lost* for pellets thrown on the floor at the back of the staircase test, thereby being unreachable.<sup>21</sup> Based on those observations the parameters *pellets taken* (*pellets originally placed* minus *pellets remaining*) and *pellets eaten* (*pellets originally placed* minus [*pellets remaining* plus *pellets misplaced* plus *pellets lost*]) were calculated. The parameters *pellets remaining* and *pellets taken* are considered to measure general reaching activity and reaching motivation, whereas the parameters *pellets misplaced*, *pellets lost*, and *pellets eaten* provide more specific information about forelimb reaching and grasping success.<sup>21</sup> Third, using video footage, reaching attempts with the preferred limb were quantified based on absence or presence of 12 typical behaviors observed in a successful reaching action, similar to earlier research (VCL media player, VideoLAN Organization, Paris, France).<sup>22</sup>

Because we observed a large number of reaching attempts that did not result in a pellet being grasped, reaching behavior to be included for qualitative assessment was defined as bringing the forelimb toward the pellets and either touching the steps or performing palpitating movements in search of pellets. For each rat, five such reaches were analyzed for the absence or presence of typical reaching behaviors after surgery, and scores from the five reaches were averaged at baseline, and at 2 and 6 weeks, respectively. Each behavior was scored 1 when present, 0.5 when abnormal, and 0 when absent or severely abnormal.<sup>22</sup> Individual components of a typical reaching action included: 1) Inserting the snout into the staircase compartment, 2) advancing the limb toward the steps, 3) pronating the paw, while 4) extending the digits, 5) palpitating on the steps in search of pellets, 6) flexing the digits, 7) closing all digits to form a tight fist ("power grip"), 8) directing the limb to the snout, 9) supinating the paw 90 degrees toward the body, 10) bringing the paw to the mouth, 11) supinating the paw another 90 degrees for the palm to face the mouth, and 12) eating the pellet from the paw.

Data are presented showing the number of pellets with regard to the parameter at hand for each forelimb separately and by calculating the *difference score*, which is again calculated by subtracting the score of the unpreferred forelimb from the score of the preferred forelimb. Qualitative scores of reaching attempts were assessed for the preferred forelimb only.

Changes in gait were measured by the CatWalk XT (Noldus, Wageningen, the Netherlands), an automated gait analysis system. Rats were habituated to the system and trained to run down the

walkway daily for 1 week. If a rat successfully crossed the entire walkway, it was rewarded with sugar pellets (TestDiet, Richmond, USA) at the end of the walkway. On the last day of training, baseline recordings for each rat were taken. In general, one successful test recording consisted of an average of three uninterrupted runs having a comparable running speed with a maximum variation of 30%.

The following 20 static and dynamic parameters assessing individual paw functioning and gait patterns were analyzed: stand, mean intensity, print area, print length, print width, swing mean, swing speed, stride length, maximum intensity at maximum contact, maximum intensity, minimum intensity, step cycle, duty cycle, regularity index, base of support of the forelimbs, base of support of the hindlimbs, three limb support, speed, cadence, and couplings between the ipsilateral forelimb (unpreferred) and the contralateral hindlimb. Data are presented for each forelimb as well as using the *difference score*, defined as the score of the unpreferred forelimb subtracted from the score of the preferred forelimb in the respective units.

### Tissue processing

The day after the last behavioral testing session, 6 weeks after CCI, rats were euthanized by transcardial perfusion with Tyrode's buffer, followed by fixative containing 4% paraformaldehyde, 15% picric acid, and 0.05% glutaraldehyde in 0.1M phosphate buffer (pH 7.6). Brains were post-fixed in the same fixative lacking glutaraldehyde for 2 h at 4°C, followed by immersion in 15% and 20% sucrose for cryoprotection. Brains were frozen using CO<sub>2</sub> and stored at -80°C until further processing. For histochemical analysis, brains were cut in 10 series of 30 μm thick coronal sections, mounted on glass slides and fixed with 4% paraformaldehyde for 20 min. To measure the CCI lesion volume, one series of sections was stained with standard hematoxylin-eosin (Merck, Darmstadt, Germany).

### Assessment of lesion volume

The effect of CCI on the motor cortex was assessed by quantifying lesion volumes using an Olympus BX50 microscope (Zoeterwoude, The Netherlands) and StereoInvestigator software (MBF Bioscience, Magdeburg, Germany). First, both hemispheres were delineated. The area of the hemisphere, which received a CCI or a craniotomy, was subtracted from the area of the contralateral hemisphere to calculate the lesion area in mm<sup>2</sup> of the CCI rats (in control rats the same calculation was performed, although there was no lesion). The lesion volume in mm<sup>3</sup> per rat was calculated based on the sum of the lesion areas of all sections in mm<sup>2</sup> (*A*) and the distance between consecutive brain sections (300 μm, *D*) using the following formula:  $V = \Sigma A \times D$ . In case one brain section could not be delineated because of tissue damage or folding, the average of the preceding and subsequent section was used to replace the single missing value. Animals were excluded from the analysis if more than one brain section in a row was missing or could not be delineated. Groups consisted of  $n=8$  for CCI rats and  $n=5$  for control rats, with 10 brain sections being analyzed for each rat.

### Statistical analysis

The behavioral data were analyzed with SPSS statistical software (version 20, IBM) using repeated measures ANOVA. Behavioral scores at the three time points of testing were treated as repeated measures for each parameter, whereas the two groups (CCI vs. control rats) served as a between-subjects factor. In case repeated measures ANOVA showed that CCI and control rats differed significantly from each other after surgery, but not at baseline, one way ANOVA was performed comparing both groups at each separate time point. To illustrate the differences in lesion size

between CCI and control rats at the tissue level, average lesion volumes were compared between groups using one way ANOVA.

Data are presented as mean  $\pm$  standard error of the mean (SEM). Differences were considered significant at  $p < 0.05$ . Values below or above 1.5 interquartile ranges were identified as outliers by SPSS, and were removed from the final data set belonging to each individual parameter. Therefore, when a test had more than one outcome parameter, the removal of outliers belonging to one single parameter did not influence the data of the other parameters belonging to the same test. After detection of an outlier, all data of the respective animal were excluded from the subsequent analysis.

## Results

### CCI led to severe cortical and striatal tissue damage

CCI on the forelimb area of the motor cortex contralateral to the preferred paw caused a substantial lesion at the motor cortex and striatum 6 weeks after CCI (Fig. 1 A, B). Lesion volumes differed significantly between CCI and control rats, which only received a craniotomy above the target brain region ( $p < 0.001$ , Fig. 1 C).

### Deficits in tactile recognition

Sensorimotor deficits were evaluated using the adhesive removal test. After CCI, the *time to contact* the sticker underneath the preferred paw was significantly increased compared with control rats at both time points after surgery ( $p < 0.05$  and  $p < 0.05$ , Fig. 2 A2; Video S1). However, there were no differences observed between CCI and control rats in the *time to remove* the sticker (Fig. 2 B1-3). Although rats were distributed randomly between the groups, after excluding outliers we observed a significant difference in baseline values between the groups with regard to the *time to contact* the sticker underneath the unpreferred paw ( $p < 0.05$  and  $p < 0.01$ , Fig. 2 A1 and 2 A3, respectively). Therefore, data including the *time to contact* the sticker underneath the unpreferred paw should be interpreted with caution.

### Using the unpreferred paw for vertical exploration

With the cylinder test, paw use during exploration of a vertical wall was measured. After CCI, rats relied mainly on their unpreferred,

unaffected paw during vertical exploration and differed in their behavior significantly from control rats, which made use of both forelimbs at 2 and 6 weeks after surgery ( $p < 0.05$  and  $p < 0.01$ , Fig. 2 C1-3; Video S2).

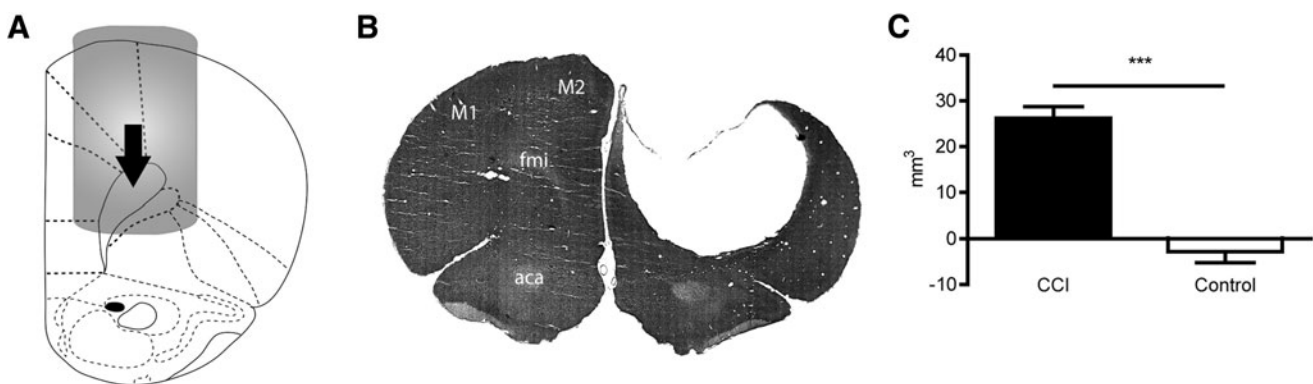
### CCI did not cause gait-related motor deficits

The automated gait analysis system CatWalk XT was used to detect gait-related motor deficits after CCI. No significant differences in functioning of the individual forelimbs were found when comparing CCI rats with control rats at all time points. Data of representative parameters such as mean paw print intensity and print dimensions are shown (Fig. 3 A–H), which were significantly changed in a study using a different model of TBI in rats.<sup>23</sup> One exception was the parameter maximum paw print intensity of the unpreferred paw, which was significantly higher in CCI rats 6 weeks after surgery ( $p < 0.05$ , Fig. S1 A1) (see online supplementary material at <http://www.liebertpub.com/neu>).

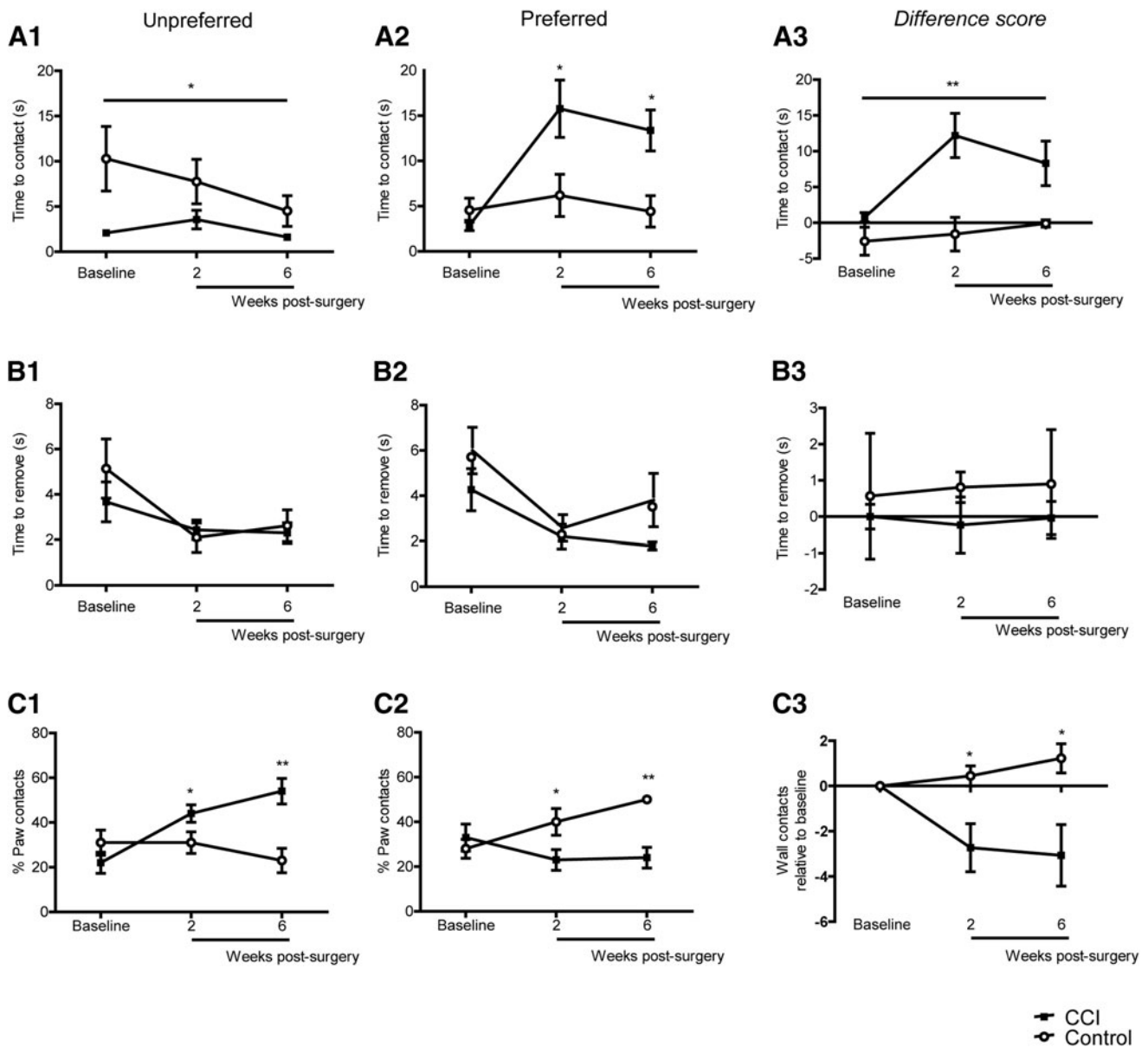
The unpreferred paw may compensate for the impairment in the preferred paw, but given the rather small difference in intensity values, the biological relevance of this result is questionable. Analysis of the *difference score* did not show any significant changes after CCI, either. Values related to the majority of parameters increased over time for both paws; however, this increase was equally present in CCI and control rats. Additionally, no significant differences in gait were found between the groups. All analyzed general gait parameters are shown in Figure 4 and correspond to parameters significantly changed up to 7 days after TBI in an earlier study.<sup>23</sup>

### Severe disturbance of reaching and grasping behavior

The Montoya staircase test was used to assess grasping of small objects and coordinated paw-to-mouth movements. Two and six weeks after CCI, rats ate significantly fewer sucrose pellets with their preferred paw across all stairs (both  $p < 0.001$ , Fig. 5 A2). Analyzing the color composition of the pellets for each individual step revealed that significantly fewer pellets were eaten with the preferred paw from each individual step at 6 weeks after CCI, indicating that the height of the steps was unimportant for the number of *pellets eaten* ( $p \leq 0.05$ , Fig. 5 B2). CCI rats



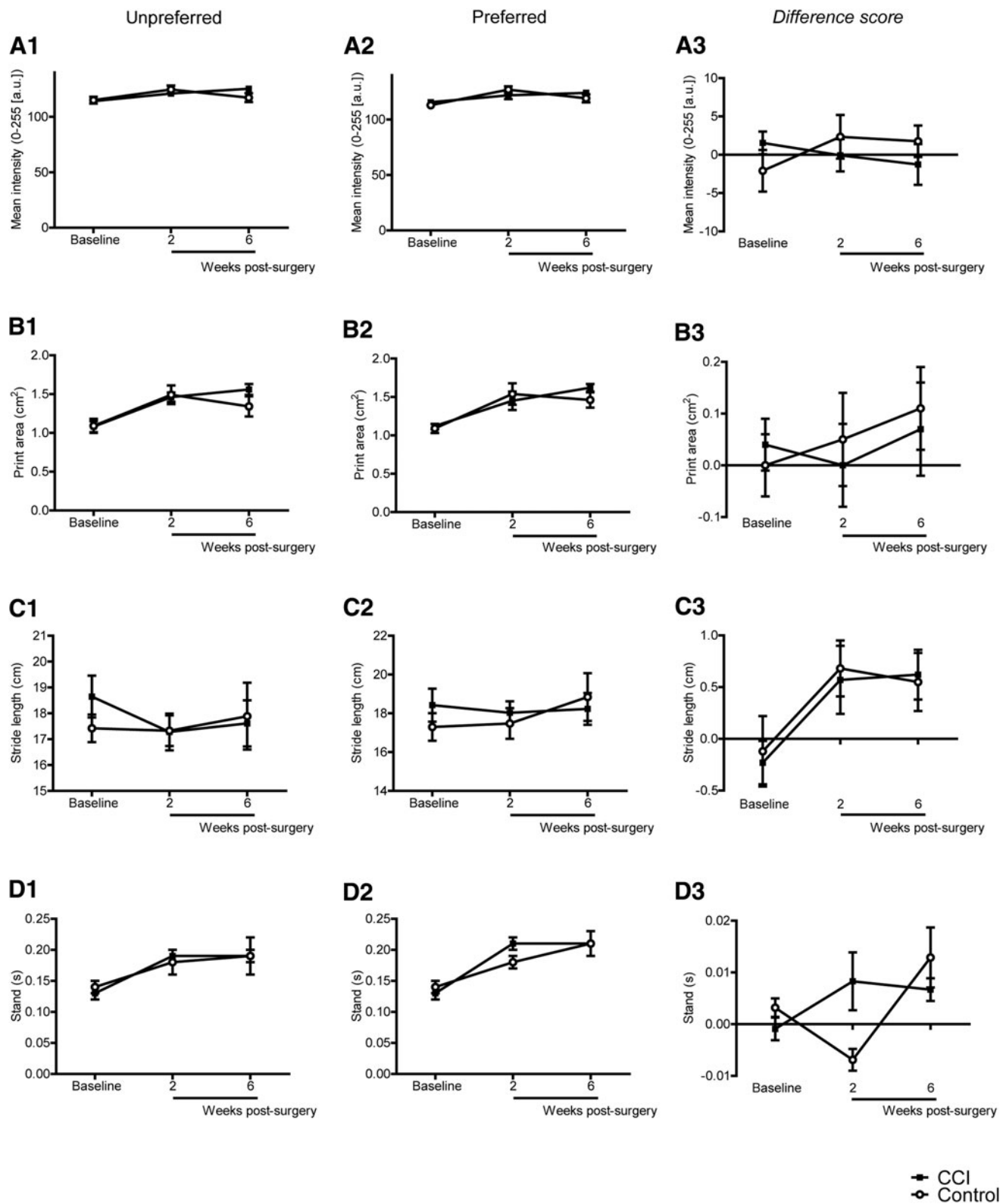
**FIG. 1.** Controlled cortical impact (CCI) on the forelimb motor cortex causes severe tissue damage. The impactor tip with a diameter of 3 mm was positioned on top of the exposed forelimb area of the motor cortex contralateral to the preferred limb, and hit the tissue with a target depth of 5 mm (scheme created at 3.2 mm anterior to bregma, **A**). A representative hematoxylin-eosin stained section showing the severe tissue damage 6 weeks after CCI ( $\sim 3.2$  mm anterior to bregma; M1, primary motor cortex; M2, secondary motor cortex; fmi, forceps minor of the corpus callosum; aca, anterior part of the anterior commissure, **B**) further supported by quantitative analysis of the lesion volume in CCI rats ( $n = 8$ ) compared with control rats ( $n = 5$ ) with a craniotomy ( $***p < 0.001$ ) **C**.



**FIG. 2.** Controlled cortical impact (CCI) impairs tactile recognition of adhesive stickers underneath the preferred paw, and causes reliance on the unpreferred paw during vertical exploration. In the adhesive removal test, rats designated to the CCI group were significantly faster in contacting the sticker underneath their unpreferred paw at all three time points; that is, even before CCI (CCI  $n=9$ , control  $n=7$ ), **A1**. The time to contacting the sticker underneath the preferred paw was significantly increased 2 and 6 weeks after CCI (CCI  $n=10$ , control  $n=6$ ), **A2**. Using the *difference score*, both animal groups again differed significantly from each other at all three time points (CCI  $n=10$ , control  $n=7$ ), **A3**. CCI did not affect the time taken to remove the stickers (unpreferred paw: CCI  $n=10$ , control  $n=7$ ; preferred paw: CCI  $n=8$ , control  $n=6$ ; *difference score*: CCI  $n=8$ , control  $n=7$ ), **B1–3**. After CCI, rats exhibited an increased percentage of wall contacts with their unpreferred paw while exploring the cylinder (CCI  $n=9$ , control  $n=7$ ), **C1**. At the same time, the percentage of wall contacts with the preferred paw was significantly lower than that of the control group (CCI  $n=10$ , control  $n=5$ ) (**C2**). Also, the *difference score* (here set relative to baseline values) showed a progressive asymmetry in paw use in the CCI group over time (CCI  $n=10$ , control  $n=7$ )  $*p < 0.05$ ,  $**p < 0.01$ ), **C3**.

exhibited a general decrease in reaching attempts with their preferred paw, reflected by an increase in *pellets remaining* for lower steps 5–7 ( $p < 0.001$ , Fig. 5 C2) together with a decrease in *pellets taken* for steps 5–7 ( $p < 0.001$ , Fig. S2.4 C2) (see online supplementary material at <http://www.liebertpub.com/neu>). The number of *pellets misplaced* with the preferred paw was higher in CCI rats for the upper steps 3 and 4 ( $p < 0.001$  and  $p < 0.01$ , Fig. 5 D2), whereas the number of *pellets lost* with the preferred paw

was lower only at step 7 ( $p < 0.01$ , Fig. S2.3 C2) (see online supplementary material at <http://www.liebertpub.com>). Increases in *pellets misplaced* and *pellets lost* both reflect unsuccessful reaching performance; however, *pellets lost* are thought to result from more undirected searching for pellets. On the other hand, *pellets misplaced* may also result from successfully grasping a pellet with an inability to eat the pellet using the preferred paw.



**FIG. 3:** CatWalk XT analysis did not reveal any impairment in the preferred forelimb. Eight selected parameters (out of 13 parameters analyzed) describing individual paw functioning are shown (mean intensity unpreferred paw: controlled cortical impact [CCI]  $n = 10$ , control  $n = 7$ ; preferred paw: CCI  $n = 9$ , control  $n = 7$ ; difference score: CCI  $n = 9$ , control  $n = 7$ , **1 A1-3**; print area unpreferred paw: CCI  $n = 10$ , control  $n = 7$ ; preferred paw: CCI  $n = 10$ , control  $n = 7$ ; difference score: CCI  $n = 10$ , control  $n = 7$ , **1 B1-3**; stride length unpreferred paw: CCI  $n = 10$ , control  $n = 7$ ; preferred paw: CCI  $n = 9$ , control  $n = 7$ ; difference score: CCI  $n = 9$ , control  $n = 6$ , **1 C1-3**; stand unpreferred paw: CCI  $n = 10$ , control  $n = 7$ ; preferred paw: CCI  $n = 9$ , control  $n = 7$ ; difference score: CCI  $n = 8$ , control  $n = 7$ , **1 D1-3**; swing mean unpreferred paw: CCI  $n = 8$ , control  $n = 6$ ; preferred paw: CCI  $n = 7$ , control  $n = 6$ ; difference score: CCI  $n = 7$ , control  $n = 7$ , **2 E1-3**; swing speed unpreferred paw: CCI  $n = 10$ , control  $n = 7$ ; preferred paw: CCI  $n = 9$ , control  $n = 7$ ; difference score: CCI  $n = 7$ , control  $n = 7$ , **2 F1-3**; step cycle unpreferred paw: CCI  $n = 9$ , control  $n = 7$ ; preferred paw: CCI  $n = 8$ , control  $n = 7$ ; difference score: CCI  $n = 9$ , control  $n = 5$ , **2 G1-3**; duty cycle unpreferred paw: CCI  $n = 10$ , control  $n = 7$ ; preferred paw: CCI  $n = 9$ , control  $n = 7$ ; difference score: CCI  $n = 7$ , control  $n = 7$ , **2 H1-3**). No significant differences between groups could be detected after CCI concerning the individual forelimbs and the difference score.

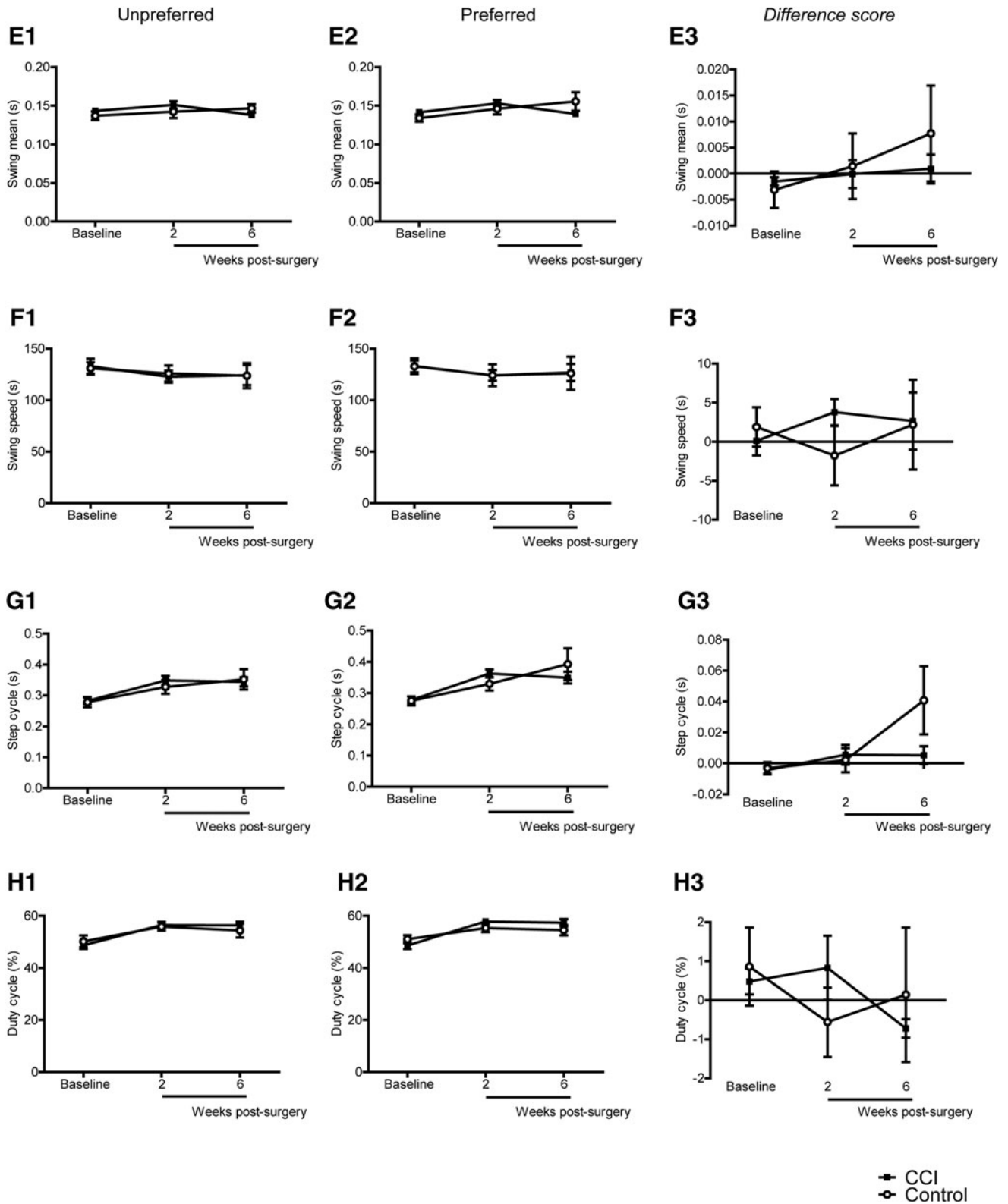
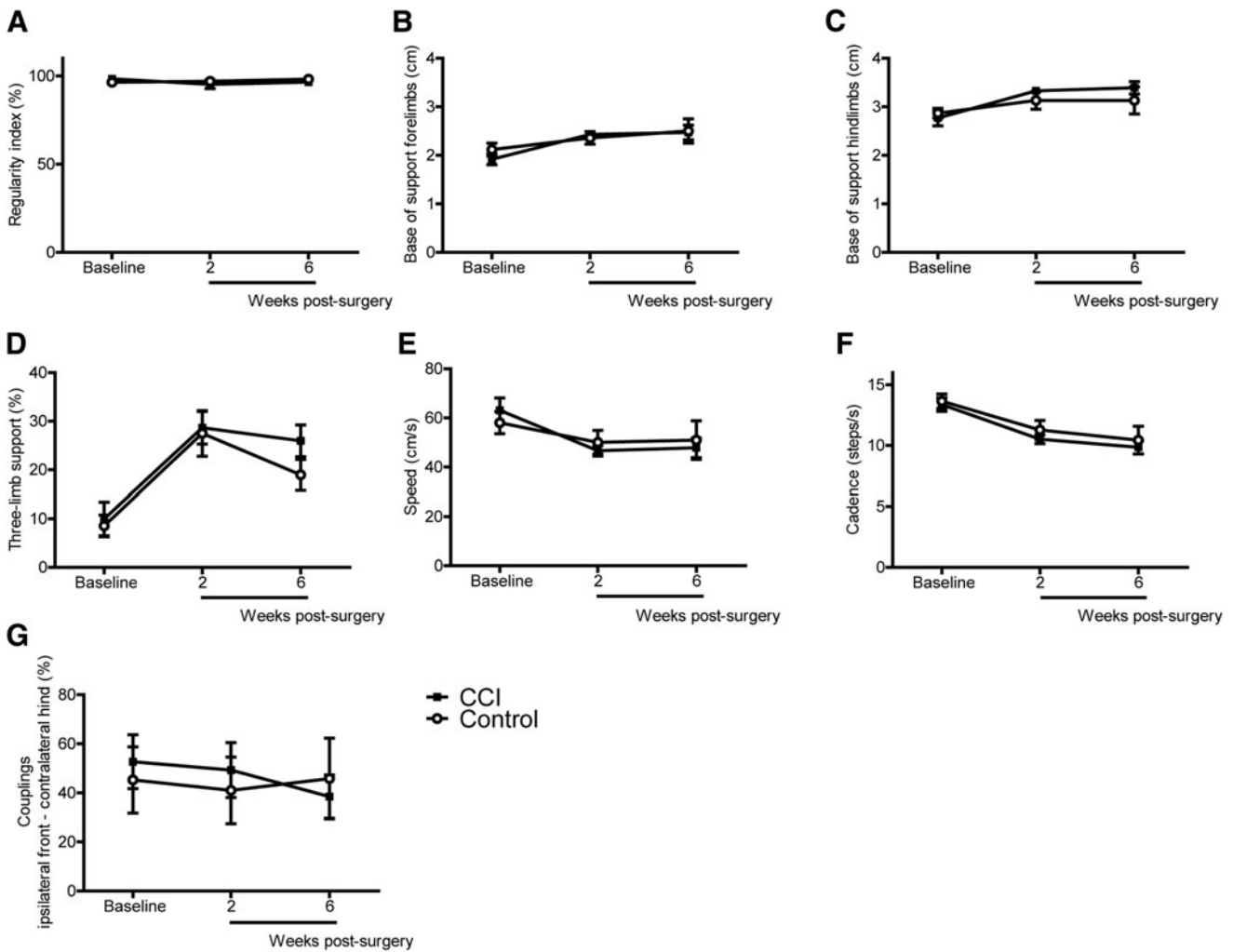


FIG. 3. (Continued).

To determine which exact component of a typical reaching action prevented the rats from successfully eating a pellet after CCI, slow motion video analysis was conducted of reaching attempts with the preferred paw. Two weeks after CCI, rats exhibited specific grasping impairments shown by abnormal digit flexing and

holding the pellet in a closed fist ("power grip";  $p < 0.05$ , Fig. S3 F and  $p < 0.01$ , Fig. 6 A) (see online supplementary material at <http://www.liebertpub.com/neu>). Often an inability to flex the digits when touching a pellet was observed, or, in cases in which digits could be flexed, the pellet was either dropped or held by fewer than





**FIG. 4.** No changes in gait were measured after controlled cortical impact (CCI) using the CatWalk XT. All analyzed gait-related parameters, which describe coordinated movements of the four paws in concert, are shown (regularity index: CCI  $n=10$ , control  $n=6$ , **A**; base of support forelimbs: CCI  $n=10$ , control  $n=7$ , **B**; base of support hindlimbs: CCI  $n=10$ , control  $n=7$ , **C**; three-limb support: CCI  $n=10$ , control  $n=6$ , **D**; speed: CCI  $n=10$ , control  $n=7$ , **E**; cadence: CCI  $n=10$ , control  $n=7$ , **F**; couplings ipsilateral front–contralateral hind: CCI  $n=10$ , control  $n=7$ , **G**). None of the parameters was significantly changed after CCI when comparing CCI rats with controls.

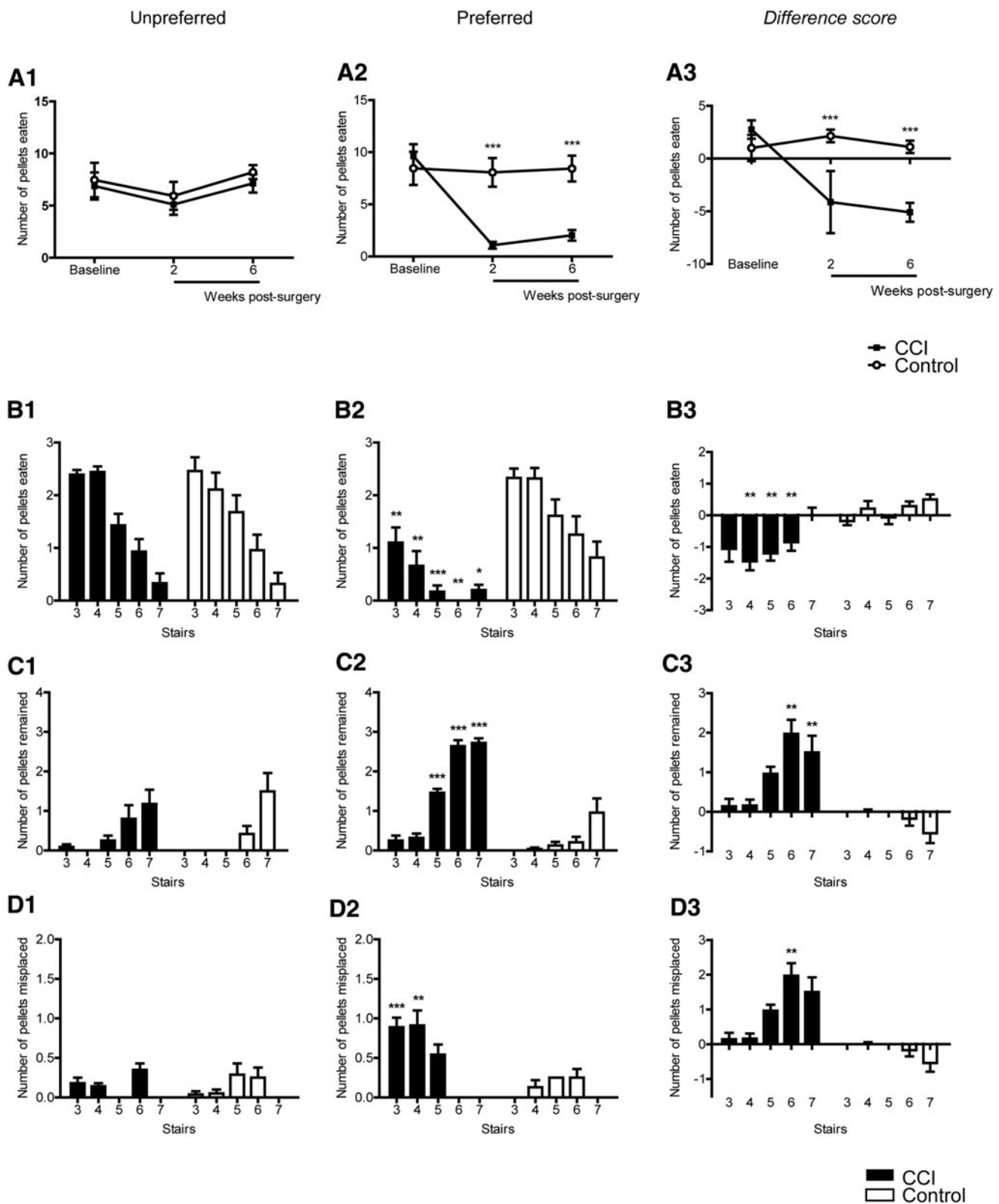
five digits. Also, 2 weeks after CCI, rats showed deficits in bringing the paw toward the mouth and eating the pellet ( $p < 0.01$ , Fig. 6 B). Instead of bringing the paw toward the mouth, rats advanced their head toward the paw that was holding the pellet. The most prominent observation was that CCI rats showed severe impairments in opening the grip to release the pellet in order to eat it, an observation still present at 6 weeks after CCI ( $p < 0.01$  and  $p < 0.001$ , Fig. 6 C). In many cases, the unpreferred paw had to hold the preferred limb in place while rats tried to grab the pellet from the paw with their teeth.

## Discussion

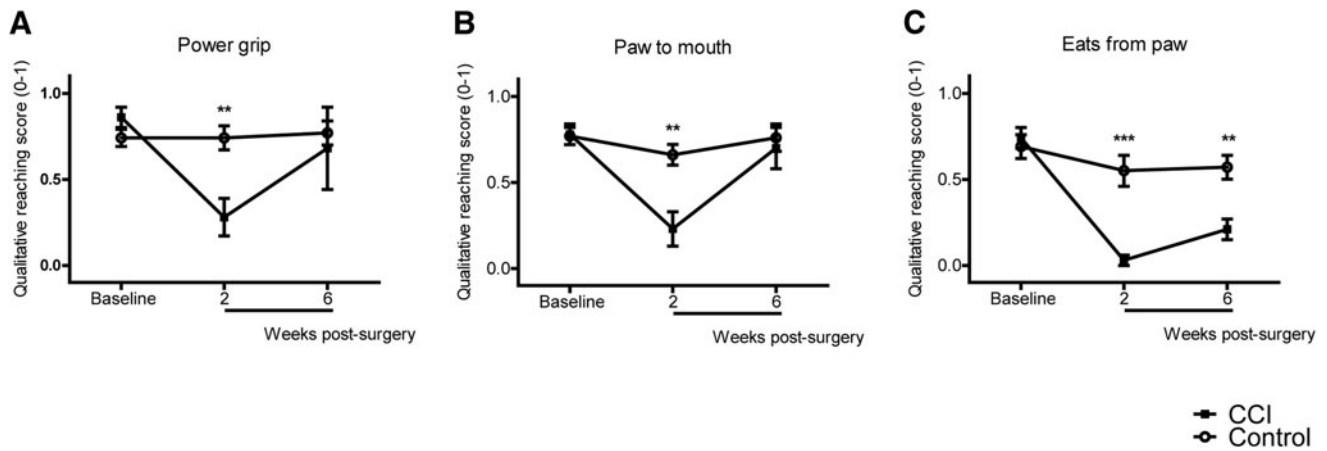
Effective treatment opportunities that target motor deficits are essential, given the high number of patients with TBI. A wide variety of animal models for TBI and tests for post-lesional motor performance are available;<sup>24</sup> however, only a very limited number of studies were able to detect behavioral deficits exceeding 2–4 weeks. In the present study, behavioral deficits were detected during the entire period of post-lesional assessment, which lasted 6

weeks. Being able to measure behavioral deficits that are present for at least 6 weeks is a considerable improvement on previous studies investigating only short-term functional impairment. As TBI patients can experience motor deficits during several months, animal studies that measure behavioral impairments in a comparable time frame, even  $>6$  weeks, might be desirable. However, extending the duration of animal experiments might raise ethical conflicts, because increasing the amount of time an animal is used for research purposes also increases the level of discomfort experienced. Animal models of TBI with motor deficits, which are constant for at least 6 weeks, may already provide a phenotype of impairment more suitable for testing new treatment opportunities that have to be delivered repeatedly.

In order to use a standardized animal model of TBI that induces chronic motor deficits, we chose to damage the motor cortex by a CCI, as this manner of lesion induction is well defined in terms of impact location, depth, width, and velocity making it highly reproducible. In the present study, a severe lesion was created to increase the intensity of long-lasting behavioral deficits, because motor deficits in rats induced by a CCI are positively correlated to



**FIG. 5.** Pellet eating with the preferred paw was significantly deteriorated after controlled cortical impact (CCI). In the Montoya staircase test, pellet eating with the unpreferred paw did not differ between CCI and control rats (CCI  $n = 10$ , control  $n = 6$ ; **A1**), whereas after surgery, CCI rats ate significantly fewer pellets from the staircase close to their preferred paw (CCI  $n = 10$ , control  $n = 7$ ; **A2**). The pellet consumption deficit in CCI rats was also measurable when analyzing the *difference score* comparing the number of eaten pellets between the preferred and unpreferred paws (CCI  $n = 10$ , control  $n = 7$ ; **A3**). Three additional parameters are shown at 6 weeks after surgery that provide more details about deficits in pellet retrieval (**B1–3**, number of pellets eaten; **C1–3**, number of pellets remaining; **D1–3**, number of pellets misplaced). After CCI, rats ate significantly fewer pellets from each individual step with their preferred limb (step 3: CCI  $n = 10$ , control  $n = 6$ ; step 4: CCI  $n = 8$ , control  $n = 7$ ; step 5: CCI  $n = 6$ , control  $n = 7$ ; step 6: CCI  $n = 7$ , control  $n = 7$ ; step 7: CCI  $n = 10$ , control  $n = 7$ ; **B2**) while leaving more pellets untouched at their original location (step 3: CCI  $n = 8$ , control  $n = 4$ ; step 4: CCI  $n = 7$ , control  $n = 6$ ; step 5: CCI  $n = 7$ , control  $n = 5$ ; step 6: CCI  $n = 7$ , control  $n = 7$ ; step 7: CCI  $n = 8$ , control  $n = 7$ ; **C2**). When rats reached for the pellets with the preferred limb, there was an increase in the number of pellets misplaced after CCI (step 3: CCI  $n = 7$ , control  $n = 5$ ; step 4: CCI  $n = 8$ , control  $n = 6$ ; step 5: CCI  $n = 7$ , control  $n = 5$ ; step 6: CCI  $n = 4$ , control  $n = 7$ ; step 7: CCI  $n = 8$ , control  $n = 6$ ;  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ; **D2**).



**FIG. 6.** Reaching behavior with the preferred paw was severely altered after controlled cortical impact (CCI). Rats showed overt abnormalities in a number of reaching behaviors related to pellet grasping and eating after CCI. Two weeks after CCI, rats had deficits in forming a “power grip”; that is, closing all digits around the pellet when grasping a pellet (CCI  $n=8$ , control  $n=7$ ; **A**), followed by a problematic return of the paw toward the mouth (CCI  $n=8$ , control  $n=7$ ; **B**). Pellet eating from the paw was significantly impaired both 2 and 6 weeks after CCI (CCI  $n=7$ , control  $n=7$ ;  $**p < 0.01$ ,  $***p < 0.001$ ; **C**).

lesion severity.<sup>25</sup> In a previous study, behavioral deficits could be detected at 8 weeks after a CCI with an impact depth of 5 mm, whereas a lower impact depth of 2.5 mm did not result in any measurable deficit.<sup>9</sup> Therefore, an impact depth of 5 mm was also chosen in this study to induce long-term motor deficits.

A single lesion severity was used to compare the reliability and sensitivity of different rodent motor tests. Given the location of the craniotomy, the current CCI lesion induced the maximal amount of tissue damage possible. Choosing a larger tip diameter would have resulted in increased bleeding and post-surgical discomfort as a result of damaging the temporal muscle, and a deeper impact depth is lethal through compressing the cerebellum and the brainstem. In a number of previous studies, milder CCI lesions have been created by using lower impact depths of 2 mm; however, in those studies, motor impairment was not detectable after a period between 2 and 4 weeks using comparable tests.<sup>8,11,26–29</sup> Therefore, groups with milder CCI injuries were not included in the present study on long-term motor impairment to prevent unnecessary discomfort of experimental animals.

At the level of the cortex and the dorsal striatum, a considerable lesion was still evident 6 weeks after CCI, whereas the craniotomy performed in control animals did not cause measurable damage to the parenchyma. Using several fine motor skill tests, a clear phenotype of unilateral forelimb impairment could be identified. Quantifying forelimb function with a combination of fine motor skill tests is essential to describe impairment from several angles. As in humans, fine motor skills in rodents are controlled by the motor cortex and, therefore, have the same underlying neural basis. However, the selected muscle groups create behavior overtly different from that of humans, which stresses the need for distinct specialized tests.<sup>30</sup> Each of the fine motor skill tests used in the present study described different manifestations of the CCI-induced unilateral forelimb impairment. The adhesive removal test allows a distinction between sensory deficits caused by a unilateral lesion; that is, a decreased ability to feel the adhesive underneath the paw, and deteriorated motor skills, such as holding the paw in front of the mouth and extending the digits to remove the adhesive.<sup>20,31,32</sup> However, it should be noted that assessment of sensory deficits in the adhesive removal test requires intact gross motor function of the forelimb, because for it to count as a contact,

the rat has to shake its limb or raise the paw toward the nose. Therefore, a significantly worsened sensory ability after CCI highlights the necessity of using more than one behavioral test to describe impairment.

The cylinder test measures forelimb usage during exploration of unknown vertical surfaces, with a special focus on weight-bearing wall touches used to keep balance while standing on the hindlimbs.<sup>33</sup> We showed that rats no longer relied on their preferred paw to explore the cylinder walls after CCI. This asymmetry may either reflect a weaker muscle tone causing a decreased ability to stabilize weight while standing, or a sensory deficit, meaning a decreased capacity to feel the cylinder wall, or a combination of both types of deficits. The Montoya staircase test provides a detailed evaluation of pellet grasping and eating, which requires actions such as digit extension, flexion, and coordinated paw-to-mouth movement.<sup>22,34</sup> After CCI, rats consumed fewer pellets on the steps contralateral to the lesion. Further analyses showed that this deviation was caused by potentially less frequent attempts to reach with their preferred paw as well as behavioral deficits while trying to obtain a pellet. A number of interrelated behavioral components were previously identified that together result in a typical reaching action of a rat, among which are forming a closed fist around a pellet, bringing the paw toward the mouth, and eating the pellet from the paw.<sup>22</sup> Because of the relationship between the individual behaviors, performance in one behavior may influence the performance in a subsequent behavior. For example, when it was impossible for a rat to direct its paw toward the mouth, it also could not eat the pellet from this paw. We either observed an absence or severe abnormalities in the “paw to mouth” action at 2 weeks after CCI (Fig. 6 B), which may have influenced the impairment in “eating from paw” at 2 weeks after CCI (Fig. 6 C). However, at 6 weeks after CCI, we still detected abnormal behaviors in “eating from paw” (Fig. 6 C), suggesting that an impairment in “eating from paw” remained present even when preceding behaviors, such as “power grip” and “paw to mouth” had improved (Fig. 6 A, B).

The parameters *pellets eaten*, *remaining*, *misplaced*, *taken*, and *lost*, through which pellet reaching and grasping were assessed by counting the number of colored pellets on each step of the staircase, show a comparable relationship with each other. In a testing session, a

fixed number of three pellets was lying on each step; therefore, a decrease in the number of *pellets eaten* consequently leads to an increased number of either *pellets remaining*, *misplaced*, or *lost*.

For example, 6 weeks after CCI, rats ate fewer pellets from steps 5–7 (Fig. 5 B2), whereas at the same time more *pellets remained* at these steps (Fig. 5 C2). Behavioral deficits of the preferred forelimb, which were assessed in the Montoya staircase test by counting the number of pellets on each step of the staircase, could be caused by two types of impairment. On the one hand, after CCI, rats might make fewer attempts to reach for pellets, but in cases in which they did reach, pellet grasping and eating was successful. On the other hand, the number of reaching attempts was unaffected after CCI, but because of deficits in fine motor skills, rats could not retrieve pellets successfully.

Changes in the parameters *pellets remaining*, *misplaced*, and *lost* (Fig. 5 C, D and Fig. S2.3) provided a first indication of unsuccessful pellet retrieval after CCI. In order to relate a specific motor deficit to changes in these parameters, individual components of typical reaching actions were evaluated based on the filmed performance of the rats during test sessions in the Montoya staircase test. At 2 weeks after CCI, deficits were present in behavioral components of a typical reaching action. However, deficits in some of these behaviors were transient, and could not be detected at 6 weeks after CCI (Fig. 6), whereas the number of pellets located on the staircases was still indicative of a behavioral impairment (Fig. 5).

The relationship between an absence of long-term impairment based on the qualitative analysis of typical reaching actions and a presence of long-term impairment based on counting the number of pellets on the staircases might be explained as follows: The analysis of individual behavioral compounds was based on a fixed number of five typical reaching actions for every rat at each time point of assessment, whereas quantification of the pellet number and location occurred after a fixed duration of 15 min per test session, during which the number of reaches may slightly vary between rats. Therefore, after CCI the quality of individual reaching actions may improve over time (Fig. 6), whereas the total number of reaches is still lower compared with that in control animals (Fig. 5). The observed improvement in individual components of a typical reaching may be indicative of both recovery of function or learning effects, which might be intermingled, and could not be differentiated based on data obtained with the Montoya staircase test.

No behavioral deficits were detected with the automated gait analysis system CatWalk XT. Occasionally, a change in parameter values occurred over time, but this effect could be attributed to maturation of the rats, because no significant differences between the groups were detected. The CatWalk XT measures changes in gait, focusing on coordinated movement of all four limbs together, as well as gait-related attributes of each paw separately.<sup>35</sup> Undisturbed gait in the presence of severe brain damage can be explained by central pattern generators (CPGs) located in the spinal cord, which regulate locomotion without conscious intention to move, whereas goal-directed actions such as grasping require conscious integration of spatial and proprioceptive cues as provided by the motor cortex.<sup>36,37</sup> Additionally, a considerable portion of the striatum was spared in the present study. In cases in which some connections from the motor cortex remain intact, the striatum is still able to guide gross movements, which explains the absence of deficits in gait-related parameters. In the majority of studies using animal models for cortical damage, behavioral abnormalities are assessed with fine motor skill tests comparable to the ones used in the present study.<sup>38</sup> Still, the CatWalk XT is an attractive option

because of its objectiveness in processing variables automatically as opposed to manual scoring necessary for fine motor skill tests.

So far, one study on TBI in rats reported behavioral deficits using the CatWalk XT, but failed to provide evidence that this test is suitable to measure long-term impairment after the lesion.<sup>23</sup> In this study, TBI was induced in rats using a different model, penetrating ballistic-like brain injury, which created a considerable lesion at the level of the striatum while leaving the majority of the motor cortex intact. Using this particular model of TBI, the authors were able to measure effects in the same parameters as presented here for up to 2 weeks post-surgery. After 1 month, forelimb deficits seemed to be resolved when using the Catwalk XT, whereas a significant impairment was still measurable using a manual scoring method, the 12 point neuroscore.<sup>23</sup> To date, our study is the first to investigate whether long-term deficits are measurable by the CatWalk XT in comparison with fine motor skill tests during a period of 6 weeks after CCI in rats. Severe CCI on the motor cortex caused robust histological lesions and persistent fine motor skill impairment in a number of specified motor tests; however, no gait-related abnormalities were detected using the CatWalk XT. These findings are in striking contrast to previous results obtained after CCI in mice.<sup>12–14</sup> In mice, it was possible to detect functional impairments after CCI up to 31 days post-surgery using the CatWalk XT.<sup>12–14</sup>

Our findings are an important indication that automated gait analysis systems such as the CatWalk XT may not be suitable in rat models of TBI in order to analyze the long-term effects of potential treatments on motor recovery. Our extensive report of negative data should prevent researchers from opting for the CatWalk XT as the only behavioral assessment method after TBI in rats to prevent false negative results and decrease the number of unnecessary animal experiments.

On the contrary, fine motor skill tests such as the adhesive removal test, the cylinder test, and the Montoya staircase test appear to be reliable and sensitive testing methods. Ideally, a combination of those fine motor skill tests should be used to measure long-lasting motor impairments.

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## Author Disclosure Statement

No competing financial interests exist.

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