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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

Controlled cortical impact; behavioral assessments; traumatic brain injury; locomotor function; adult brain injury

Introduction

Brain 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 treat cortical lesions may include electrical stimulation, stem cell transplantation or neuroprotection.[1-3] 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 what is observed in the clinics. A chronic behavioral phenotype is essential to test new treatments relevant for the patient population, since 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 pre-defined depth, velocity and dwell time. Advantages of this model are the well-defined lesion and high reproducibility due to a low mortality and low variability.[4, 5] However, it is a notorious problem to reliably detect behavioral deficits that last longer than two to four weeks after the lesion.[6]

Previously, Zörner *et al.* thoroughly profiled impairments in gross locomotion and coordinated limb movements after different kinds of central nervous

system damage, among which unilateral cortical stroke.[7] 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, which behavioral testing methods can reliably detect behavioral deficits after CCI, but 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 and specific deviations in grasping small objects can be evaluated with the Montoya staircase test. These three tests have been used in previous publications on CCI in rats to detect motor deficits.[8-11] To increase readability, we refer to the tests as “fine motor skill tests” in the remainder of the manuscript although the cylinder test also contains 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.[12-14] 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 six weeks after injury induced by a standard model of TBI and which behavioral tests can be used to reliably measure those deficits.

Materials & Methods

Subjects

All animal experiments were conducted according to the EU 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), four months of age and weighing 416 ± 8 g at the time of surgery, were housed in pairs under a reversed 12 h light/dark cycle. Only male rats were used since the prevalence of TBI is highest in young adult men[15] and the immune system, which determines the extend of secondary damage after TBI, is substantially influenced by the estrogen cycle.[16] Animals received standard laboratory chow (Sniff, Soest, Germany) and acidified water (pH 2.3 - 2.7) *ad libitum*. Animal housing took place under IVC conditions 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.[17] Anesthesia was induced and maintained with 1.5 - 3% isoflurane and rats were fixed in a stereotactic frame (Stoelting, Wood Dale, IL, USA). A craniotomy of 3.5 x 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 description below), at the coordinates AP 0 - 3.5 mm anterior to bregma and 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.[18, 19] CCI was

created by an electromagnetically driven impactor device (Leica Impact One, Leica Biosystems, Richmond, IL, USA). 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/s. Seven rats received only a craniotomy using the same coordinates as the CCI rats to function as controls taking the potentially damaging effect by the craniotomy itself into account. The order of CCI and control surgeries was randomly alternated throughout the day. After receiving CCI or a craniotomy, 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 two weeks. The mortality rate among the 17 operated rats was zero and in the course of the experiment no rat had to be excluded due to other reasons.

Behavioral testing

Behavioral testing was performed at three timepoints: Before surgery, two weeks and six weeks after surgery. All behavioral assessments took place during the dark phase of the reversed night-day cycle (between 7 am and 7 pm), which is the active period of the rats.

Before conducting the adhesive removal test, rats first were habituated to the testing environment, a Perspex cage measuring 40 x 40 x 50 cm, by placing them inside the cage for an increasing amount of time once daily during one week. On testing days, adhesive color coding 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 (Supplementary Movie 1; Logitech HD Pro C920, Lausanne, Switzerland; Debut Video Capture Software, NCH Software, Greenwood Village, Colorado, USA). 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 achieved to remove 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 non-preferred forelimb from the score of the preferred forelimb.

Asymmetry of paw use was assessed using the cylinder test as previously described.[20] In short, rats were placed in 37.5 cm high Perspex cylinders with a diameter of 19 cm and recorded from above during 10 minutes (Supplementary Movie 2; EthoVision XT, Noldus, Wageningen, the Netherlands). The first 20 wall contacts with the forepaws were counted and used for analysis (VCL media player, VideoLAN Organization, Paris, France). It was differentiated between individual paw contacts of either the contralesional or ipsilesional paw or 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. In a case where

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 non-preferred paw after surgery, post-surgery scores were set relative to their corresponding baseline scores, followed by subtracting the score of the non-preferred paw from the score of the preferred paw.[11] A lower value thus 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 on the right hand side of a central, narrow platform. In the first week, rats were familiarized to 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 minutes. During 3 weeks, rats were trained twice daily to reach a stable grasping performance that was considered sufficient when more than 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, since 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 minutes and the average score from the four trials was calculated at baseline, two weeks and six weeks after surgery. Individual test trials took place with a time interval between trials of at least 3 hours, 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 (Supplementary Movie 3; 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, Colorado, USA). After the rats finished the test, grasping performance was assessed three-fold. 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 rats, i.e. the side where more pellets were eaten. Second, based on pellet colors, the following parameters were assessed for each separate step: *pellets remained* for pellets still lying on their original step, *pellets misplaced* for pellets, which 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.[21] Based on those observations the parameters *pellets taken* (*pellets originally placed* minus *pellets remained*) and *pellets eaten* [*pellets originally placed* minus (*pellets remained* plus *pellets misplaced* plus *pellets lost*)] were calculated. The parameters *pellets remained* 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.[21] 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).[22] Since we observed a high number of reaching attempts, which did not result in a pellet being grasped, reaching behavior to be included for qualitative assessment was defined as the forelimb being brought towards 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, two and six weeks, respectively. Each behavior was scored 1 when being present, 0,5 when being abnormal and 0 when being absent or severely abnormal.[22] Individual components of a typical reaching action included: (1) Inserting the snout into the staircase compartment, (2) advancing the limb towards 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 first (“power grip”), (8) directing the limb to the snout, (9) supinating the paw 90 degrees towards 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 non-preferred 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 one 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 twenty 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 (non-preferred) and the contralateral hindlimb. Data are presented for each forelimb as well as using the *difference score,* defined as the score of the non-preferred forelimb subtracted from the score of the preferred forelimb in the respective units.

Tissue processing

The day after the last behavioral testing session, thus six weeks after CCI, rats were sacrificed 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_2 and stored at -80°C until further processing. For histochemical analysis, brains were cut in ten series of 30 µm thick coronal sections, mounted on glass slides and fixed with 4% paraformaldehyde for 20 minutes. 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^2 of the CCI rats (in control rats the same calculation was performed although there was no lesion). The lesion volume in mm^3 per rat was calculated based on the sum of the lesion areas of all sections in mm^2 (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 due to tissue damage or folding, the average of the preceding and subsequent section was used to replace the single missing value. Animals were excluded from 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 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, Oneway ANOVA was performed comparing both groups at each separate timepoint. To illustrate the differences in lesion size between CCI and control rats at the tissue level, average lesion volumes were compared between groups using Oneway ANOVA.

Data are presented as mean ± 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 dataset 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 six 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 to control rats at both timepoints after surgery ($p < 0.05$ and $p < 0.05$, Fig. 2 A2; Supplementary Movie 1). 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 non-preferred 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 non-preferred paw should be interpreted with caution.

Using the non-preferred 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 non-preferred, unaffected paw during vertical exploration and differed in their behavior significantly from

control rats, which made use of both forelimbs at two and six weeks after surgery ($p < 0.05$ and $p < 0.01$, Fig. 2 C1-3; Supplementary Movie 2).

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 timepoints. 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.[23] One exception was the parameter maximum paw print intensity of the non-preferred paw, which was significantly higher in CCI rats six weeks after surgery ($p < 0.05$, Supplementary Fig. 1 A1). The non-preferred 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* neither showed any significant changes after CCI. Values related to the majority of parameters increased over time for both paws, but 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 seven days after TBI in an earlier study.[23]

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 sigificantly less pellets were eaten with the preferred paw from each individual step at six weeks after CCI, indicating that the height of the steps was unimportant for the number of *pellets eaten* ($p < 0.05$ or lower, Fig. 5 B2). CCI rats exhibited a general decrease in reaching attempts with their preferred paw, reflected by an increase in *pellets remained* for lower steps 5 until 7 ($p < 0.001$, Fig. 5 C2) together with a decrease in *pellets taken* for steps 5 until 7 ($p < 0.001$, Supplementary Fig. 2.4 C2). 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 ($p < 0.01$, Supplementary Fig. 2.3 C2). Increases in *pellets misplaced* and *pellets lost* both reflect unsuccessful reaching performance, however, *pellets lost* is 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.

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 done 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”; $p < 0.05$, Supplementary Fig. 3 F and $p < 0.01$, Fig. 6 A). Often an inability to flex the digits when touching a pellet was observed, or, in the case where digits could be flexed, the pellet was either dropped or held by fewer than five digits. Also two weeks

after CCI, rats showed deficits in bringing the paw towards the mouth and eating the pellet ($p < 0.01$, Fig. 6 B). Instead of bringing the paw towards the mouth, rats advanced their head towards 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 six weeks after CCI ($p < 0.01$ and $p < 0.001$, Fig. 6 C). In many cases, the non-preferred 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, which target motor deficits are essential, given the high number of patients suffering from TBI. A wide variety of animal models for TBI and tests for postlesional motor performance are available[24], however, only a very limited number of studies were able to detect behavioral deficits exceeding two to four weeks. In the present study, behavioral deficits were detected during the entire period of postlesional assessment, which lasted six weeks. Being able to measure behavioral deficits that are present for at least six weeks is a considerable improvement to previous studies investigating only short-term functional impairment. As TBI patients can suffer from motor deficits during several months, animal studies that measure behavioral impairments in a comparable timeframe, thus even exceeding six weeks, might be desirable. However, extending the duration of animal experiments might raise ethical conflicts, since 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 six weeks, may already provide a phenotype of impairment more suitable to test 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 way of lesion induction is well defined in terms of impact location, depth, width and velocity and therefore 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 lesion

severity.[25] In a previous study, behavioral deficits could be detected at eight 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.[9] 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 due to damaging the temporal muscle and a deeper impact depth is lethal due to compression of the cerebellum and the brain stem. In a number of previous studies, milder CCI lesions have created by using lower impact depths of 2 mm; however, in those studies motor impairment was not detectable after a period between two and four weeks using comparable tests.[8, 11, 26-29] 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 six 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 humans, which stresses the need for distinct specialized tests.[30] 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, i.e. 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.[20, 31, 32] However, it should be noted that assessment of sensory deficits in the adhesive removal test requires intact gross motor function of the forelimb, since to count as a contact the rat has to shake its limb or raise the paw towards the nose. A significantly worsened sensory ability after CCI highlights the necessity of 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.[33] 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 like digit extension, flexion and coordinated paw-to-mouth movement.[22, 34] After CCI, rats consumed less pellets on the steps contralateral to the lesion. Further analysis 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, amongst which are forming a closed fist around a pellet, bringing the paw towards the mouth and eating the pellet from the paw.[22] Due to 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 towards 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 two weeks after CCI (Fig. 6 B), that may have influenced the impairment in 'eating from paw' at two weeks after CCI (Fig. 6 C). However, at six 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*, *remained*, *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 between 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 remained*, *misplaced* or *lost*. For example, six weeks after CCI, rats ate fewer pellets from steps 5 until 7 (Fig. 5 B2), whereas at the same time more *pellets remained* at these steps (Fig. 5 C2). Behavioral deficits of the preferred forelimb, that were assessed in the Montoya staircase 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 case they did reach, pellet grasping and eating was successful. On the other hand the number of reaching attempts was unaffected after CCI, but due to deficits in fine motor skills, rats could not succeed to retrieve pellets successfully. Changes in the parameters *pellets remained, misplaced* and *lost* (Fig. 5 C-D, Supplementary Fig. 2.3) 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 two 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 six weeks after CCI (Fig. 6), while the number of pellets located on the staircases was still indicative of a behavioral impairment (Fig. 5). The relation 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: Analysis of individual behavioral compounds was based on a fixed number of five typical reaching actions for every rat at each timepoint of assessment, whereas quantification of the pellet number and location occurred after a fixed duration of 15 minutes 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 to 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, since 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.[35] 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.[36, 37] Additionally, a considerable portion of the striatum was spared in the present study. In the case where 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.[38] Still, the CatWalk XT is an attractive option due to its objectiveness in processing variables automatically as opposed to manual scoring as done with fine motor skill tests. So far, one study on TBI in rats reported behavioral deficits using the CatWalk XT, but also failed to provide evidence that this test is suitable to measure

long-term impairment after the lesion.[23] 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 two weeks post-surgery. After one 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.[23] 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 six 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.[12-14] In mice it was possible to detect functional impairments after CCI up to 31 days post-surgery using the CatWalk XT.[12-14]

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 that researchers opt 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 like 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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Figure legends

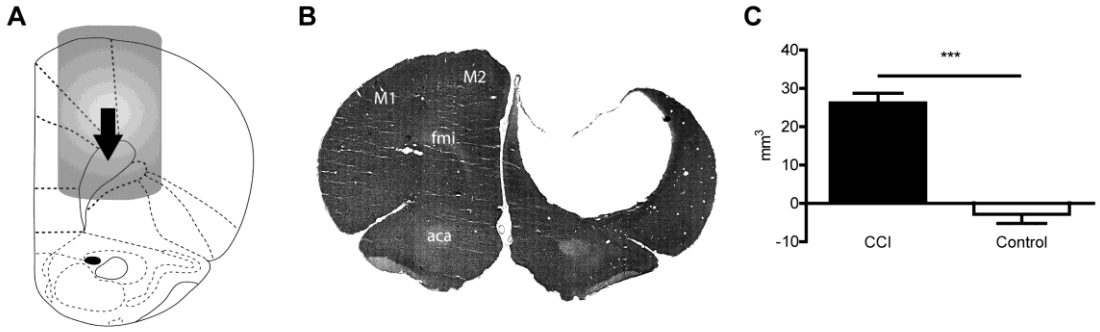


Figure 1

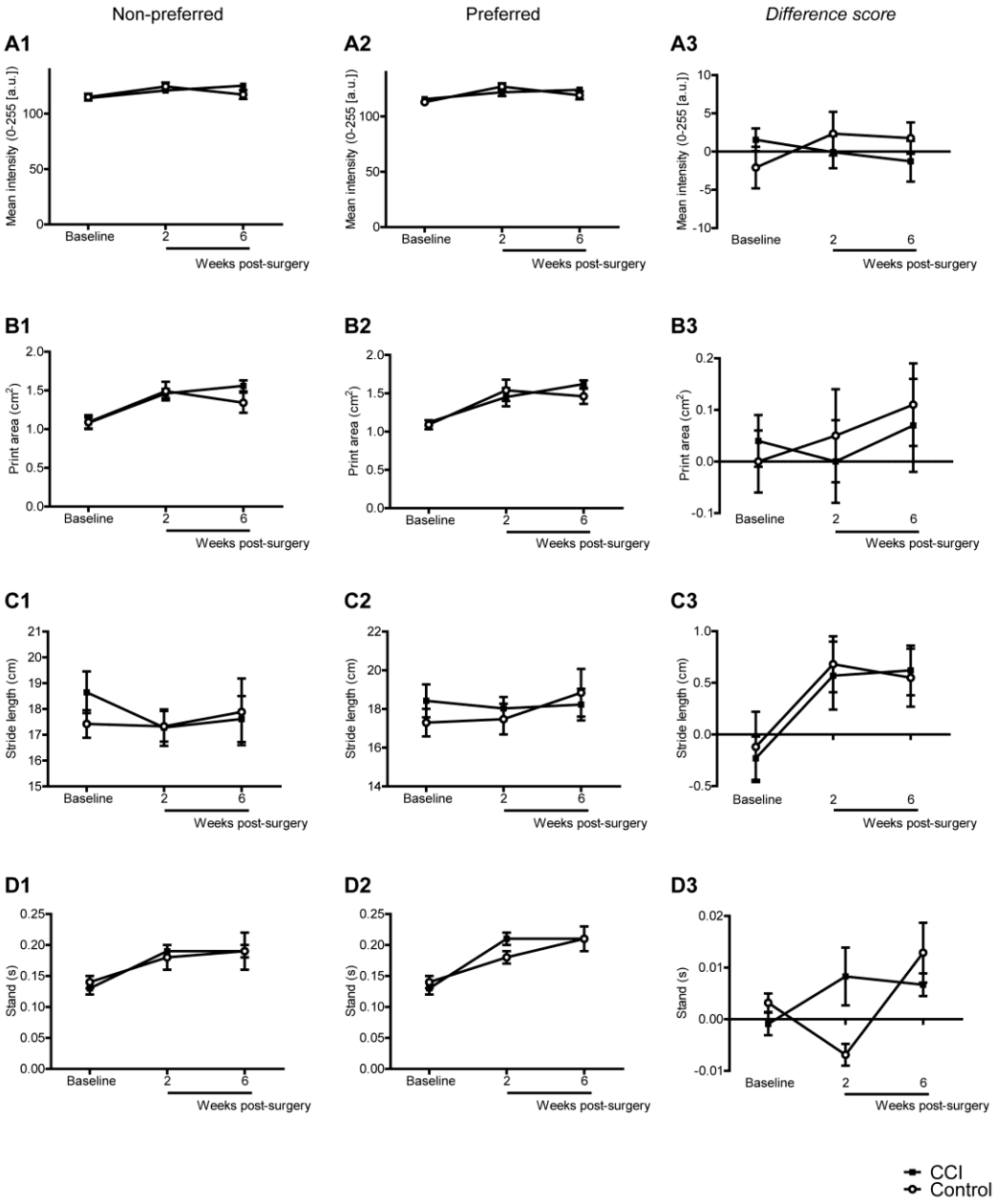


Figure 3.1

Figure 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 six weeks after CCI (approximately 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 to control rats (n=5) with a craniotomy (*** $p < 0.001$, **C**).

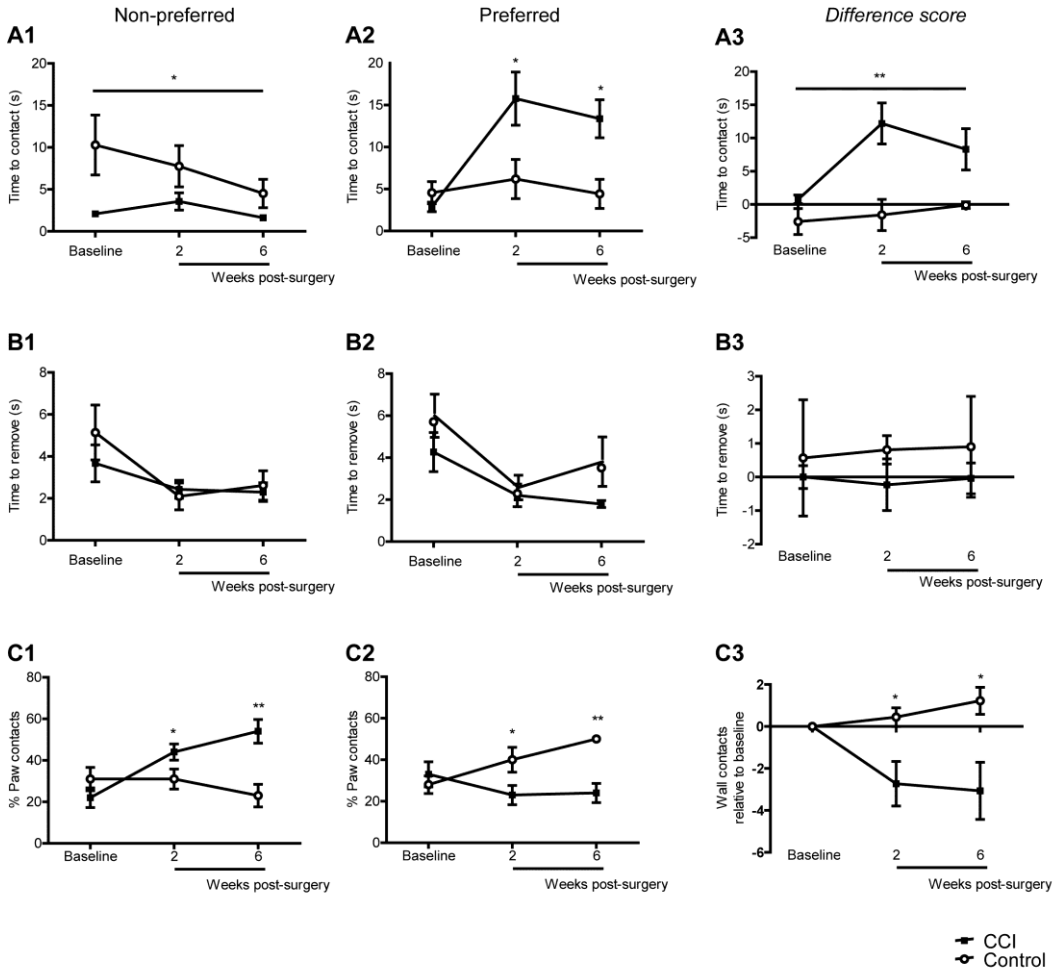


Figure 2

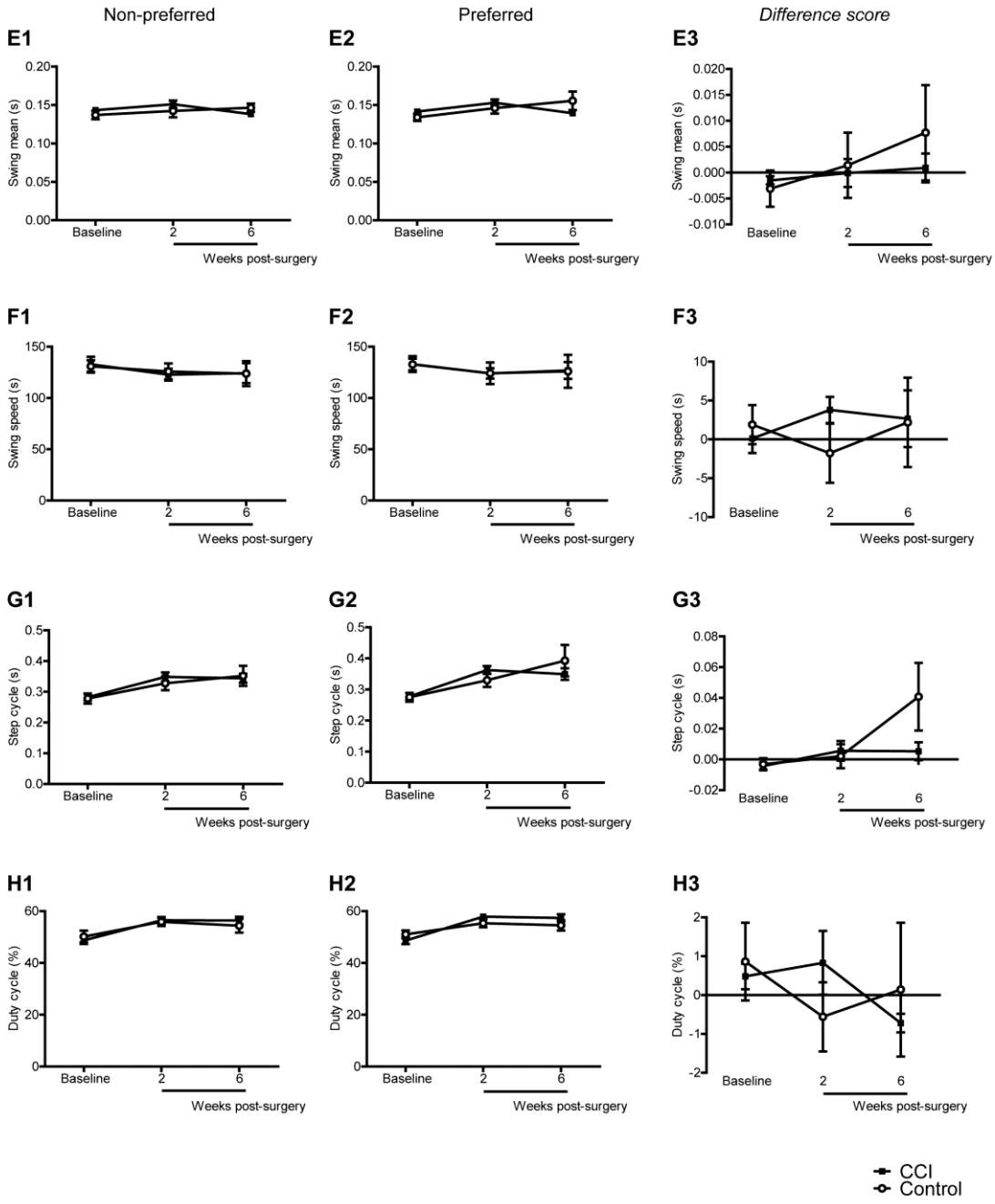


Figure 3.2

Figure 2: CCI impairs tactile recognition of adhesive stickers underneath the preferred paw and causes reliance on the non-preferred paw during vertical exploration

In the adhesive removal test, rats designated to the CCI group were significantly faster in contacting the sticker underneath their non-preferred paw at all three timepoints, thus even before CCI (CCI n=9, control n=7; **A1**).

The time to contact the sticker underneath the preferred paw was significantly increased two and six 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 timepoints (CCI n=10, control n=7; **A3**). CCI did not affect the time taken to remove the stickers (non-preferred 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 exhibit an increased percentage of wall contacts with their non-preferred 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 sigificantly lower compared to 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**).

Figure 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 non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=7; *difference score*: CCI n=9, control n=7; **Fig. 3.1 A1-3**; print area non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=10, control n=7; *difference score*: CCI n=10, control n=7; **B1-3**; stride length non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=7; *difference score*: CCI n=9, control n=6; **C1-3;** stand non-preferred paw: CCI n=10, control n=7; preferred paw: CCI

n=9, control n=7; *difference score*: CCI n=8, control n=7; **D1-3**; swing mean non-preferred paw: CCI n=8, control n=6; preferred paw: CCI n=7, control n=6; *difference score*: CCI n=7, control n=7; **Fig. 3.2 E1-3**, swing speed non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=7; *difference score*: CCI n=7, control n=7; **F1-3**; step cycle non-preferred paw: CCI n=9, control n=7; preferred paw: CCI n=8, control n=7; *difference score*: CCI n=9, control n=5; **G1-3**; duty cycle non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=7; *difference score*: CCI n=7, control n=7; **H1-3**). No significant differences between groups could be detected after CCI concerning the individual forelimbs and the *difference score*.

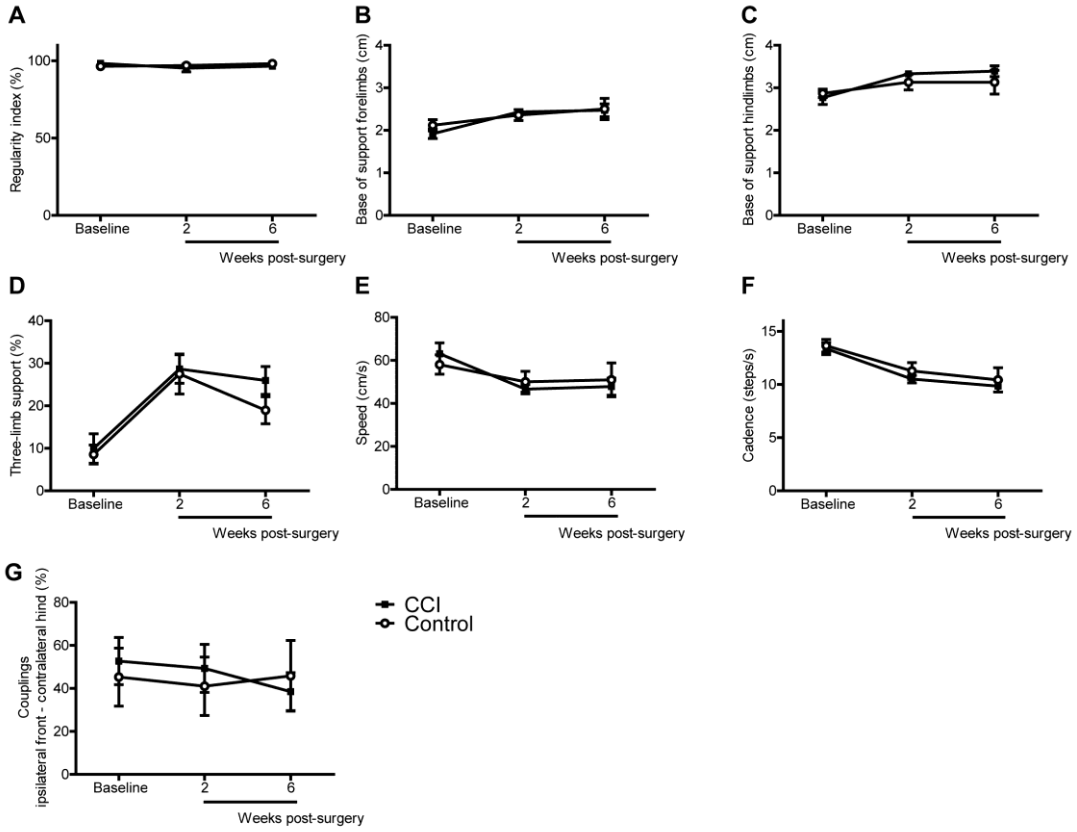


Figure 4

Figure 4: No changes in gait were measured after 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 isilateral front – contralateral hind: CCI n=10, control n=7; **G**). None of the parameters was significantly changed after CCI when comparing CCI rats to controls.

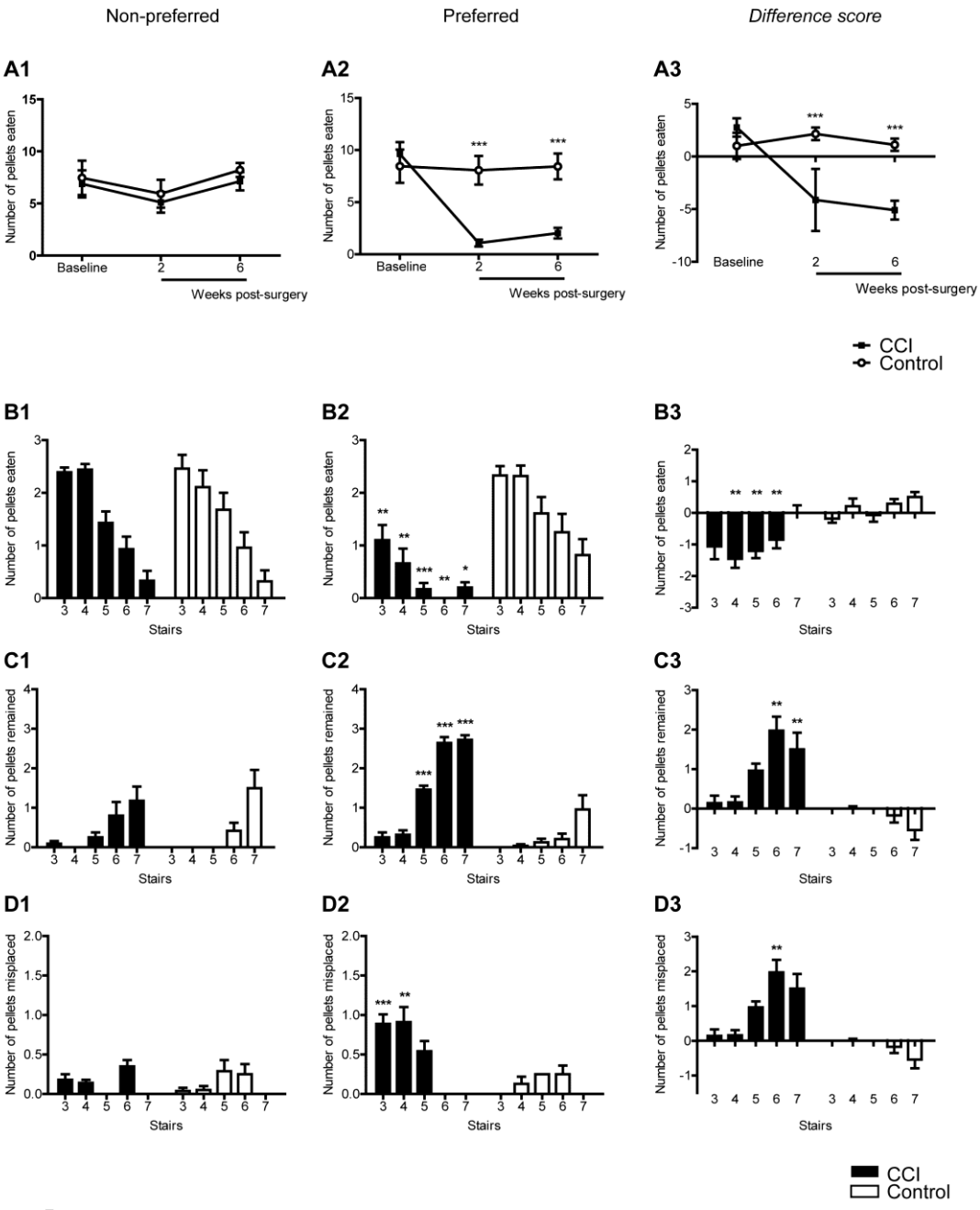


Figure 5

Figure 5: Pellet eating with the preferred paw is significantly deteriorated after CCI

In the Montoya staircase test pellet eating with the non-preferred 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 non-preferred paw (CCI n=10, control n=7; **A3**).

Three additional parameters are shown at six weeks after surgery that provide more details about deficits in pellet retrieval (**B1-3**: number of pellets eaten, **C1-3**: number of pellets remained, **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 reaching 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**).

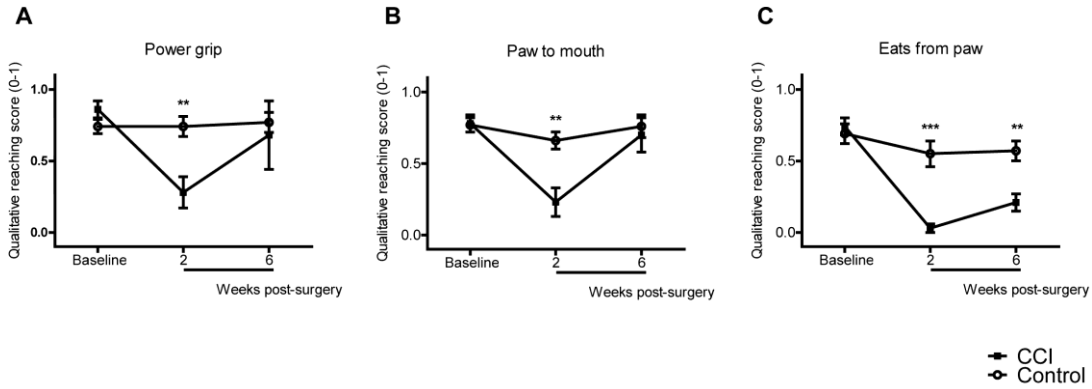


Figure 6

Figure 6: Reaching behavior with the preferred paw is severely altered after 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”, i.e. closing all digits around the pellet when achieving to grasp a pellet (CCI n=8, control n=7; **A**), followed by a problematic return of the paw towards the mouth (CCI n=8, control n=7; **B**). Pellet eating from the paw was significantly impaired both two and six weeks after CCI (CCI n=7, control n=7; ** $p < 0.01$, *** $p < 0.001$, **C**).

schnfeld.supplementary movie 1.mov

schnfeld.supplementary movie 2.mov

schnfeld.supplementary movie 3.mov

Supplementary material

Supplementary Movie 1: CCI impairs tactile recognition of adhesive stickers underneath the preferred paw

Rats are shown six weeks after surgery; the rat on the left received a craniotomy above the forelimb area of the motor cortex contralateral to the preferred paw (in this case: the right paw), whereas the rat on the right received a controlled cortical impact (CCI) at the corresponding location. A blue adhesive sticker is placed underneath the non-preferred paw (left) and a yellow adhesive sticker is placed underneath the preferred paw (right). The control rat simultaneously contacts and removes both stickers with no substantial time delay between the paws. The CCI rat readily contacts and removes the blue sticker underneath the non-preferred paw, but shows no immediate reaction towards the yellow sticker underneath the preferred paw, which is impaired after CCI.

Supplementary Movie 2: CCI causes reliance on the non-preferred paw during vertical exploration

Six weeks after surgery, the control rat uses both forelimbs for vertical exploration, whereas the CCI rat uses its non-preferred, healthy limb (left) to lean against the cylinder walls.

In this movie, optical enhancement (red circles) is used to facilitate the visibility of all wall contacts with the forelimbs.

Supplementary Movie 3: Reaching for pellets with the preferred paw is significantly deteriorated after CCI

Upon entering the staircase box, the control rat immediately starts reaching for pellets using its preferred forelimb (left, indicated by an arrow), whereas the CCI rat starts to reach for pellets with its non-preferred forelimb (right).

Supplementary Movies are included as separate files.

Supplementary Figure 1: CatWalk XT analysis did not reveal any impairment in the preferred forelimb

The five remaining parameters (additionally to the eight parameters shown in Fig. 3) describing individual paw functioning are shown (maximum intensity non-preferred paw: CCI n=10, control n=6; preferred paw: CCI n=10, control n=6; *difference score:* CCI n=9, control n=7; **A1-3**; minimum intensity non-preferred paw: CCI n=10, control n=6; preferred paw: CCI n=9, control n=7; *difference score:* CCI n=9, control n=7; **B1-3**; maximum intensity at maximum contact non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=7; *difference score:* CCI n=9, control n=7; **C1-3**; print length non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=7; *difference score:* CCI n=9, control n=6; **D1-3;** print width non-preferred paw: CCI n=10, control n=7; preferred paw: CCI n=9, control n=5; *difference score:* CCI n=9, control n=7; **E1-3**). One significant difference between CCI and control rats was detected in the maximum paw print intensity of the non-preferred paw six weeks after surgery (**A1**). Concerning all other parameters, no significant differences between groups could be detected after CCI when analyzing scores of the individual forelimbs and the *difference score* (* $p < 0.05$).

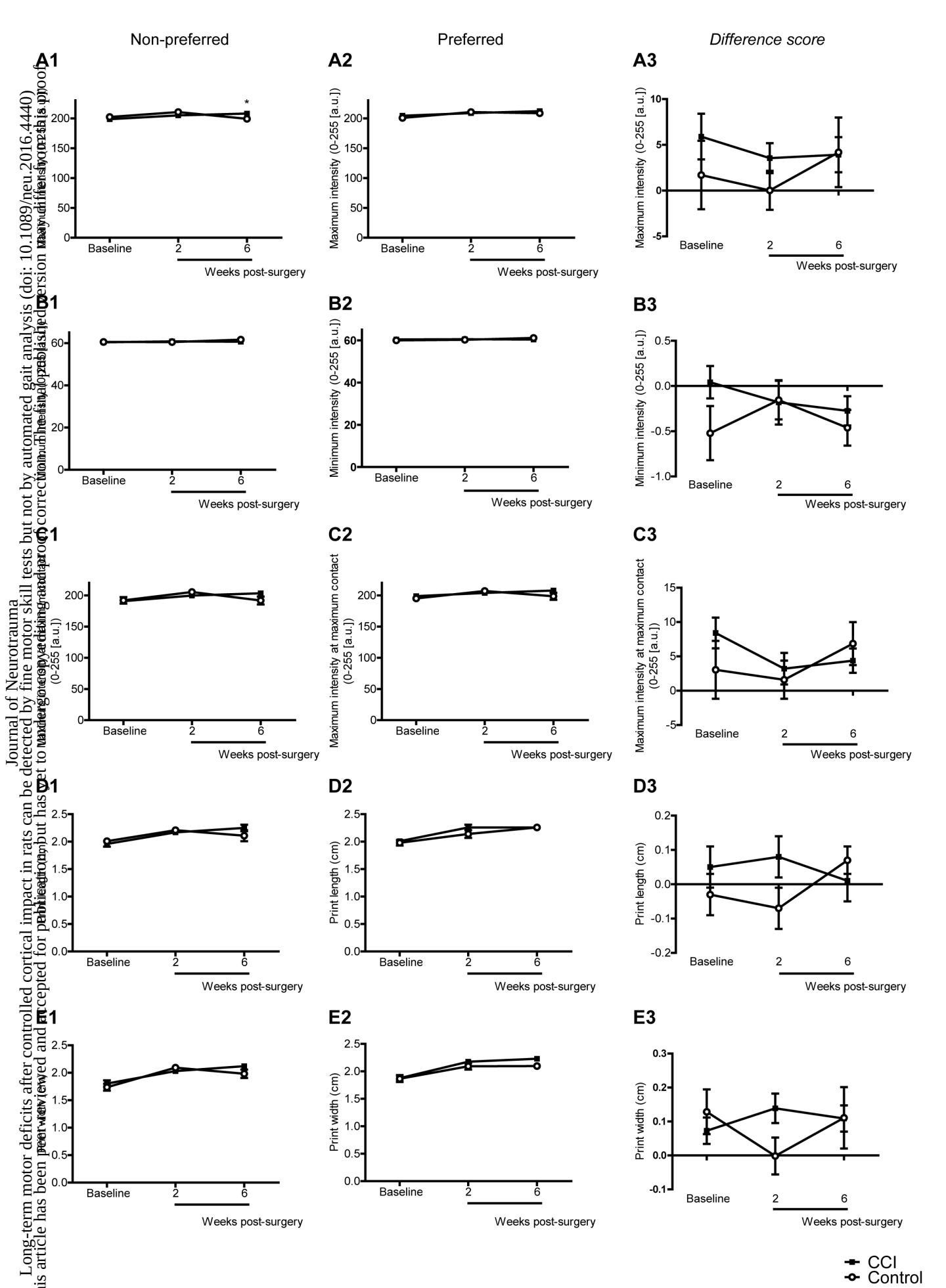
Supplementary Figure 2: Pellet retrieval in the Montoya staircase test is significantly altered after CCI

Data of five parameters measuring pellet retrieval are shown separately for baseline, two weeks and six weeks after surgery (**Supplementary Fig. 2.1 A1-3**: number of pellets remained at baseline, **B1-3**: number of pellets remained 2 weeks after surgery, **C1-3**: number of pellets remained 6 weeks after surgery; **Supplementary Fig. 2.2 A1-3**: number of pellets misplaced at baseline, **B1-3**: number of pellets misplaced 2 weeks after surgery, **C1-3**: number of pellets misplaced 6 weeks after surgery; **Supplementary Fig. 2.3 A1-3**: number of pellets lost at baseline, **B1-3**: number of pellets lost 2 weeks after surgery, **C1-3**: number of pellets lost 6 weeks after surgery; **Supplementary Fig. 2.4 A1-3**: number of pellets taken at baseline, **B1-3**: number of pellets taken 2 weeks after surgery, **C1-3**: number of pellets taken 6 weeks after surgery; **Supplementary Fig. 2.5 A1-3**: number of pellets eaten at baseline, **B1-3**: number of pellets eaten 2 weeks after surgery, **C1-3**: number of pellets eaten 6 weeks after surgery). Overall, after CCI more pellets remained on the individual steps (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; **Supplementary Fig. 2.1 B2**) or were misplaced to another step (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; **Supplementary Fig. 2.2 B2**), whereas less pellets were lost (step 3: CCI n=8, control n=7; step 4: CCI n=8, control n=7; step 5: CCI n=8, control n=7; step 6: CCI n=7, control n=7; step 7: CCI n=8, control n=7; **Supplementary Fig. 2.3 B2**), taken (step 3: CCI n=9, control n=5; step 4: CCI n=10, control n=6; step 5: CCI n=9, control n=5; step 6: CCI n=8, control n=7; step 7: CCI n=9, control n=7; **Supplementary Fig. 2.4 B2)** and eaten

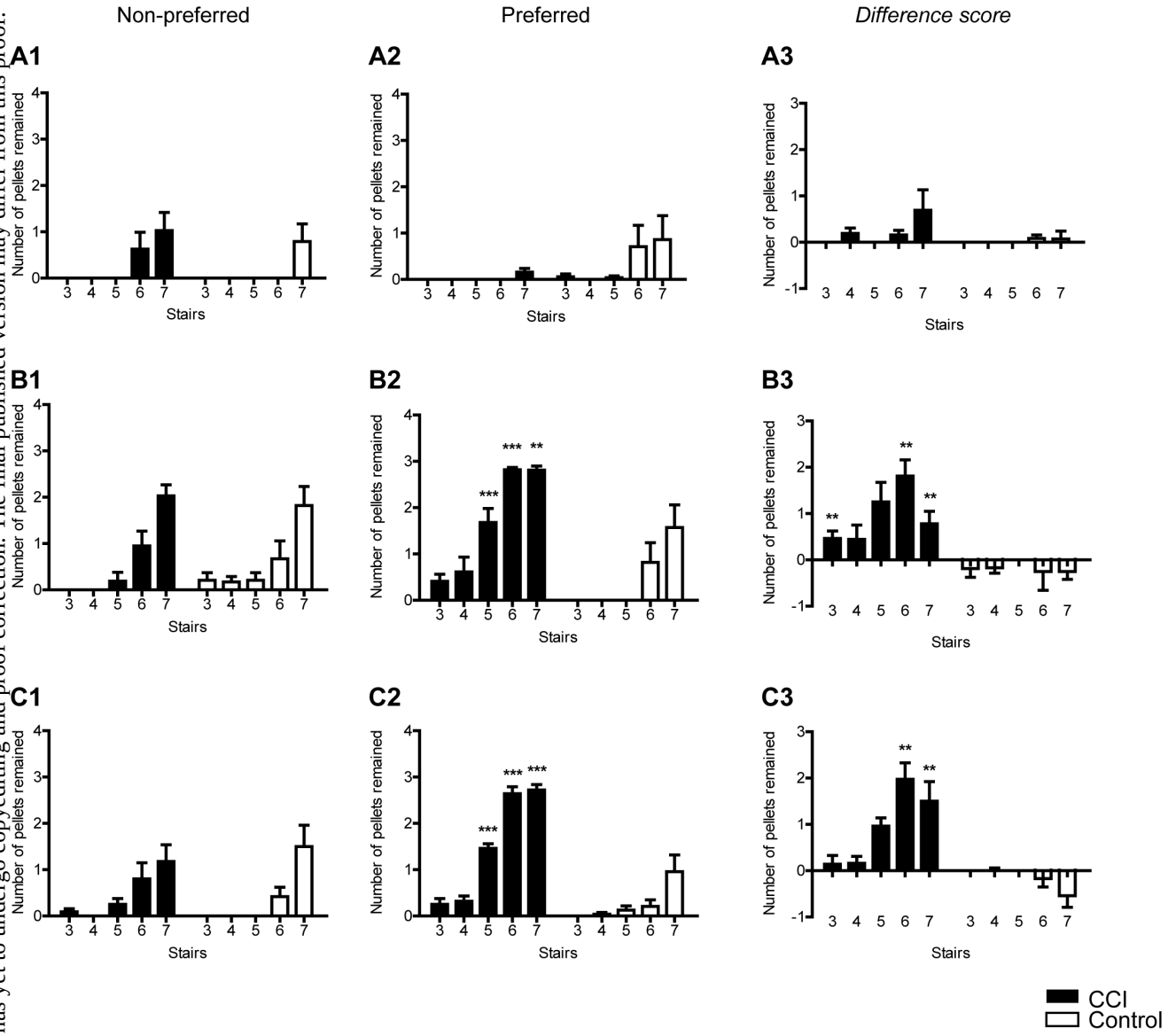
(step 3: CCI n=10, control n=6; step 4: CCI n=8, control n=7; step 5: CCI n=6, control n=7; step 6: CCI n=7, control n=7; step 7: CCI n=10, control n=7; **Supplementary Fig. 2.5 B2)** at the side of the preferred paw (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Supplementary Figure 3: Pellet retrieval with the preferred paw is severely altered after CCI

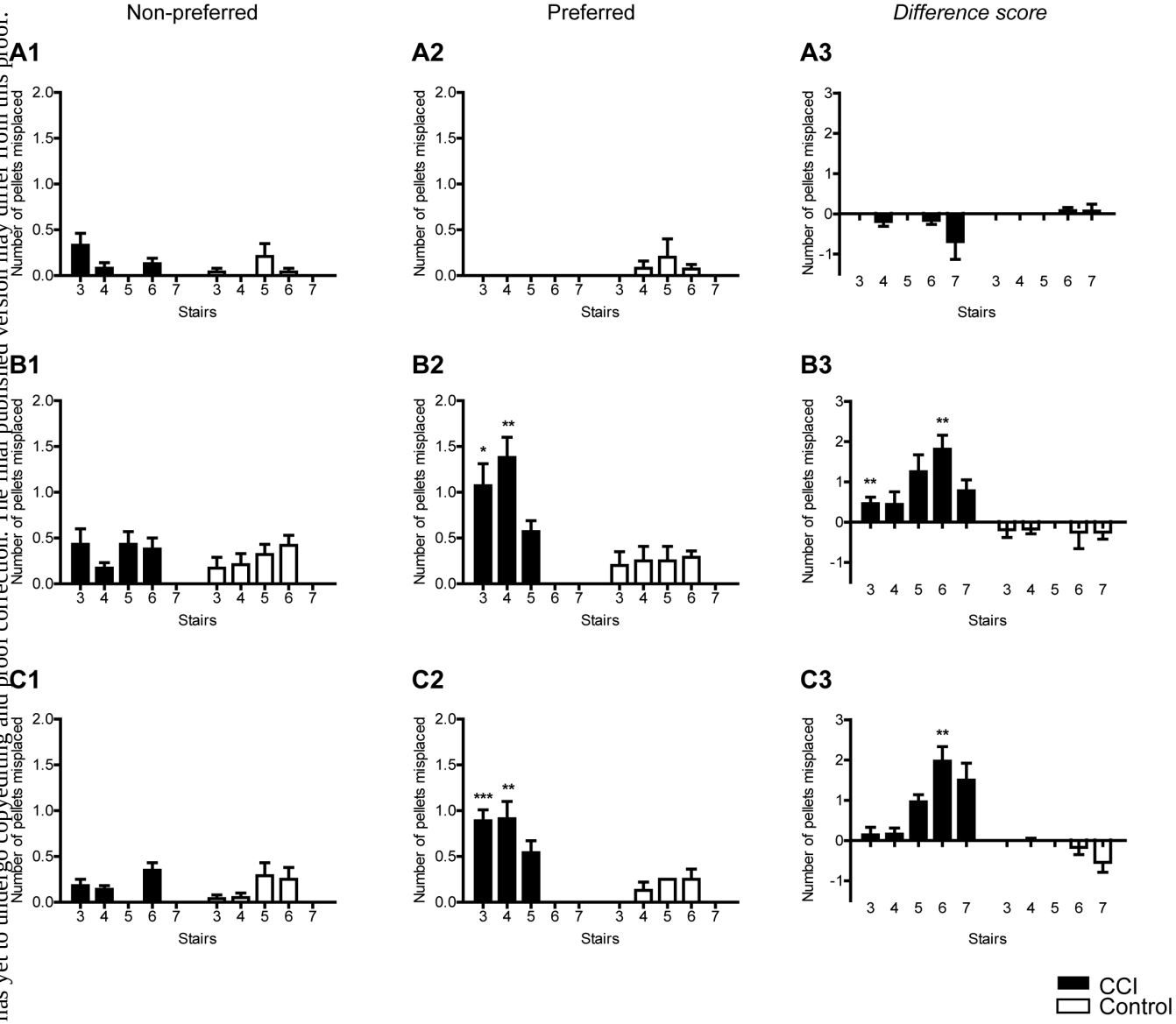
In addition to the three parameters presented in Fig. 6, nine remaining components of a typical reaching action are shown here [inserts snout: CCI n=8, control n=7; **A**; advances limb: CCI n=8, control n=5; **B**; paw pronates: CCI n=5, control n=7; **C**; digits extend: CCI n=7, control n=7; **D**; palpitates: CCI n=8, control n=6; **E;** digits flex: CCI n=8, control n=6; **F**; limb to snout: CCI n=8, control n=7; **G;** supinates paw (1): CCI n=8, control n=7; **H**; supinates paw (2): CCI n=6, control n=7; **I**]. Two weeks after CCI, rats exhibited abnormalities in flexing the digits when trying to hold onto a pellet (**F**) and when supinating the paw (**I**; * $p < 0.05$, *** $p < 0.001$).



Supplementary Figure 1: CatWalk XT analysis did not reveal any impairment in the preferred forelimb



Supplementary Figure 2.1: After CCI more pellets remain at their original step



Supplementary Figure 2.2: More pellets are misplaced after CCI

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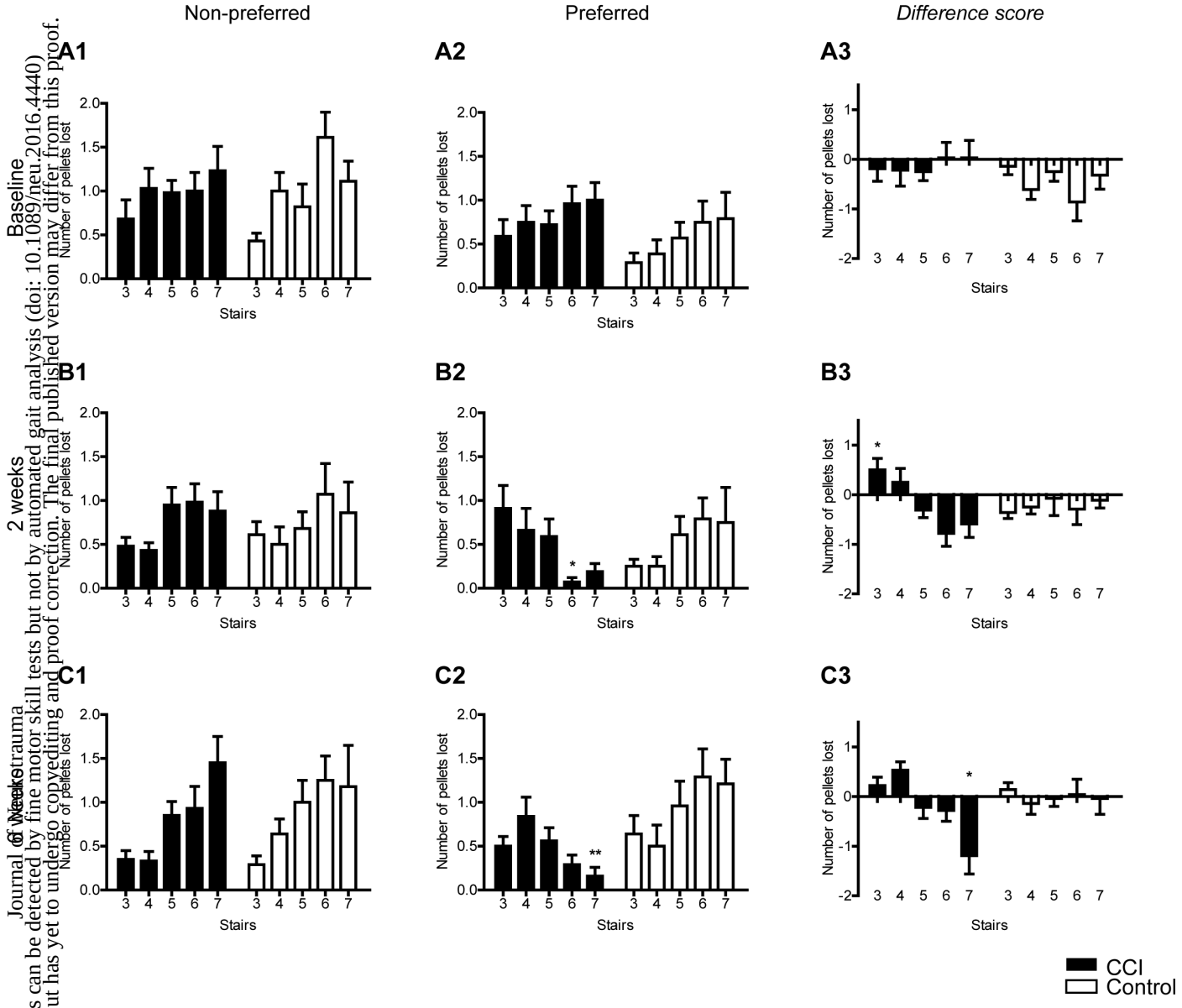
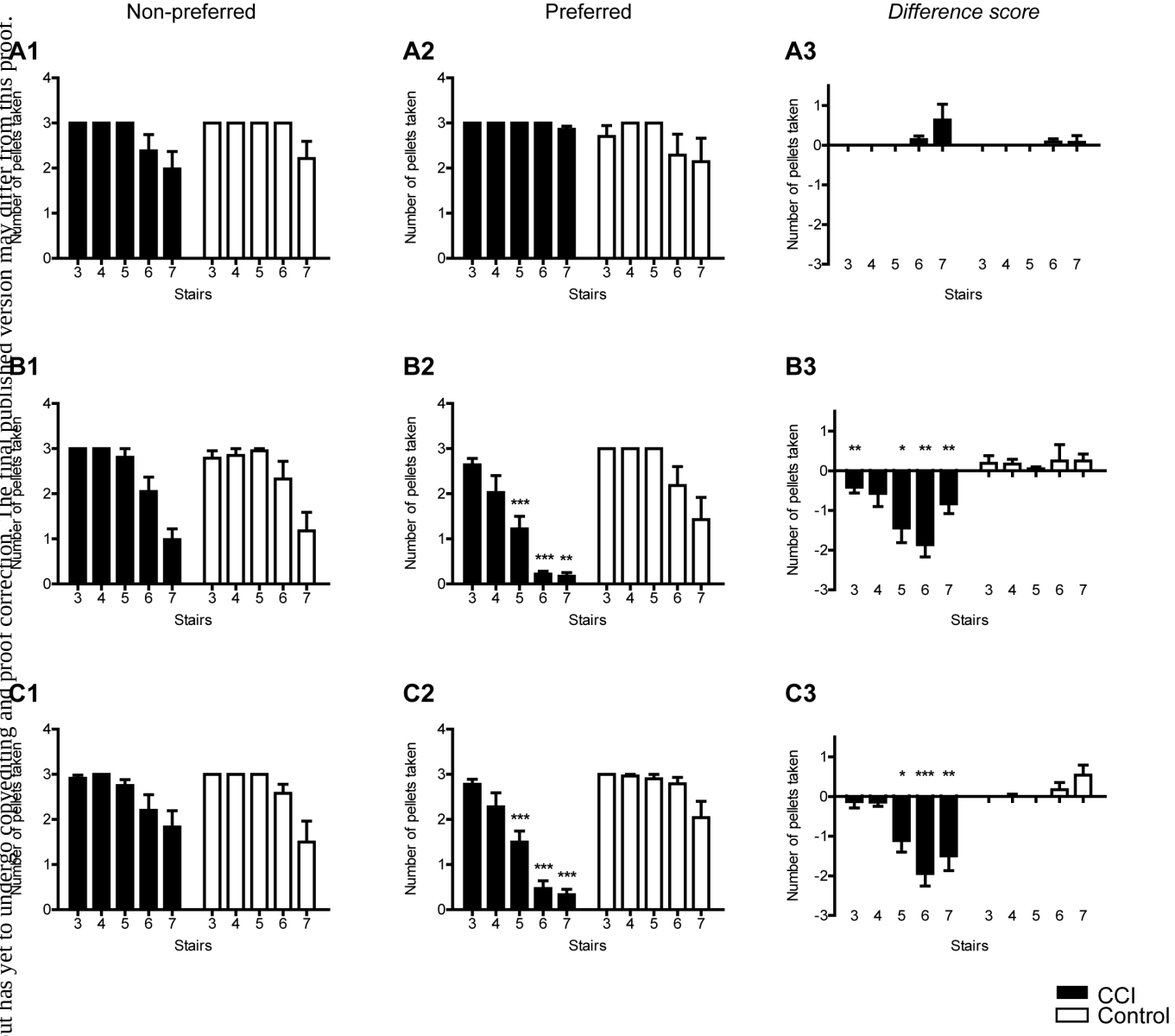
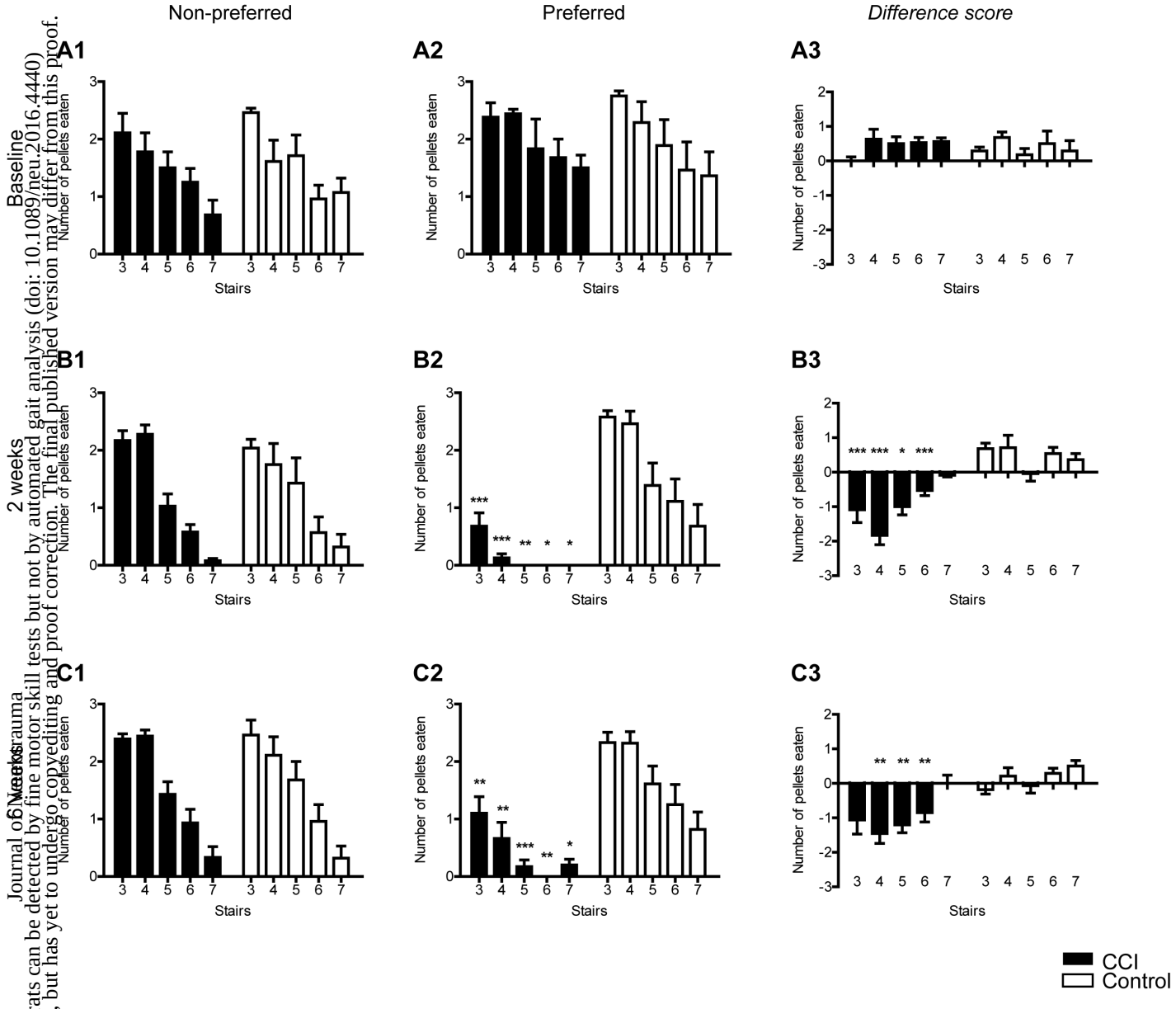


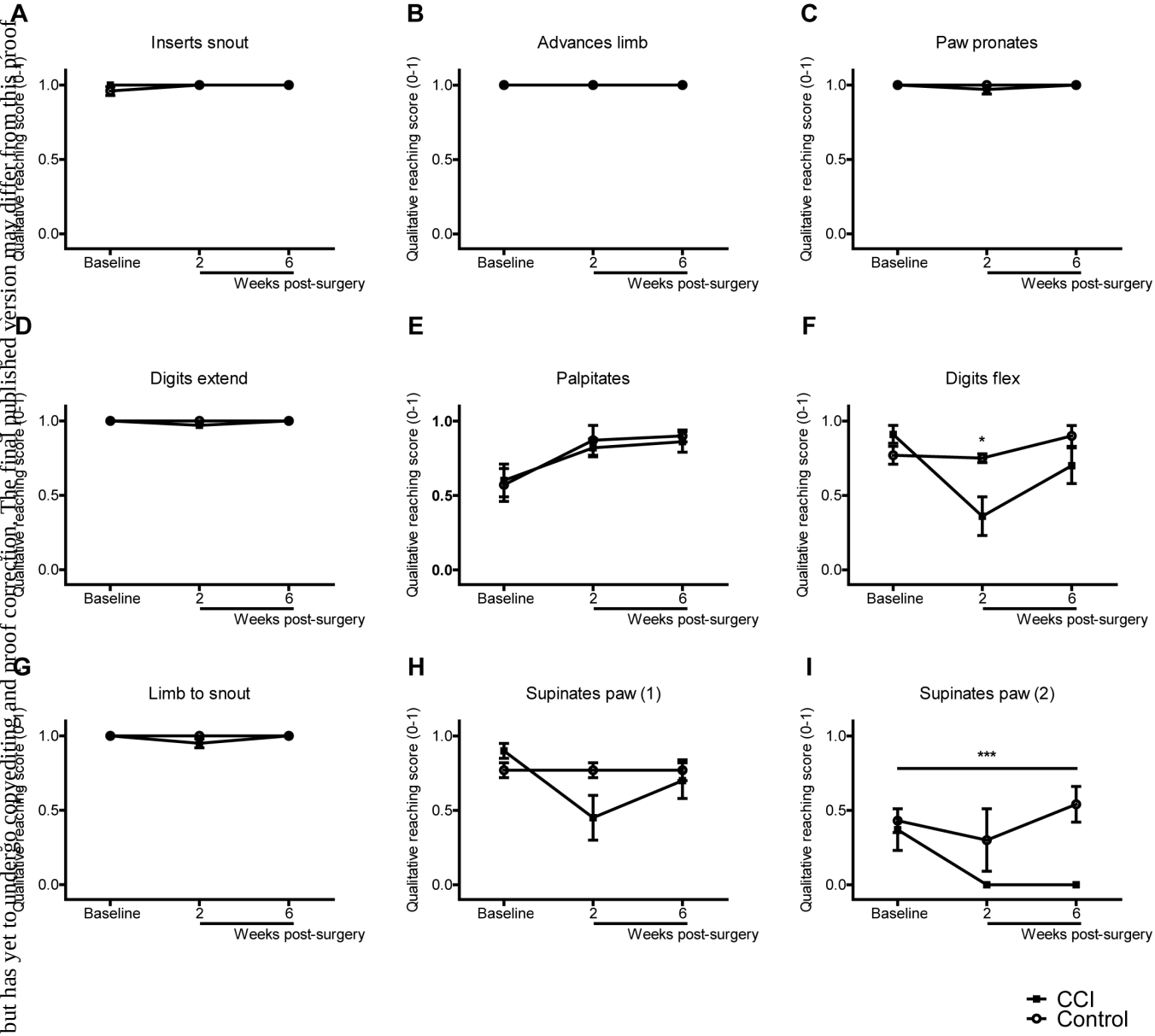
Supplementary Figure 2.3: More pellets are lost when rats reached for lower steps



Supplementary Figure 2.4: After CCI less pellets are taken from the lower steps



Supplementary Figure 2.5: After CCI rats ate less pellets from each single step



Supplementary Figure 3: Pellet retrieval with the preferred paw is severely altered after CCI