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Voluntary and evoked behavioral correlates in neuropathic pain states under different social housing conditions

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Abstract

Background: There is an urgent need to develop and incorporate novel behavioral tests in classically used preclinical pain models. Most rodent studies are based upon stimulus-evoked hindpaw measurements even though chronic pain is usually a day and night experience. Chronic pain is indeed a debilitating condition that influences the sociability and the ability for voluntary tasks, but the relevant behavioral readouts for these aspects are mostly under-represented in the literature. Moreover, we lack standardization in most behavioral paradigms to guarantee reproducibility and ensure adequate discussion between different studies. This concerns not only the combination, application, and duration of particular behavioral tasks but also the effects of different housing conditions implicating social isolation.

Results: Our aim was to thoroughly characterize the classically used spared nerve injury model for 12 weeks following surgery. We used a portfolio of classical stimulus-evoked response measurements, detailed gait analysis with two different measuring systems (Dynamic weight bearing (DWB) system and CatWalk), as well as observer-independent voluntary wheel running and home cage monitoring (Laboras system). Additionally, we analyzed the effects of social isolation in all behavioral tasks. We found that evoked hypersensitivity temporally matched changes in static gait parameters, whereas some dynamic gait parameters were changed in a time-dependent manner. Interestingly, voluntary wheel running behavior was not affected in spared nerve injury mice but by social isolation. Besides a reduced climbing activity, spared nerve injury mice did not showed tremendous alterations in the home cage activity.

Conclusion: This is the first longitudinal study providing detailed insights into various voluntary behavioral parameters related to pain and highlights the importance of social environment on spontaneous non-evoked behaviors in a mouse model of chronic neuropathy. Our results provide fundamental considerations for future experimental planning and discussion of pain-related behavioral changes.

Keywords

spared nerve injury, stimulus-evoked behavior, voluntary wheel running activity, gait analysis, home cage monitoring, housing condition, isolation

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Background

For decades, rodent models have been used to investigate diverse clinically relevant pain conditions. It is generally agreed that we need these models to investigate mechanisms and to develop novel treatments,[1] although currently used rodent pain models are often criticized for not fully reflecting clinical pain characteristics.[2–4] Chronic pain is mostly of spontaneous nature and experienced throughout the day and night. Furthermore, pain-attacks affect sociability and often the ability for voluntary behavioral tasks. These aspects are severely under-investigated in rodents. While patients can verbally describe their pain, most rodent studies rely on stimulus-evoked unilateral hindpaw measurements. More importantly, we should also consider that most rodent pain studies are performed over short durations, on restrained animals or during the day when rodents are naturally inactive. These studies are, therefore,

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limited and cannot represent the full pain picture. An important aspect is, therefore, to comprehensively characterize existing models, assess changes in pain-related daily-life well-being[5] and to focus on measurements of voluntary behavior in unrestrained animals. In most studies, longitudinal measures including behavioral changes in the circadian rhythm are missing. Moreover, the emotional and affective components of pain and the general well-being are considered to play an immense role for the overall pain picture.[6] However, these are also not well-studied in rodent models.[7] Hence, there is a growing interest and need to access new parameters that may reflect impairments in the quality of life.[8] Additionally, we should consider that experimental aspects can also influence behavioral readouts, such as applying too many tests, or restraining of the animals, both which can lead to stress[7] and thereby stress-induced analgesia[9] or stress-induced hyperalgesia.[10] It has been shown that physical and social enrichment[11,12] affects behavioral outcomes and that social isolation harbors stress conditions and can thereby also affect pain.[13,14]

Recently, a variety of non-evoked measures have been introduced to investigate changes in the animal well-being as potential readouts for the affective component of pain and spontaneous pain. Among these measures are voluntary wheel running,[2] home-cage monitoring,[4] DWB,[2,3,15] or gait analysis.[16] Although these tests are increasingly reported, they are still subject of controversy and do not work consistently across laboratories[5] due to a lack of standardization leading to different results reported between laboratories.

In this study, we first aimed to provide a standardized and longitudinal investigation including a portfolio of classical stimulus-evoked tests and voluntary, observer-independent behavioral tasks to assess pain and pain-related behavioral changes in a preferably observer independent manner. We performed detailed analyses of static and dynamic gait alterations, using two different measuring systems, and we investigated long-term voluntary wheel running behavior and detailed home cage monitoring to assess changes in the circadian rhythm. As a model, we used the longstanding classical spared nerve injury (SNI) model for neuropathic pain.[17] Additionally, because animal housing is not performed uniformly between different laboratories, we analyzed animal behavior under grouped and individually housed conditions. Our main objective was to provide a detailed characterization of a portfolio of longitudinal voluntary behavioral measurements in SNI mice and provide behavioral results taken into account different generally practiced housing conditions without the intention to directly compare these results. Most of these behavioral tests have not been published in SNI mice before. Moreover, the effect of social isolation has only been studied with respect to stimulus evoked response thresholds over one week in SNI rats.[18]

Methods

Animals and social housing conditions

C57BL/6N male mice were purchased from Charles River Laboratories (Sulzfeld, Germany) at the age of eight weeks.

Immediately after delivery, mice were divided into two different housing conditions. For the grouped-housed condition, mice were housed in groups of three per cage (named herewith "grouped mice"). Either SNI or sham animals were caged together.

For the isolated-housing condition animals were housed individually (named herewith "isolated mice").

All animals were housed with food and water ad libitum under a standard 12-h light/dark cycle (light on between 7 a.m.–7 p.m.) with regulated ambient temperature of $\pm 22^{\circ}$C and at relative humidity of 40%–50%.

All animal experiments were conducted in agreement with national and international guidelines. Care was taken to minimize suffering for the animals. Animal experiments were approved by the Regierungspräsidium Karlsruhe, Germany.

Experimental design and groups

All behavioral experiments started two weeks after arrival of the mice and behavioral tests were applied up to 84 days (12 weeks) following surgery. Behavioral tests of grouped and isolated animals were not performed in parallel.

Behavioral testing was split in different cohorts because of the large number of behavioral tests and to avoid over-handling of the animals. We investigated four cohorts of animals per housing condition. One cohort was analyzed using stimulus-evoked behavioral tests (Coldplate and von Frey test) and DWB test. A second cohort was analyzed for their voluntary wheel running behavior, a third cohort was monitored in the LABORAS home cage monitoring system, and another cohort was investigated using the CatWalk system.

Cohort	1	2	3	4
Behavioral test	Von Frey test Coldplate DWB (only grouped mice)	Voluntary wheel running (continuously 24 h at different timepoints)	LABORAS home cage monitoring (continuously 24 h at different timepoints)	CatWalk

Common animal husbandry and behavioral experiments were performed by females and in an observer-independent manner, to limit experimenter-sex dependent influences.[19] All behavioral experiments were conducted in a completely randomized and blinded fashion and separation of experiment conduction from data analyses.

Neuropathic SNI model

The SNI model for neuropathic pain following lesions of two of the three terminal branches of the sciatic nerve (tibial and common peroneal nerves), leaving the remaining sural nerve intact, has been described in detail previously.[17]

Reflexive pain tests

Von Frey test. All animals were acclimatized on four consecutive days for 1.5 h to the behavioral compartments. The von Frey test was performed in the morning. Mechanical sensitivity was determined using graded von Frey filaments with bending forces of 0.07, 0.16, 0.4, 0.6, 1, and 1.4 g on the plantar surface of the hindpaws. For the SNI mice, the filaments were applied only within the sural nerve territory (lateral part of the hind paw). Filaments were applied with increasing forces, and each filament was tested five times with adequate resting periods between each application, and the number of withdrawals was recorded. Mechanical thresholds were defined as the minimum pressure required for eliciting a 40% withdrawal responses out of five stimulations and measured in grams (force application).

Coldplate. The Coldplate test was performed with a Hot/Cold Plate (Bioseb, Vitrolles, France) at 2°C in the morning. The latency until the first withdrawal response of the injured hindpaw was recorded, and mice were removed immediately. Cutoff latencies were set at 30 s.

Non-reflexive behavioral tests

Dynamic weight bearing. We used the DWB system (Bioseb, Boulogne, France) for incapacitance testing in freely moving mice. The system consists of a Plexiglas enclosure (11 × 11 cm) with a floor composed of 1,936 pressure transducers. A digital camera was placed at one side of the enclosure. Mice were allowed to move freely within the apparatus for 5 min. Pressure data and live video were transmitted via an USB interface to a PC containing DWB software v1.3. Following completion of the test, mice were removed, and the test chamber was cleaned with alcohol wipes. For data analysis, the raw pressure data were automatically synchronized with time-lapse video images. Each test segment was manually validated ensuring each weight zone corresponded to the appropriate assigned paw. The system enabled the analysis of the paw weight distribution and the paw print area. Animals were acclimatized for two sessions before basal measurement.

"CatWalk"-based analysis. The CatWalk XT version 10.6 gait analysis system (Noldus, Netherlands system) consists of an enclosed 1.3 m black corridor on a glass plate, which is illuminated inside with a green LED. This light is internally reflected, except at those areas where the animal makes contact with the glass plate. Wherever the paws touch the glass, light is refracted on the opposite side. Using the Illuminated footprints™ technology, paws are captured by a high speed video camera that is positioned underneath the glass. The mouse is placed on one end of the corridor and allowed to transverse it voluntarily. The brightness of a pixel depends on the amount of light received from a paw area by the camera. The system enables an automatic footprint classification, error correction, interactive footprint measurements, and data segmentation profiling.

We used the following parameters:

- Paw print area represents the surface of the complete print of a paw.
- Maximal contact maximal intensity of a paw. The intensity of a print that depends on the degree of contact between a paw and the glass plate.
- Swing phase is the duration of no contact of a paw with the glass plate in a step cycle.
- Stride length is the distance between successive placements of the same paw.
- Stance is the duration of ground contact for a single paw.
- Duty cycle expresses the stand as a percentage of a step cycle (step cycle is the time between two consecutive initial contacts of the same paw. Step cycle = stand + swing). Duty cycle = stand/(stand + swing) × 100%.

Mice were habituated to the CatWalk setup and allowed to cross the corridor for three sessions. On each testing day, animals were allowed to cross the corridor for three times.

Home cage monitoring. The Laboras home cage observation (Metris B.V., Netherlands) is a system that uses a carbon fiber platform to detect behavior-specific vibration patterns produced by the animal. A home cage is placed on top of the platform, and the specific Laboras software version 2.6. processes the produced vibrations into various behavioral parameters. Over 24 h, we analyzed climbing, grooming, rearing, locomotion, and immobility. These behavioral parameters were calculated over time or as frequency counts. Additional tracking

information like travelling distance, average, or maximal speed was collected. Animals were placed individually in the calibrated cage under standard housing condition with free access to food and water at all measuring days in the morning, usually around 8 a.m.

Voluntary wheel running activity. Animals were placed individually in cages containing a running wheel and free access to food and water. We usually started this measurement in the morning around 9 a.m. for a total duration of 24 h. Unrestricted voluntary wheel running activity was digitally recorded using the AWM counter (Lafayette Instrument, Louisiana, USA), which uses an optical sensor to detect the total revolutions of the wheel and is connected to an USB Interface and PC running an AWM Software (Lafayette Instrument, Louisiana, USA).

Statistical analysis

For all measurements, data were calculated and presented as mean ± SEM. Unless stated otherwise, two-way repeated measures ANOVA followed by post hoc Tukey's tests was used to assess statistical significance. Changes with $p < 0.05$ were considered to be significant.

Results

Stimulus-evoked behavior and bodyweight

We investigated mechanical and cold sensitivity up to 72 days following nerve injury in SNI or sham-operated control animals. SNI mice of the grouped cohort showed a significant drop in von Frey response thresholds three days following nerve-injury compared to sham controls (Figure 1(a)). With respect to temperature sensitivity, we measured a significant and stable cold hyperalgesia in SNI mice (Figure 1(b)) throughout the observation period, which was absent in the sham animals. Additionally, we monitored the body weight of all mice. Sham animals gained significant weight from day 14 following surgery, while SNI animals showed a significant gain in body weight at a later stage after day 28 post-surgery (Figure 1(c)). At the end of the observation period, we measured a significant difference in body weight between SNI and sham animals. SNI mice gained significantly less weight compared to sham animals at 84 days following surgery (Figure 1(c)).

We went on to measure stimulus-evoked hindpaw behavior in sham and SNI animals which were housed separately (isolated mice). Isolated SNI animals developed significant mechanical allodynia at day 3 following surgery, which lasted over the whole observation period of 72 days (Figure 1(d)). Furthermore, isolated SNI mice developed significant cold hyperalgesia compared to isolated sham mice throughout the experimental timeframe (Figure 1(e)). With respect to the body weight, isolated sham mice gained significant weight from day 14 (Figure 1(f)) but isolated SNI animals gained significant weight later, from day 28 following surgery (Figure 1(f)). Although all mice showed a constant increase in body weight over the course of the experiment, the body weight of isolated SNI mice remained significantly lower than the body weight of isolated sham mice (Figure 1(f)).

All SNI mice developed a stable mechanical allodynia and cold hyperalgesia and gained less weight that sham mice. The magnitude and timecourse of mechanical allodynia and cold hyperalgesia was similar between grouped and isolated SNI animals.

Weight distribution and gait analysis

Additionally, we were interested to monitor changes in weight distribution and different gait parameters. We used two different measuring systems, the DWB system (Bioseb) and the CatWalk system (Noldus). All measuring parameters are illustrated as ratio of the ipsilateral to the surgery (left) hindpaw (LH) over the healthy (right) hindpaw (RH).

The DWB system (Bioseb) enables the investigation of static gait parameters, as paw weight distribution and paw print area, in freely moving mice. The CatWalk system enables complete automatic gait analysis of these static parameters as well as dynamic gait parameters such as stride length, stand duration, and swing phase among others.

Using the DWB system, grouped SNI mice showed a significant decrease in the ratio of the hindpaw weight distribution between the ipsilateral and contralateral paw as compared to the basal paw distribution, and as compared to the sham mice at 1, 2, 3, and 10 weeks following surgery (Figure 2(a)). Similarly, we measured a significant drop in the ratio of the paw print area of SNI mice compared to basal and sham animals (Figure 2(b)). Owing to the high degree of comparability between results of the DWB and CatWalk system, we analyzed isolated SNI and sham animals solely with the CatWalk system.

These static weight parameters were compared using the CatWalk system where we found a significant reduction in paw intensity contact (Figure 3(a)) and paw print area (Figure 3(b)) in SNI mice at every timepoint measured (Figure 3(a) and (b)). The drop in paw intensity contact and paw print area was very prominent already at day 3 and remained constant at all other measuring timepoints (Figure 3(a) and (b)).

Isolated SNI mice showed a significant decrease in paw intensity contact (Figure 3(c)) and paw print area (Figure 3(d)) during the whole observation period as compared to their basal paw-ratios and to the isolated sham control animals at all timepoints.

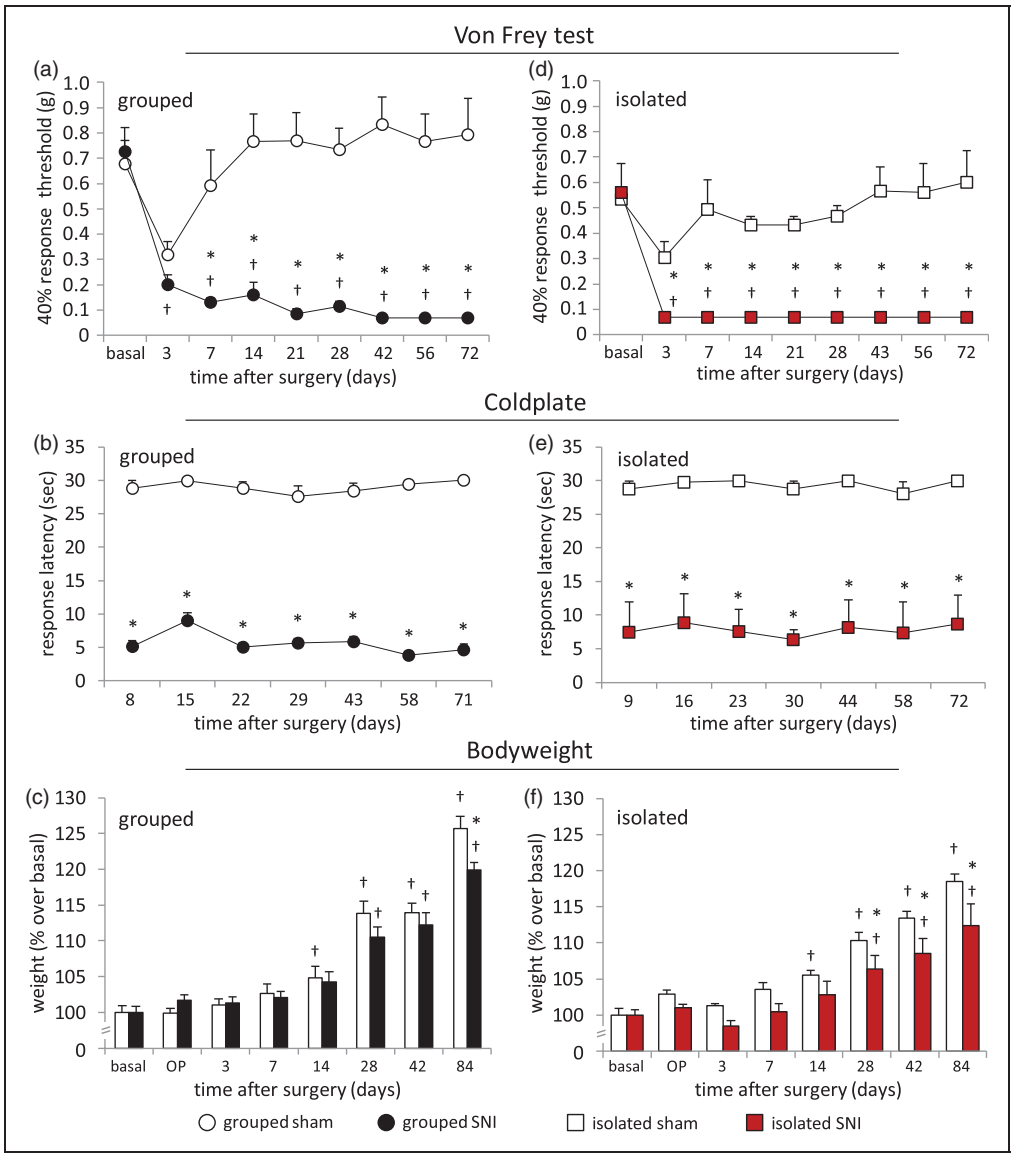

Figure 1. Analysis of nociceptive sensitivity and bodyweight in mice with spared nerve injury (SNI). All left column panels (a–c) show results from animals which were housed in groups (black circular symbols and black bars), all right column panels (d–f) show results from animals which were housed individually (red square symbols and red bars). (a, d) 40% response threshold toward the application of graded von Frey hair filaments. (b, e) response latency on a 2°C coldplate. (c, f) Analysis of bodyweight changes over basal bodyweight up to 84 days following surgery. $N = 6$ mice/group, $p < 0.05$ indicated by * as compared to control group, † as compared to basal values within a group, two-way repeated measures ANOVA with post hoc Tukey test, except for the Coldplate test (b, e), where significant differences were calculated using a *t* test. All data points represent mean ± SEM.

In addition to these prominent and steady changes in static gait parameters, we further analyzed dynamic gait parameters in SNI mice using the CatWalk system. There was a significant drop in the ratio of the stance duration between the ipsilateral and contralateral paw in grouped SNI mice compared to basal and sham animals at all timepoints (Figure 4(a)). Interestingly, the swing phase of the ipsilateral paw increased significantly during the first two weeks in SNI animals (Figure 4(b)), with a maximal peak at three days following surgery. From three weeks on, there was no difference in swing phase ratio between the ipsilateral and contralateral paw of SNI mice, as compared to their basal swing ratio or as compared to sham control mice at all measuring timepoints (Figure 4(b)). Additionally, we measured a continuous decrease in stride length of the ipsilateral paw from two weeks following surgery (Figure 4(c)). Furthermore, the paw ratio of the duty cycle was significantly reduced at all observation days in SNI animals (Figure 4(d)).

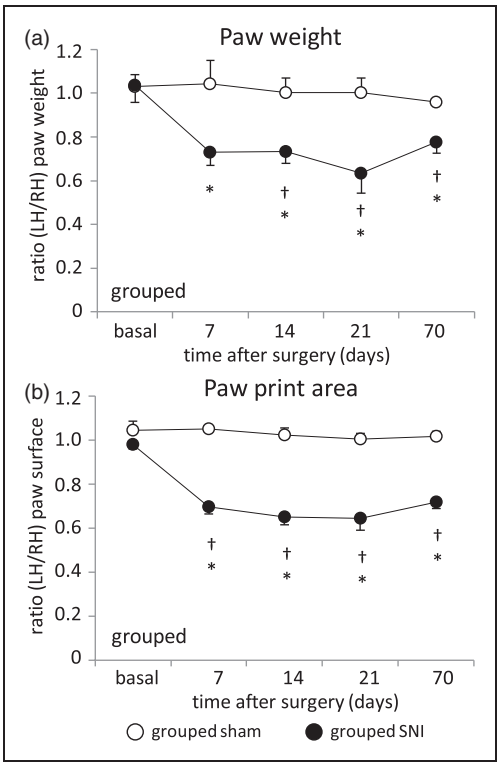

Figure 2. Changes in static weight bearing following SNI. Grouped animals were analyzed using the Dynamic weight bearing system (Bioseb). The ratio of the left over the right hindpaw is shown for (a) paw weight and (b) paw print area. $N = 6$ mice/group, $p < 0.05$ indicated by * as compared to control group, † as compared to basal values within a group, two-way repeated measures ANOVA with post hoc Tukey test. All data points represent mean ± SEM.

We obtained similar results when we investigated the same dynamic gait parameters in isolated mice. Isolated SNI mice showed a significant reduction in stance duration at every post-surgical timepoint (Figure 4(d)), and a significant increase in ratio of swing phase (ipsilateral over the contralateral paw) during the first two weeks following surgery (Figure 4(f)). This increase in swing phase peaked at seven days, dropped and stayed at a constant level that was significantly higher as compared to control mice at 21, 28, and 49 days following surgery (Figure 4(f)). Additionally, we saw a decrease in stride length of the ipsilateral paw at day 21, 28, and 49 after surgery in SNI mice, although this did not reach significance (Figure 4(g)). As seen in grouped SNI mice, isolated SNI mice displayed a significant decrease in duty cycle as expressed as the ratio of the ipsilateral over the contralateral paw at all timepoints during the post-surgical period (Figure 4(h)).

We found similar changes in static and dynamic gait pattern between grouped and isolated SNI mice. SNI mice showed very robust changes in static gait parameters throughout the whole observation timecourse, whereas some dynamic parameters were affected albeit in a temporal manner: changes in the swing phase were prominent during the first two weeks and alterations in stride length appeared only after two weeks post-injury.

Voluntary wheel running

So far, there is no report of voluntary wheel running in SNI animals. We investigated voluntary wheel running in SNI and sham mice on a longitudinal timeframe. Each voluntary wheel running session was measured over 24 h. This allowed us to assess activity changes between illuminated (day) and dark (night) periods. Mice were analyzed for their basal running behavior, directly following surgery on the day of operation (OP), at day 3, day 7, and thereafter on a weekly basis to 10 weeks following surgery.

Since the 24-h measurement required placing the animals in individual setups, grouped mice were always only separated for the measurement and immediately reunited with their cage mates at the end. On the day of operation, there was a significant reduction in total running distance of grouped SNI mice as compared to their basal running distance, whereas there was no difference observed in voluntary wheel running behavior of sham operated mice (Figure 5(a)). Grouped sham mice showed an increase in 24 h running distance on day 3 and reached a stable running distance from day 7 on. There was no further notable difference in the running behavior of grouped sham mice until the end of the observation period (Figure 5(a)). Interestingly, we did not observed any significant reduction in voluntary wheel running of grouped SNI mice following nerve injury. We saw a reduction of wheel running activity on the day of operation and at later stages, but this was not significant different as compared to their basal running distance. Interestingly, the running distance of grouped SNI mice was significantly lesser than the running distance of the sham animals at most timepoints (Figure 5(a)). A typical representative 24 h running profile of grouped sham and SNI animals at day 21 following surgery is shown in Figure 5(b). Owing to the novelty of the wheel in the cage, all mice explored and used it immediately within the first 1 to 2 h when placed into the wheel-running-cage but not subsequent more during the rest of the illuminated day time (Figure 5(b)). Typical wheel running activity occurred during the dark night time between 7 p.m. and 4 a.m. There was a clear difference in voluntary wheel running between sham and SNI mice during the night period (Figure 5(b)).

To explore whether the difference in voluntary wheel running behavior appeared because of a training effect over time of the sham mice, we tested grouped SNI and sham mice from another cohort, which were not previously subjected to the running wheel. Similarly, we found

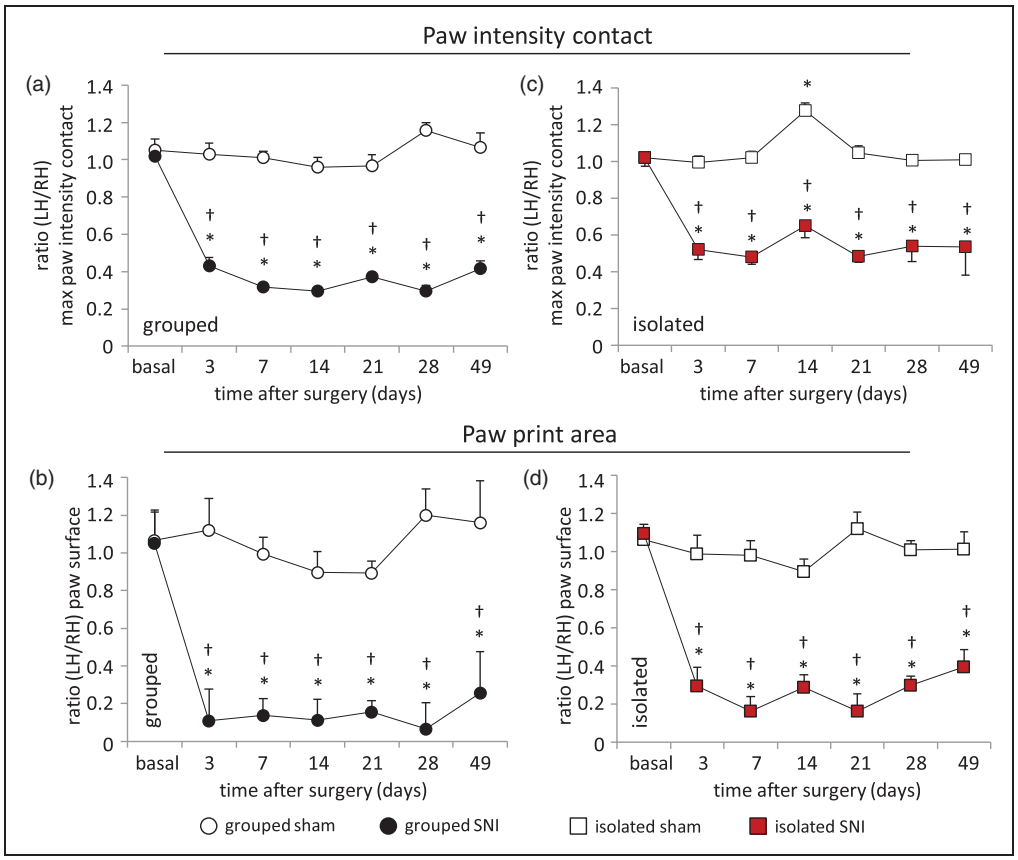

Figure 3. Changes in static weight parameters following SNI in animals which were housed in groups (black circular symbols) (a, b) and animals which were housed individually (red square symbols) (c, d) using the CatWalk system (Noldus).The ratio of the left over the right hindpaw is shown for (a, c) paw intensity contact and (b, d) paw print area. $N = 6$ mice/group, $p < 0.05$ indicated by * as compared to control group, † as compared to basal values within a group, two-way repeated measures ANOVA with post hoc Tukey test. All data points represent mean ± SEM.

a significant difference in running distances between the sham and SNI mice of the untrained cohort at 28 days following surgery (Figure 5(c)). We can thereby exclude that training of healthy control mice accounted for the observed significant difference in running distance between sham and SNI mice.

We were further interested to explore the impact of housing condition and isolation on voluntary wheel running activity and performed the same wheel running experiments with SNI and sham mice, which were housed individually already before the start of the experiment (isolated mice). To our surprise, there was no difference between isolated sham and SNI mice at any post-surgery day (Figure 5(d)). All mice showed a similar wheel running activity at all investigation timepoints. We did not detect an increase in running distance with repetitive running trials and also no decrease on the day of surgery (OP) (Figure 5(d)). There was, however, a small difference in running distance at the late stage (day 63 and day 70 following surgery) between the isolated sham and SNI mice, but this was not significant (Figure 5(d)).

We also tested untrained isolated sham and SNI mice from another cohort for their voluntary wheel running behavior and could not find a significant difference between both groups when tested at day 41 following surgery (Figure 5(e)).

Overall, we found differences in voluntary wheel running activity of operated animals depending on their housing conditions. First, wheel running was attenuated immediately after nerve injury only in mice housed in groups but not in isolated animals. Second, within the grouped animals, wheel running was significantly lower in the SNI mice compared to the sham animals over the whole investigation period. Interestingly, peripheral nerve injury did not affected running behaviors of socially isolated animals.

Home cage monitoring

To further explore innate behaviors, we investigated SNI and sham animals using the automated Laboras home cage monitoring system (Figure 6, Supplementary Table 1).

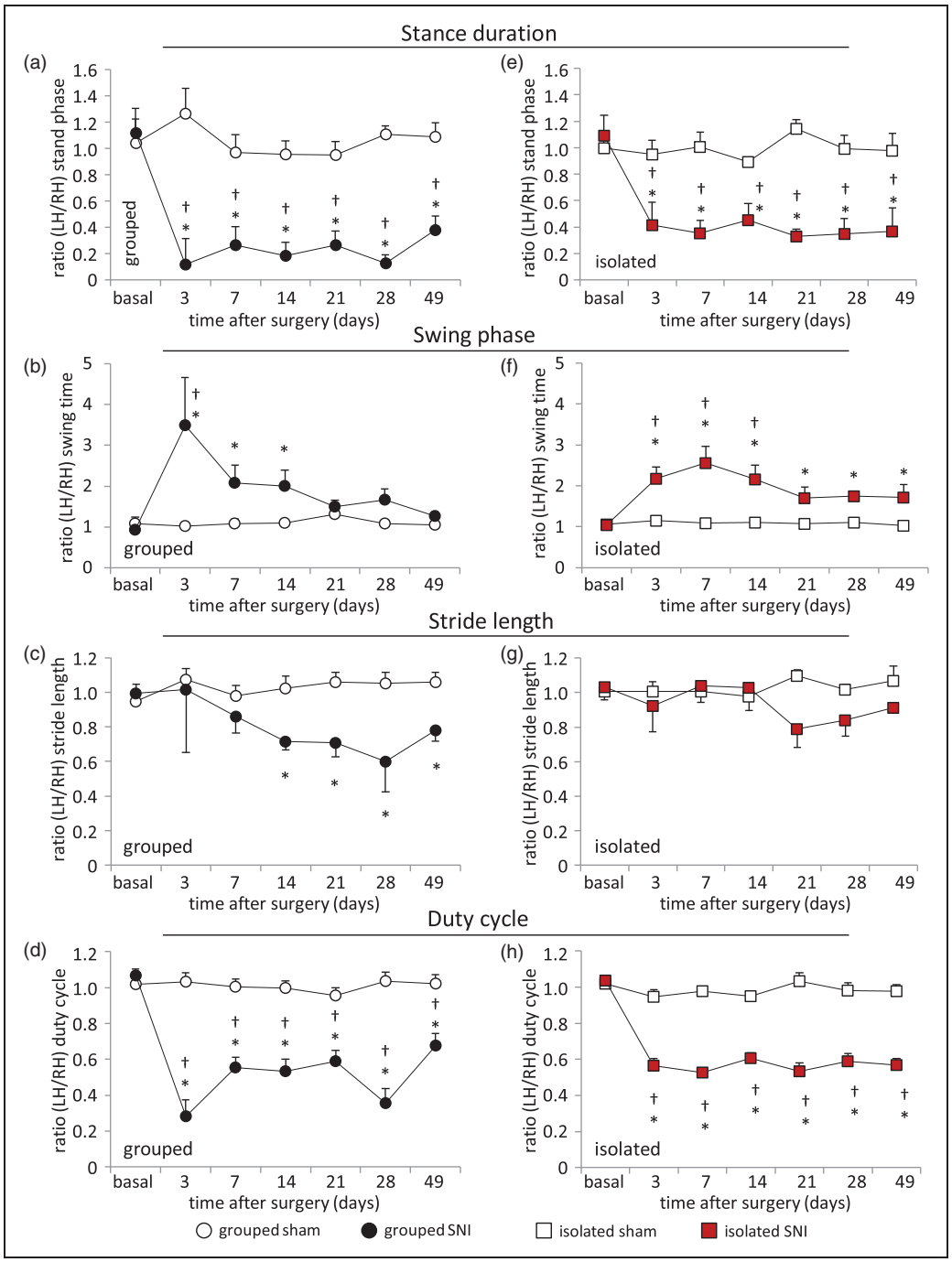

Figure 4. Changes in dynamic gait parameters following SNI in animals which were housed in groups (black circular symbols) (a–d) and animals which were housed individually (red square symbols) (e, f). The ratio of the left over the right hindpaw is shown for (a, e) stance duration, (b, f) swing phase (c, g) stride length, and (d, f) swing phase. $N = 6$ mice/group, $p < 0.05$ indicated by * as compared to control group, † as compared to basal values within a group, two-way repeated measures ANOVA with post hoc Tukey test. All data points represent mean ± SEM.

We first analyzed grouped SNI and sham animals which had to be separated into individual cages for every 24 h measuring timepoint. Measurements were performed at basal, 24 h following surgery (OP), days 3, 7, 14, 21, 28, 42, and 84 following surgery. Mice were always reunited with their cage mates immediately following measurement.

Following surgery, SNI mice showed a reduced climbing behavior as compared to the sham control mice at all timepoints (Figure 6(a), Supplementary Table 1). This

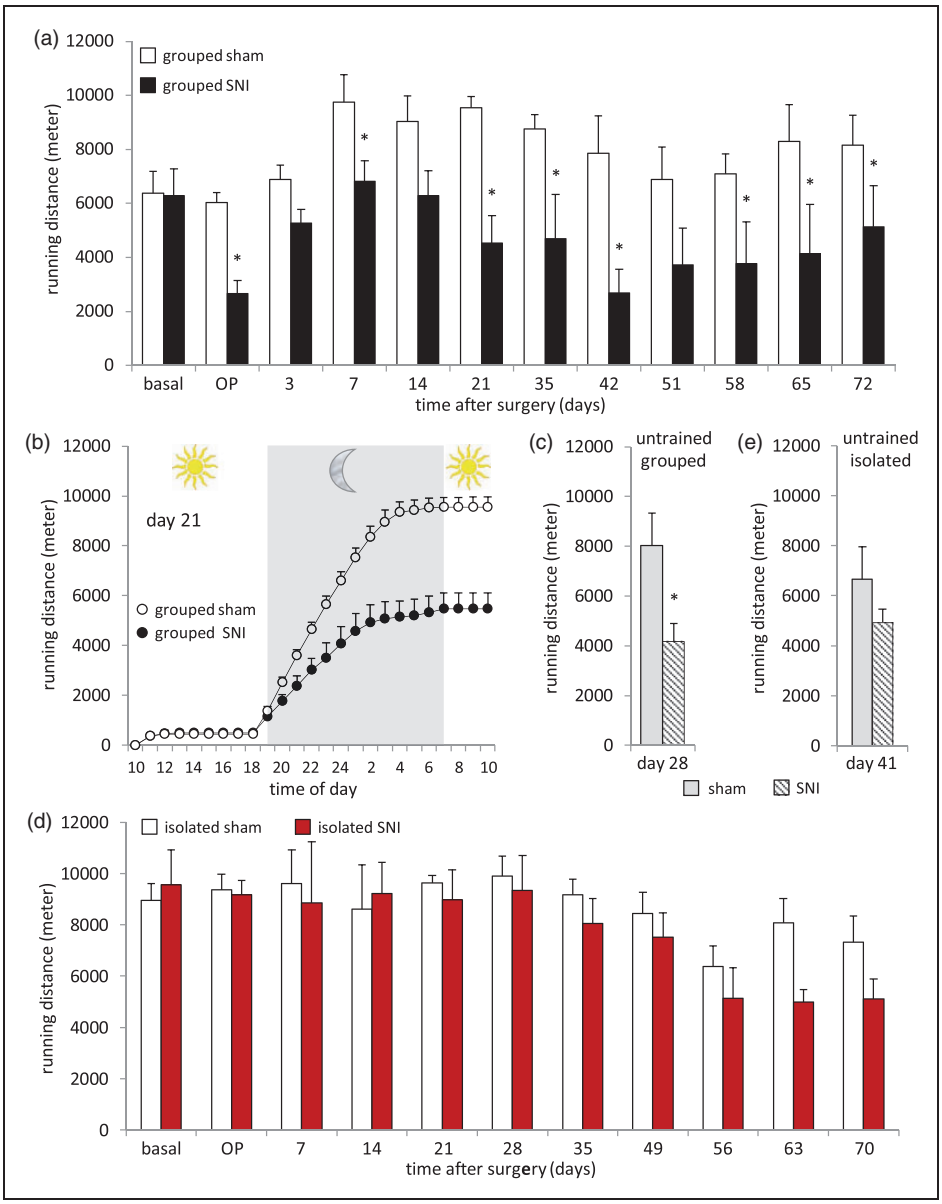

Figure 5. Consequence of neuropathic SNI on voluntary wheel running behavior. Mice were analyzed at each timepoint for their 24 h running behavior before (basal), at the day of surgery (OP) and up to 72 days following surgery. (a) Running distance (meter) of SNI and sham mice which were housed in groups except for the voluntary wheel running analysis. (b) Typical running profile of grouped mice at day 21. (c) Running distance (meter) of untrained SNI and sham mice which were housed in groups and only measured once at post-surgery day 28. (d) Running distance (meter) of SNI and sham mice which were housed individually. (e) Running distance (meter) of untrained SNI and sham mice which were housed individually and only measured once at post-surgery day 41. $N = 6$ mice/group, $p < 0.05$ indicated by * as compared to control group, † as compared to basal values within a group, two-way repeated measures ANOVA with post hoc Tukey test. All data points represent mean ± SEM.

difference was significant on the day of operation (OP), days 7, 14, and 21. Additionally, on the OP day, grouped SNI mice showed a significant reduction in locomotion (Figure 6(b)) and a significant increase in immobility time (Figure 6(c)). SNI mice showed a trend toward less locomotion (Figure 6(b)), less moving distance (Figure 6(d)), and more immobility (Figure 6(c)) than sham mice at most timepoints, but this was mostly not significant (Figure 6(c)). Other parameters such as grooming behavior, maximal and average speed were also not significantly different between SNI and sham animals and also not different before and after SNI surgery (Supplementary Table 1).

As with all other behavioral tests, we also analyzed isolated SNI and sham mice for their voluntary natural home cage behavior. We found a significant reduction in

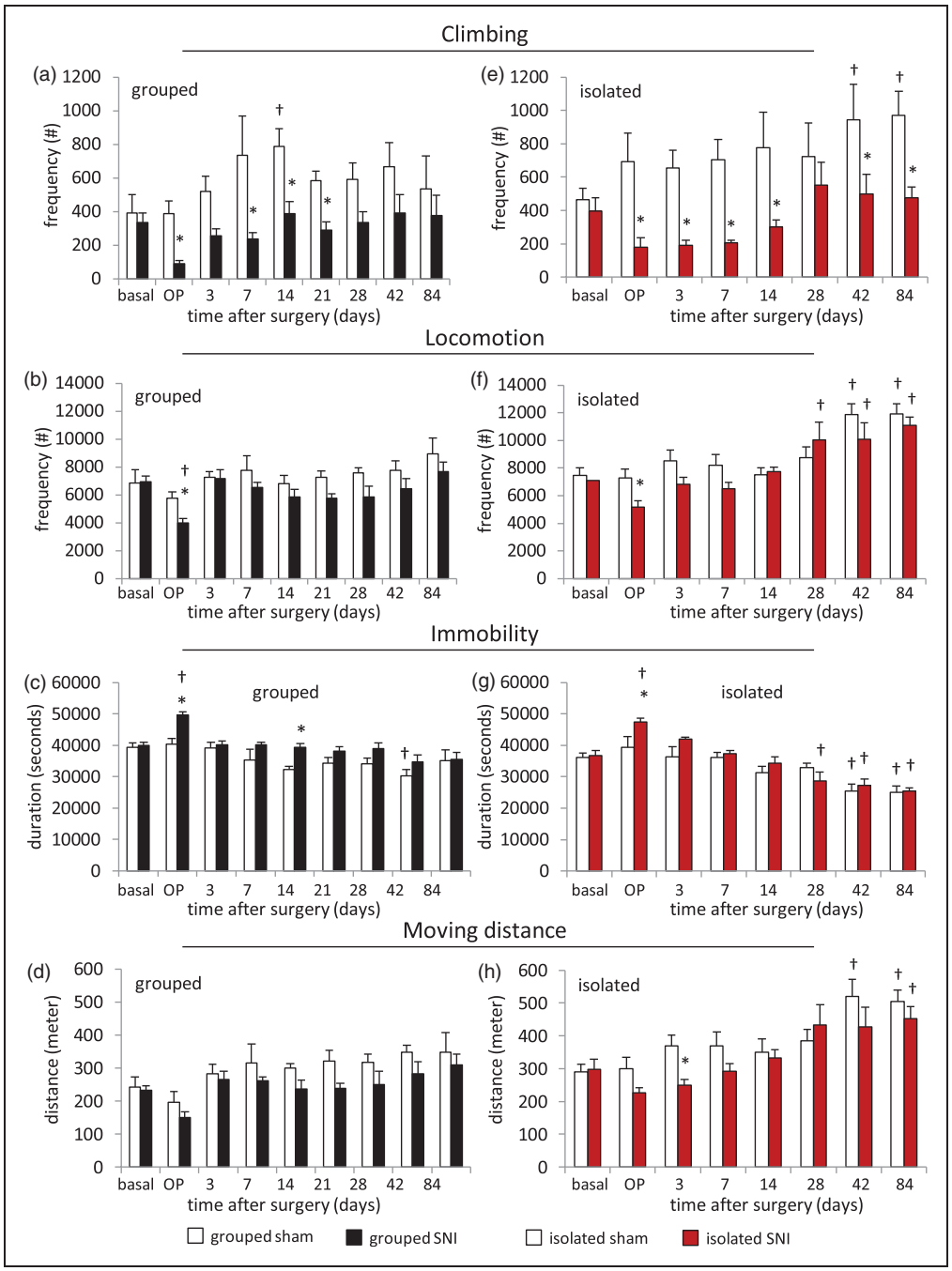

Figure 6. Home cage behavior of mice following neuropathic SNI surgery. Mice were analyzed at each timepoint for their 24 h running behavior before (basal), at the day of surgery (OP) and up to 84 days following surgery. All left column panels (a–d) show results from SNI and sham animals which were housed in groups (black bars) except for the day of home cage monitoring, all right column panels (e–h) show results from SNI and sham animals which were housed individually (red bars). (a, e) Climbing frequency counts per 24-h time period. (b, f) Locomotion frequency counts per 24 h (c, g) Total immobility time (seconds) per 24 h intervals and (d, h) total moving distance (meters) per 24 h. $N = 6$ mice/group, $p < 0.05$ indicated by * as compared to control group, † as compared to basal values within a group, two-way repeated measures ANOVA with post hoc Tukey test. All data points represent mean ± SEM.

climbing behavior in isolated SNI mice as compared to control animals at most measurement timepoints (Figure 6(e)). There was a significant reduction in locomotion (Figure 6(f)) and a significant increase in immobility (Figure 6(g)) in the isolated SNI mice on the OP day. These mice also moved less within the first week following surgery but this was only significantly different to control mice on day 3 post-surgery

(Figure 6(h)). Uniformly for most behavioral parameters, we found a continuous and significant increase over time (12 weeks) in SNI and sham animals in locomotion (Figure 6(f)), moving distance (Figure 6(h)) and average speed (Supplementary Table 1). Additionally, we measured a continuous significant decrease over time in immobility in both groups (Figure 6(g)).

Most of the home cage activity parameters we measured here were significantly altered at the day of surgery (OP) between SNI and control mice, irrespectively of their housing conditions. All SNI mice showed a reduced climbing behavior over the observation time, but hardly any other behavioral parameters were changed between SNI and sham mice. Isolated mice showed a classical age-dependent increase or decrease in most behavioral parameters over time. With increasing age, all isolated mice, SNI and sham animals, are less immobile and display more locomotion.

General observations based upon different housing conditions

Besides the obvious difference in voluntary wheel running activity, it seems that some behavioral outcomes are differently pronounced between isolated and grouped mice. Isolated mice seem to gain less weight than grouped mice, and it seems that grouped SNI mice gained more weight than isolated sham and SNI mice. It also appears that isolated mice have a higher sensitivity to von Frey hair stimulation during the first weeks. Behavioral experiments of grouped and isolated cohorts were not performed in parallel and statistical comparison of behavioral results between both housing conditions cannot be applied. Nevertheless, we will discuss some aspects later.

Discussion

Up to now, there is no behavioral study thoroughly investigating long-term behavioral changes, including circadian rhythm and the effect of the animal housing conditions. In this novel study, we thoroughly applied a portfolio of diverse behavioral paradigms in the neuropathic SNI model. Thus far, neither the voluntary wheel running behavior nor the DWB or CatWalk behavior has been investigated concurrently in SNI mice. Moreover, the effect of social isolation has only been studied with respect to stimulus-evoked response thresholds over one week in SNI rats.[18]

We focused mainly on voluntary behavioral changes and their timely correlation with results from the stimulus-evoked measures and found several aspects influencing the behavioral outcomes. The different behavioral tests, their usefulness to assess changes in the daily well-being and the effect of the housing condition in the SNI model are discussed below in detail. Potential significant differences between grouped and isolated mice are discussed in the general section at the end.

Stimulus-evoked behaviors and body weight

Irrespective of the housing conditions, SNI mice developed significant mechanical and cold hyperalgesia. Moreover, SNI mice of both groups gained significantly less weight than the corresponding sham mice.

Weight distribution and gait analysis

The analysis of gait changes in chronic pain models is controversially discussed. While some people argue that gait changes are not related to pain,[3] others assign them to pain.[20–23] Many factors, including differences in the rodent strain, the pain model, the investigation timepoint, and the measuring system might influence the results. There is evidence that gait changes in inflammatory models are based upon inflammatory pain,[23] whereas such changes reportedly result from motor system perturbations in neuropathic pain models.[23] Other studies showed that changes in gait parameters in neuropathic pain models do not only arise from motor deficits but neuropathic pain.[16,24] With respect to pharmacological interventions, there are opposite effects published. In some publications, the application of analgesics led to a reversal of static weight parameters as well as dynamic gait alterations[22] or only of dynamic but not static weight changes[25] whereas they did not improve deficits in other studies.[3] These differences may occur upon differences in the mouse strain or pain model.

The DWB system (Bioseb) and the CatWalk system (Noldus) have not previously been compared simultaneously as described in this study, using the SNI model. We revealed stable and reproducible results throughout the observation period of 12 weeks post-surgery. Irrespective of the housing conditions, we monitored very robust changes in static weight parameters in all SNI cohorts which were congruent with the duration of hyperalgesia and allodynia (both mechanical and cold). Interestingly, we observed a dynamic time-dependent increase in swing phase during the first two weeks following surgery. Changes in weight bearing can be based on spontaneous pain, increased contact sensitivity, or pain-avoidance behavior.[2] Here, the described increased swing phase in the initial phase of the SNI model were timely congruent with successful measures of spontaneous pain using the CPP test.[5,26] This increased limping behavior resulting in abnormal gait is likely due to spontaneous pain. Although it has been previously suggested that different mechanisms underlie spontaneous and stimulus-evoked pain,[3,27] based on our

current study, we would recommend the gait analysis as a meaningful tool to investigate pain-related behavioral changes in the SNI model.

Voluntary wheel running

Voluntary wheel running activity has been proposed as an observer independent measure for ongoing pain in inflammatory models,[2,28] but it has not been investigated in SNI mice so far. Taking into account the circadian rhythm, we performed 24 h day and night measurements to comprehensively analyze voluntary wheel running activity. We were very surprised to see that SNI animals did not show any significant reduced wheel running activity compared to their basal running activity. Interestingly, there was a significant difference in the wheel running activity between SNI and sham mice of the grouped cohort following surgery throughout the whole observation timecourse, whereas no difference between SNI and sham mice of the isolated cohort appeared. It is likely that the necessity of isolation of normally grouped mice into individual cages for the duration of the measurement induces stress. Recently, it has been shown that stress aggravates chronic pain in rodents (Lomazzo et al.[29]), and this might contribute to the difference in wheel running activity between SNI and sham mice. Additionally, wheel running has been described as a reward,[30] and recently Schwarz et al.[31] showed that SNI mice showed a reduced motivation in a reward task, based upon changes in synaptic transitions in the nucleus accumbens. It is likely that these mechanisms are accountable for the observed phenotype here. Social isolation stress, provoked by individual housing condition leads to anxiety- and depression-like phenotypes[32,33] and might explain why isolated sham mice do not show any increase in voluntary wheel running activity. Isolated SNI mice showed a trend toward decreased running behavior at late phases of SNI (day 63, day 70). It is possible that there are later effects of nerve injury leading to different pain sensation or altered motivation, thereby resulting in reduced wheel running activity. It has been shown that there are long-term functional reorganization in pain-related brain regions,[34,35] which might account for the observed behavioral differences. Furthermore, an impact of age on the interaction between chronic pain and affective/cognitive behavior has been demonstrated. Leite-Almeida et al.[36] showed that mid-aged animals (40 weeks) seem to be more susceptible to depression associated with chronic pain than young (8 weeks) or old animals (91 weeks). At 63 and 70 days following surgery, the mice in our study were considered mid-aged and this could also account for the observed reduced wheel running activity. From our data, it appears that voluntary wheel running may not be an effective measure to examine voluntary behavioral changes associated with neuropathy in mice. Importantly, the animal housing condition has a strong influence on the behavioral outcome on a 24 h longitudinal measurement, requiring separation of the animals.

Home cage monitoring

We used home cage monitoring to assess objective, observer independent behavioral parameters in a familiar cage environment. Voluntary movements have been investigated in SNI animals before, using activity cages[37] or the open field test.[18,36] These tests are limited because of the maximal measurement duration and the artificial environmental conditions. The Laboras System is a validated automated system to register diverse behavioral parameters over a prolonged time period.[38] We performed 24 h measurements at each timepoint, implicating circadian changes. At the day of surgery (OP), we observed significant differences between SNI and sham animals for most behavioral parameters. These behavioral alterations are likely caused by post-operative pain. All SNI mice showed significantly reduced climbing frequency over the whole observation time, irrespective of their housing condition. Apart from the climbing behavior, we rarely measured any differences between SNI and sham mice. We observed behavioral changes with increasing age in SNI or sham animals, but only under isolated conditions. These mice, sham or SNI, showed a classical age-dependent increase or decrease in behavioral parameters over time, which might be hampered in grouped mice by isolation stress during the recording time, as discussed above. With respect to unaffected moving distance, our results are consistent with previous reports.[4,18,37] Concerning the climbing analysis, Urban et al.[4] reported no impairment of climbing duration in SNI mice. It is herewith important to consider that Urban et al.[4] used a separate climbing paradigm test, whereas we analyzed climbing in the home cage. Noteworthy, we observed significant differences in climbing frequency but not climbing duration. The observed significant difference between sham and SNI mice might be elicited by pain from jumping up and down the grid lid or motor deficits, whereas the ability to hang under the grid is not affected. On the other hand, it is possible that sham mice climb more to escape from this cage.

General observations based upon different housing conditions

Beside the pronounced behavioral differences for voluntary wheel running activity between grouped SNI versus sham and isolated SNI versus sham mice, which has been

discussed above, there seem to be other differentially pronounced parameters. Isolated mice seem to gain less weight than grouped mice. Interestingly, it seems that grouped SNI mice gained more weight than isolated sham and SNI mice. This is consistent with a previous report in which grouped mice gained more weight following surgical intervention, than isolated mice.[12] It is possible that social isolation aggravates the difference in body weight between diseased and healthy animals and is an indication of impaired well-being. It also appears that isolated mice have a higher sensitivity to von Frey hair stimulation during the first weeks. Norman et al.[18] showed that social isolation decreased the paw withdrawal threshold at one week following surgery through a mechanism that might involve oxytocin.

Summary

Using a portfolio of stimulus-evoked and voluntary behavioral paradigms, we could show that the SNI model does not implicate clear alteration in voluntary wheel running or home cage activity. We observed time-dependent changes in DWB parameters in the gait analysis, and this highlights the importance of this behavioral paradigm in studying chronic pain elicited in the SNI model. Additionally, from the above described data, it appears that the housing condition has an influence on the animal behavior, especially voluntary wheel running.

Outlook

This study focused on detailed behavioral analysis of voluntary paradigms. It would be interesting in future studies to further include tests that examine the affective and emotional components of pain to generate a more complete picture of pain. It has been demanded that we need to develop better animal pain models mimicking human pain conditions.[4] To step along that direction, there is first an urgent need to expand the portfolio of behavioral measurements beyond the classical stimulus-evoked tests to have a comprehensive description of symptoms associated with our current existing pain models that are clinically relevant to pain patients.

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Appendix

Parameter	Housing condition: grouped	Time after SNI surgery (days)							
		OP	3	7	14	21	28	42	84
Climbing duration	Sham								
	SNI	*							
Climbing frequency	Sham				↑				
	SNI	*		*	*	*			
Locomotion duration	Sham								
	SNI								
Locomotion frequency	Sham								
	SNI	↓*							
Immobility duration	Sham							↓	
	SNI	↑*			*				
Immobility frequency	Sham								
	SNI	↑*							
Grooming duration	Sham								
	SNI								
Grooming frequency	Sham								
	SNI								
Maximal speed	Sham								
	SNI								
Average speed	Sham								
	SNI								
Distance travelled	Sham								
	SNI								

Parameter	Housing condition: isolated	Time after SNI surgery (days)						
		OP	3	7	14	28	42	84
Climbing duration	Sham							
	SNI							
Climbing frequency	Sham						↑	↑
	SNI	*	*	*	*		*	*
Locomotion duration	Sham						↑	↑
	SNI	*				↑	↑	↑
Locomotion frequency	Sham						↑	↑
	SNI	*				↑	↑	↑
Immobility duration	Sham						↓	↓
	SNI	↑*				↓	↓	↓
Immobility frequency	Sham							↓
	SNI	*					*	↓
Grooming duration	Sham							↑
	SNI						↓	*
Grooming frequency	Sham							↑
	SNI							
Maximal speed	Sham							
	SNI							
Average speed	Sham						↑	↑
	SNI		*				↑	↑
Distance travelled	Sham						↑	↑
	SNI		*				↑	↑