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Comparison of Rat Sensory Behavioral Tasks to detect Somatosensory Morbidity after Diffuse Brain-Injury

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Abstract

Brain injury disrupts neuronal circuits, impacting neurological function. Selective and sensitive behavioral tests are required to explore neurological dysfunction, recovery and potential therapy. Previously we reported that the Whisker Nuisance Task (WNT), where whiskers are manually stimulated in an open field, shows sensory sensitivity in diffuse brain-injured rats. To further explore this somatosensory morbidity, we evaluated three additional whisker-dependent tasks: Gap Cross Test, a novel Angle Entrance Task and Whisker Guided Exploration Task. Brain-injured (n=11) and sham (n=8) rats were tested before midline fluid percussion brain injury (moderate: 2.0 atm) and one and four weeks after injury. For the WNT, we confirmed that brain-injured rats develop significant sensory sensitivity to whisker stimulation over 28 days. In the Gap Cross Test, where rats cross progressively larger elevated gaps, we found that animals were inconsistent in crossable distance regardless of injury. In the Angle Entrance Task, where rats enter 30°, 40°, 50° or 80° corners, rats performed consistently regardless of injury. In the Whisker Guided Exploration Task, where rats voluntarily explore an oval circuit, we identified significant decreases in the number of rears and reversals and changes in the predominant location (injured rats spend more time in the inside of the turn compared to the outside) after injury and increased thigmotaxis after sham and brain-injury. Both the WNT and Whisker Guided Exploration Task show injury-induced somatosensory behavioral morbidity; however, the WNT remains more sensitive in detecting brain injury, possibly due to imposed whisker stimulation that elicits agitation similar to the human condition.

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Keywords

whisker nuisance task; gap cross test; whisker guided exploration task; angle entrance task; diffuse brain injury

Section 1 - Introduction

Traumatic brain injury (TBI) effects neuronal circuits throughout the brain, possibly impacting long-term cognitive [1] and behavioral [2] function. In fact, TBI has been associated with increased risk for developing neurodegenerative diseases [3] and/or psychiatric disorders [4]. It is therefore pivotal to investigate the neuroanatomical changes consequent to injury and develop therapeutic strategies to minimize long-term consequences. Animal models of diffuse brain injury enable investigation into the anatomical underpinnings of post-traumatic symptoms and then evaluate potential therapeutic interventions. Reliable behavioral outcome measures remain essential to evaluate post-injury recovery.

To this end, our laboratory has focused on somatosensory behaviors in the rat to investigate the causes and consequences of TBI. In particular, the somatosensory whisker circuit has been shown to be susceptible to anatomical and functional damage after diffuse brain injury [5–8]. This highly developed circuit in rats mediates tactile perception, particularly for object recognition [9, 10] and position [11]. Experimentally, investigators have evaluated tactile discrimination using a modified Sunderland jumping box [12] and depth perception using a visual cliff [13]. Tactile navigation through subterranean burrows requires sensory information from the whiskers, which play a similar role in above-ground thigmotaxis behavior [14]. In novel environments, rats tend to maintain whisker contact with walls or other vertical surfaces as a means to reduce natural anxiety associated with being in an open field environment [15]. Experimentally, shaving or plucking the whiskers disrupts behavior, as has been demonstrated by unilateral removal of the vibrissae effecting the predominant side of the face kept against the walls [16].

Recently, our group has reported late-onset somatosensory behavioral morbidity involving the facial whiskers as a result of experimental diffuse brain injury [17]. Repeated manual stimulation of the whiskers of brain-injured animals causes increased freezing, changes in body stance and changes in breathing [17]. In contrast, the same whisker stimulation of uninjured rats has little or no influence on affective behavior, with the animal ignoring or being soothed by the stimulus. Interestingly, these aberrant behavioral responses develop over time post-injury, rather than showing an immediate onset, suggesting underlying circuit reorganization [6]. Behavioral responses to whisker stimulation are quantified in the Whisker Nuisance Task and indicate injury-related disruption of somatosensory perception.

Here we compare the efficacy of the Whisker Nuisance Task in detecting behavioral deficits after diffuse brain injury to three other whisker-dependent behavioral tasks. The three other tasks require whisker somatosensation without the necessity for training, which would confound the performance results due to documented cognitive deficits in brain-injured rats [18, 19]. In the Gap Cross Test, rats use their whiskers to judge whether the distance of an elevated gap is crossable, as they are exposed to gaps of increasing distances. This task has previously been used to investigate the chronic effects of whisker removal during development [20] and the effect of cerebral peduncle lesions [21]. In the Angle Entrance Task, a novel task developed here, rats enter and retreat from corners of varying angles. This task differs from the corner test [22] in that it measures how far the rat moves into the corner rather than recording the direction animals turn away from a corner. In the Whisker Guided

Exploration Task, the location and whisker preference of the rat are quantified during an extended exploration period. This task has previously been used to investigate therapeutic efficacy of sensory stimulation after an ischemic stroke [23] and the effect of unilateral and bilateral lesions on thigmotaxic scanning [24]. The results demonstrate that the Whisker Nuisance Task remains most sensitive to detect brain injury-induced behavioral deficits, even with repeated testing.

Section 2 - Methods

Midline Fluid Percussion Brain Injury (2.1)

Adult male Sprague-Dawley rats (325–375 g) were subjected to midline fluid percussion injury (FPI) consistent with methods described previously [17, 25]. Briefly, the rats were anesthetized with 5% isoflurane in 100% oxygen and maintained at 2% via nose cone. During surgery, body temperature was maintained with a Deltaphase® isothermal heating pad (Braintree Scientific Inc., Braintree, MA). With the head held in position with a head holder assembly (Kopf Instruments, Tujunga, CA), a midline scalp incision exposed the skull. A 4.8-mm circular craniotomy was performed (centered on the sagittal suture midway between the bregma and the lambda), without disrupting the underlying dura or superior sagittal sinus. An injury hub was fabricated from the female portion of a Luer-Lock needle hub, which was cut, beveled, and scored to fit within the craniotomy. A skull screw was secured in a 1-mm hand-drilled hole into the right frontal bone. The injury hub was affixed over the craniotomy using cyanoacrylate gel and then methyl-methacrylate (Hygenic Corp., Akron, OH) was applied around the injury hub and screw and allowed to harden. The incision was sutured at the anterior and posterior edges and topical lidocaine ointment was applied. Then the animals were returned to a warmed holding cage and monitored until ambulatory.

For injury induction 60–90 min after surgery, the animals were re-anesthetized with 5% isoflurane for 5 minutes to standardize anesthesia levels at the time of injury. The dura was inspected through the injury-hub assembly, which was then filled with normal saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). As reflexive responses returned, an injury of moderate (1.9–2.0 atm) severity was administered by releasing the pendulum onto the fluid-filled cylinder. The animals were monitored for the presence of a forearm fencing response and the return of the righting reflex as indicators of injury severity [25]. Sham animals were connected to the FPI device, but the pendulum was not released. The injury-hub assembly was removed *en bloc*, integrity of the dura was observed, bleeding was controlled with Gelfoam (Pharmacia, Kalamazoo, MI), and the incision was stapled closed. Moderate brain-injured animals ($n = 12$) had righting reflex recovery times >6 min and a positive fencing response; sham brain-injured animals ($n = 8$) recovered within 15 sec without a fencing response. No animals died as a result of the surgical or injury procedures. After recovery of the righting reflex, the animals were placed in a warmed holding cage before being returned to the vivarium. Surgical recovery was monitored post-operatively for 3 days, for which no overt differences (e.g., weight, coat, movement, and grooming) were observed between the animal groups. Staples were removed 7–10 days post-injury. The experiments were conducted in accordance with National Institutes of Health and institutional guidelines concerning the care and use of laboratory animals. Adequate measures were taken to minimize pain or discomfort.

Behavioral Tasks (2.2)

Prior to injury (days 3–6 pre-injury), 1 week (days 6–9 post-injury) and 4 weeks (days 27–30 post-injury) after midline fluid percussion or sham injury, 20 animals underwent four

different behavior tasks: Whisker Nuisance Task, Gap Cross Test, Angle Entrance Task and Whisker Guided Exploration Task. Tasks were carried out on four consecutive days (one task/day), in a non-random order, starting midmorning each day and continuing until testing was completed on all animals. Tasks which required more than one trial (Gap Cross Test and Angle Entrance Task) had a half hour break between trials in which the animals were returned to the vivarium. All behaviors were video recorded on to digital video disk for analysis by one individual blinded to injury status of the animals.

WHISKER NUISANCE TASK (2.2.1)—Protocols were conducted as described previously [17]. A plastic test cage ($16.5 \times 38.1 \times 55.9$ cm) lined with an absorbent pad was used (Figure 1A).

Rats were acclimated to the test cage for 5 minutes prior to testing. Testing involved manually stimulating the whiskers of both mystacial pads with a wooden applicator stick for three periods of 5 minutes with periods (≤ 1 min) of non-stimulation between periods. Animals were tested individually and the absorbent pad and wooden applicator stick were replaced for each animal after cleaning the test cage. For each five minute period, observations were made regarding the predominant observed behavior. These observations were categorized as (1) movement, (2) stance and body position, (3) breathing quality, (4) whisker position, (5) whisking response, (6) evading stimulation, (7) response to stick presentation, and (8) grooming response. Normal behavior for each category consisted of those typically seen in uninjured animals during stimulation and was recorded as a score of zero. For example, an animal that was relaxed and looking upward during stimulation was given a score of 0 for stance and body position. Meaningful abnormal behaviors expressed in response to whisker stimulation were assigned scores of 1–2, depending on degree of expression. For example, an animal that cowered and showed a guarded position was given a score of 2 for stance and body position. The maximum whisker nuisance score was 16 (two points for each of eight categories). Higher scores indicate abnormal responses to the stimulation overall, in which the rat freezes, becomes agitated or is aggressive. Lower scores indicate normal responses, in which the rat is either soothed or indifferent to the stimulation. The scores for the three periods at each time point were averaged for individual animals and incorporated into a repeated measures Kruskal-Wallis non-parametric analysis of variance (ANOVA), because ordinal integer values are assigned to behavioral performance. Specific effects were evaluated by a Dunn's multiple comparison post-test with Bonferroni correction and $p < 0.05$ was considered statistically significant.

GAP CROSS TEST (2.2.2)—An apparatus consisting of a Plexiglas box ($75 \times 29 \times 16$ cm) 30 cm off the ground was used (Figure 1B). One half of this box was white and the other black, which served as a darkened goal box (length of 37 cm). The floor of the goal box slides out to create a gap of up to 150 mm. An awning was positioned on top of this section of the apparatus. The white side of the box had a movable wall to create an alley guiding the rat to the goal box. A checkerboard mat (55×25 cm made up of 5×5 cm black and white squares) was placed lengthwise underneath the gap for the purpose of depth perception while crossing.

Animals were trained to cross the gap one day prior to pre-injury testing. This consisted of each rat being placed in the apparatus with no gap, followed by gaps increasing in 5 mm increments up to 25 mm. Animals were encouraged to cross the gap with the use of steady light and intermittent white noise. The gap distance was increased only when the animals could comfortably cross the gap within 5 seconds. After each successful gap cross, the rats were allowed to rest in the goal box for 30 seconds. Testing was completed in a similar manner, but the gaps were increased in 10 mm increments. Testing was stopped after each

rat failed to cross a gap on three consecutive attempts. The test was conducted twice on each test day. The apparatus was cleaned with Rocal between animals.

The maximum distance (gap) crossed by each animal was averaged for both trials at each time point. Means were calculated within each injury group and incorporated into a two way repeated-measures parametric ANOVA (injury $\times$ time). Specific effects were evaluated by a Dunn's multiple comparison post-test with Bonferroni correction and $p < 0.05$ was considered statistically significant.

ANGLE ENTRANCE TASK (2.2.3)—An apparatus consisting of a wooden box ($50 \times 30 \times 30$ cm) with one of the shorter walls replaced by an adjustable angle was used. The adjustable angle was created by two additional walls, connected by a hinge (Figure 1C). As the two walls were adjusted, the angle ranged from 30° to 180° . The walls and floor of the apparatus were painted black and markings on the floor indicated both the angle and distance from the corner.

Animals were first acclimated to the apparatus for 5 minutes prior to testing. Acclimation consisted of each rat being placed in the apparatus for 5 minutes with the movable walls set at an angle of 50° . Each animal was then tested with the adjustable angle set at 30° , 50° , 80° and 40° (in that order) at each of the post-injury time points. These angles represent widths where the whiskers would make contact with both walls of the corner at 5 cm intervals from the vertex of the angle. The test was conducted three times on each of the test days. Testing consisted of placing the animal in the apparatus and recording its first movement going into and away from the corner on a digital video disk. The apparatus was wiped clean between animals.

The maximum distances entered into the vertex of each adjustable angle were measured from the video recordings as the distance between the start point (center of the angle entrance) to the final position of the rat's nose before it turned around. The results were calculated as the maximum distance travelled into the adjustable angle as a percentage of the corner length (distance from the 180° angle to the vertex of the adjustable angle). The percentages allowed comparisons between the testing angles, as the corner length changed with each angle. The results from the three trials per animals were averaged and incorporated into a repeated measures multiple analysis of variance (MANOVA; injury time point $\times$ angle). Specific effects were evaluated by Dunn's multiple comparison post-test with Bonferroni correction and $p < 0.05$ was considered statistically significant.

WHISKER GUIDED EXPLORATION TASK (2.2.4)—An apparatus consisting of a wooden oval track 25 cm wide and with outer dimensions of $100 \times 80 \times 30$ cm was used (Figure 1D). The whole apparatus was painted black with white geometric shapes placed at regular intervals along the walls. A start box was marked on one of the longer sides of the apparatus.

Prior to testing, animals were acclimated to the apparatus by individually placing the animals in the apparatus for 5 minutes. On test days, each rat was placed in the apparatus for 10 minutes and their movements recorded using EZ Video DV video tracking program (AccuScan Instruments, Inc., Columbus, Ohio). Data were collected in terms of the total distance moved, the amount of time moving and time spent in defined areas of the apparatus (e.g. straight lengths, inner corner, outer corner). The apparatus was wiped clean after each animal. After testing, the videos were viewed in order to quantify the frequency of rears and reversals and the amount of time spent scanning the walls with the whiskers. The data were incorporated into a two way repeated measures parametric ANOVA followed by followed

by a Dunn's multiple comparison post-test with Bonferroni correction and $p < 0.05$ was considered statistically significant.

Animal exclusion due to an abnormal brain structure (2.3)

One brain-injured animal was removed from the study after testing due to evidence of abnormal brain structure. In the right hemisphere, between bregma -3.50 mm and bregma -5.00 mm, the hippocampal region adjacent to the barrel fields of somatosensory cortex was enlarged. Further, this area had reduced definition of the hippocampal areas. These findings are inconsistent with all other brain-injured animals processed in our laboratory. Upon review of the behavioral data, this animal had significantly different behavioral results prior to and following brain injury compared to all other animals. The animal was excluded from the study based on an outlier analysis using a GraphPad QuickCalc Outlier Calculator which is based on a Grubbs' test. Brains from all other animals, whether uninjured or brain-injured, were unremarkable in terms of gross neuropathology, as would be expected from a diffuse brain injury in the absence of contusion or cavitation [8, 26, 27].

Section 3 - Results

Whisker Nuisance Task discriminates sham from brain-injured rats over four weeks post-injury (3.1)

A longitudinal evaluation of the uninjured sham animals ($n = 8$) tested prior to, one week and four weeks after a sham injury showed a continued absence of aberrant behaviors in response to 15 minutes of whisker stimulation, with no animal obtaining a whisker nuisance score higher than 3 at any time point (Fig. 2A), of a maximum 16 score. Alternatively, a longitudinal evaluation of moderate brain-injured animals ($n=11$), at the same time points (prior to, 1 week and 4 weeks after a diffuse brain injury), showed progressive increases in behavioral responses to the whisker stimulation, increasing from under 2 to between 4 and 11 (Fig. 2B).

The average whisker nuisance scores (Fig. 2C) indicated an absence of response in the sham animals with no significant differences between time points. Brain-injured animals had a significant increase in whisker nuisance score at one week and four weeks post-injury compared to pre-injury ($F(2,34) = 101.59$, $p < 0.0001$) with no significant difference between 1 week and 4 weeks post-injury. These scores were significant compared to sham animals ($F(1,34) = 74.986$, $p < 0.0001$).

Gap Cross Test does not discriminate between sham and brain-injured rats (3.2)

The average maximum gap crossed (Fig. 3) for sham animals ($n = 8$) remained consistent (between 6.6 and 7.6 cm) at all time points measured. For brain-injured animals ($n = 11$), the average of the maximum gap crossed did not change over time post-injury, with averages between 5.5 and 7.3 cm. Statistical tests showed no significant difference in gap cross performance after brain injury compared to both pre-injury ($F(2,34) = 2.21$, $p = 0.1240$) and sham ($F(1,34) = 0.00$, $p = 0.9875$).

Angle Entrance Task does not identify injury-induced differences (3.3)

Sham animals ($n = 8$) showed consistent performance on the Angle Entrance Task, with the average distance travelled being at least 88% of the corner length (Fig. 4A). Brain-injured animals performed similarly, with the average distance travelled being at least 90% of the corner length (Fig. 4B). No significant differences were detected in distance traveled for any of the angles ($F(3,56) = 2.45$, $p = 0.0650$) or at any time point ($F(2,80) = 1.19$, $p = 0.307$) for either group. No significant differences were found between uninjured and brain-injured animals ($F(1,3) = 0.43$, $p = 0.5110$).

Whisker Guided Exploration Task demonstrates post-injury deficits by location and behavior (3.4)

The average time spent moving (Fig. 5A) remained consistent for the sham group prior to, 1 week and 4 weeks after injury. Brain injury had no effect on the average time spent moving ($F(2,34) = 2.59$, $p = 0.0895$) between groups or over time post-injury.

After diffuse brain injury, rats significantly increased the total time spent scanning with the whiskers compared to pre-injury ($F(2,34) = 37.06$, $p < 0.0001$; Fig. 5B). For sham injured animals, this significant increase was also observed, leaving no significant differences between the two groups ($F(1,34) = 1.97$, $p = 0.1788$).

Prior to injury, animals spent an average of one third of the time in the straight portions of the apparatus (195.8 ± 12.7 seconds, $n = 19$) and the other two thirds within the corners (Fig. 5C). This ratio did not change significantly after injury ($F(2,34) = 1.15$, $p = 0.3279$). Of the time spent in the corners prior to injury, one third was spent in the outside of the corner (152.5 ± 15.5 seconds) and two thirds were spent on the inside of the corner (257.5 ± 7.6 seconds), accounting for two-fifths of the total amount of time within the apparatus (Fig. 5C). After brain injury, rats spent significantly more time in the inside of the corner ($F(2,34) = 12.03$, $p = 0.0001$), with significant increases at both 1 and 4 weeks after brain injury, which was not observed for the sham group. There were no overall significant differences between sham and brain-injured groups ($F(1,34) = 0.48$, $p = 0.4963$).

After diffuse brain injury, there was a significant reduction in the number of rears compared to pre-injury ($F(2,34) = 4.98$, $p = 0.0127$; Fig. 5D). The number of rears was significantly reduced at both 1 and 4 weeks for brain-injured animals compared to pre-injury. However, there were no significant differences between sham and brain-injured groups ($F(1,34) = 2.63$, $p = 0.1235$).

After diffuse brain injury, there was a significant decrease in the average number of reversals (change in direction) compared to pre-injury ($F(2,34) = 2.14$, $p < 0.0001$; Fig. 5D). Brain-injured animals had significant reductions in reversals at both 1 and 4 weeks after injury compared to pre-injury and between the 1 week and 4 week post-injury time points. The average number of reversals were statistically lower for brain-injured, compared to sham, animals ($F(1,34) = 5.14$, $p = 0.0367$).

Section 4 - Discussion

This study compared four behavioral tasks in terms of detecting a somatosensory morbidity after experimental diffuse brain injury. Functional morbidities were defined as delayed onset and enduring alterations in task performance. The behavioral tasks were selected specifically, because no prior training was necessary to successfully complete each task. The results showed that the Whisker Nuisance Task and the Whisker Guided Exploration Task could detect changes in somatosensory behavior after injury. However, only the Whisker Nuisance Task clearly and reliably differentiated uninjured from brain-injured animals, thereby detecting somatosensory morbidity after diffuse brain-injury. The involuntary stimulation used to elicit the whisker nuisance response may be necessary to elicit behavioral morbidity, which was absent from the other tasks evaluated in this study.

Whisker Nuisance Task detects somatosensory morbidity (4.1)

As expected, the Whisker Nuisance Task detected the delayed onset of sensory sensitivity, described previously [17]. The late onset of abnormal behavior indicated that the somatosensory morbidity is not a direct consequence of the initial injury, rather the outcome of injury-initiated processes such as inflammation and neuronal degeneration/regeneration

[28]. The behavioral responses to whisker stimulation are comparable to allodynia, a pain response to a non-painful stimulus. In this case, ant-inociception interventions may alleviate the morbidity.

In comparison to the other tasks, the Whisker Nuisance Task uniquely involves involuntary and unavoidable whisker stimulation as part of conducting the behavioral task. Methods to replicate or automate the whisker stimulation (e.g. air puffs) has not been successful. Currently, no similar task exists to deliver unavoidable whisker stimulation in the awake behaving animal. Importantly, this investigation has provided validation for the Whisker Nuisance Task between investigators, studies and animals, despite using subjective scoring criteria.

Gap Cross Test and Angle Entrance Task are ineffective in detecting post-injury deficits (4.2)

The Gap Cross Test has identified behavioral deficits in other models of neurodegenerative disease, but was ineffective in detecting alterations in behavioral performance after diffuse brain injury. Uninjured and brain-injured animals crossed gaps of up to 14 cm before and after brain injury. Lee and colleagues demonstrated an inability for rats to cross sizeable gaps when they were reared in the absence of select facial whiskers [20]. Unilateral lesions of the cerebral peduncle with ipsilateral whisker shaving [21] or unilateral (with ipsilateral whisker shaving) and bilateral infarctions to the barrel fields of the sensory cortex [29] resulted in significant deficits in the ability to cross a sizeable gap up to 46 days post-injury. These lesions result in partial destruction of the somatosensory circuit itself, which may impair performance in the Gap Cross Test. A midline fluid percussion injury does not target, but rather disrupts the somatosensory whisker circuit [5, 6], without compromising performance in the Gap Cross Test. Other reports have used food as an incentive for gap crossing, while we provided light and noise for motivation. Motivation may have influenced task performance, but without overt circuit destruction, performance is likely to be grossly unaffected.

The Angle Entrance Task was devised *de novo* for the present study. For diffuse brain-injured animals, the possibility remained for disrupted central sensory processing to impede optimal performance to walk completely into the vertex of a fixed angle corner. Brain-injured animals walked equally far in to each angled corner as uninjured animals, without hesitation, possibly due to the voluntary and passive whisker stimulation associated with task performance. While angle entrance remains an intriguing task, it was shown insensitive to detect an injury-induced deficit.

Similar tasks to the Angle Entrance Task include texture or gap width discrimination [9, 12, 21, 30]. Yet, performance in these tasks requires extensive training (upwards of three weeks with food or water deprivation). In the present study, injury-induced cognitive deficits training may have confounded training [18, 19] and training may not have improved performance given that no discrimination was evaluated.

Whisker Guided Exploration Task detects behavioral changes after diffuse brain injury (4.3)

The Whisker Guided Exploration Task detected injury-induced behavioral differences, albeit small. Brain-injured animals exhibited less time moving, fewer rearing and reversals events, increased thigmotactic scanning and more time within the inside turns of the apparatus. After an unilateral lesion or ischemic stroke, rats have increases contralateral whisker scanning in a Whisker Guided Exploration Task [23, 24], with no changes in locomotion or rearing [24]. These results likely emerge from contralateral effects to the unilateral injury. In

diffuse brain injury, deficits should manifest bilaterally, without differences between left and right whisker usage (data not shown).

Limitations to this task are the duration of observation and the lighting conditions. Animals were observed for 10 minutes, rather than 5 minutes [23, 31], which could reduce the environmental novelty and thereby exploration. No significant differences were found between animals when analyzed minute by minute (data not shown). Bright, overhead lights enabled the video recording, but may have provided visual cues or affected behavior. Other studies used dim [24] or red light illumination [31].

Whisker Nuisance Task is superior to Whisker Guided Exploration Task at detecting brain injury (4.4)

When comparing uninjured to brain-injured animals, the Whisker Nuisance Task could differentiate the groups. In the Whisker Nuisance Task, scores for brain-injured animals were separate from sham animals. In the Whisker Guided Exploration Task, however, some brain-injured rats performed within the variance of the uninjured animals, although the group averages were significantly different. In fact, for some of the behaviors (time spent moving and time spent on the inside turns), injured and sham animals were indistinguishable. We postulate that the efficacy of the Whisker Nuisance Task in delineating brain-injured animals may be due to the nature of the task. The Whisker Nuisance Task requires repetitive manual stimulation from an investigator, while the other tasks involved voluntary whisker stimulation. Voluntary use of the whiskers would be subject to variability in the animal population and modulated by the extent and duration tolerated by the brain injury.

To evaluate sensory perception, involuntary whisker stimulation has not been used before the Whisker Nuisance Task. Whisker stimulation is routinely used to examine brain activity [32] and for therapeutic rehabilitation of the facial nerve [33, 34]. It should be noted that no behavioral signs of stress are reported when the whiskers were stimulated for brain activation or rehabilitation. We have yet to explore the properties of involuntary whisker stimulation (e.g. stimulation pattern or intensity) which may be responsible for the adverse responses in brain-injured animals. Alternatively, tolerance to the stimulus could be quantified, either by measuring indicators of stress (e.g. heart rate) or by devising tolerance tests, where animals are given an incentive continue being stimulated. One such example measures orofacial pain sensitivity using a heated coil partially impeding access to a sweetened reward solution [35].

Limitations (4.5)

The longitudinal nature of the study involved repeated testing, which could have impacted the results. Animals acclimated to the testing environments may perform more poorly, because of reduced novelty and hence whisker related exploration. However, the majority of the results do not indicate issues with repeated testing. However, repeated behavioral testing precluded a meaningful histological evaluation. Diffuse brain injury occurs in the absence of overt histopathology and cavitation [8, 26, 36, 37], obviating the utility of gross histopathology. By one month post-injury, secondary injury cascades have subsided and the injured brain re-establishes homeostasis, precluding traditional neuropathological immunohistochemical staining. Lastly, repeated testing across several behavioral domains confounded meaningful interpretation of neuronal activation in response to behavioral performance, as has been published previously [6]. For instance, the task order could have imposed stress from the Whisker Nuisance Task on to performance in the Whisker Guided Exploration Task.

Conclusion (4.6)

Our objective was to compare four somatosensory behavioral tasks to detect diffuse brain injury-induced morbidity, of which only the Whisker Nuisance Task has the specificity to discriminate brain-injured from uninjured animals. Brain-injured people may not lose sensation [38], but rather experience elevated sensory sensitivity [39]. Additionally, neurological conditions, such as depression and irritability, commonly have a late-onset following brain injury [40, 41], which may be predicated by behavioral morbidity. Therefore, the Whisker Nuisance Task remains a reliable outcome measure for investigations into late onset morbidity following diffuse brain injury.

Research Highlights

- We compare behavior tasks to detect somatosensory morbidity after diffuse brain-injury
- The Whisker Nuisance Task shows a late-onset somatosensory morbidity after injury
- Neither the Gap Cross Test nor the Angle Entrance Task detect a sensory deficit
- The Whisker Guided Exploration Task identifies several behavioral changes
- Only the Whisker Nuisance Task clearly categorizes sham and injured animals

Abbreviations

WNT	Whisker Nuisance Task
TBI	Traumatic Brain Injury

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Figure 1.

Schematic illustrations of the apparatus used in the behavioral tasks. **(A)** Whisker Nuisance Task. The apparatus consists of a plastic bin lined with an absorbent pad. While exploring the environment, the rats' whiskers were stimulated using a wooden applicator stick for 15 min. During this time behavioral performance was scored using criteria described in the methods. **(B)** Gap Cross Test. The apparatus consists of a Plexiglas box, half of which is white and the other black, elevated on an aluminum frame. The black side has a movable floor to create a gap, while the white side has a movable wall to create an alley for the rats. A checkerboard mat of alternating black and white squares was placed underneath the apparatus to create depth perception. Rats were placed in the white side of the apparatus and

must move to the dark side, crossing gaps of increasing distance. Note that an opening in the apparatus has been provided to visualize the gap. **(C) Angle Entrance Task.** The apparatus consists of a black wooden box with one wall replaced by an adjustable angle. The adjustable angle was moved away from the box to create one of four angles (30°, 40°, 50° and 80°). Each rat was placed into the apparatus and observed when moving into and out of the adjustable corner. Note that an opening in the apparatus has been provided to visualize the inside of the apparatus. **(D) Whisker Guided Exploration.** The apparatus consists of a black oval track with white shapes spaced along the walls and a start box on one of the longer sides of the apparatus. Rats were placed in the start box and allowed 10 minutes for exploration. Their movements were video recorded and analyzed by a video tracking program. Schematic illustrations were designed in Google SketchUp (ver. 8.0.3117).

Figure 2.

Whisker Nuisance Task discriminates sham (A) from brain-injured (B) animals over four weeks post-injury. (A) Longitudinal evaluation of sham animals indicates consistent absence of behavioral responses to whisker stimulation (each line represents an individual animal). (B) Longitudinal evaluation of brain-injured animals indicates progressive development of behavioral responses to whisker stimulation. (C) Average whisker nuisance scores for each group at each time point demonstrate an absence of responses in sham animals. Brain-injured animals showed significant increases in the expression of whisker nuisance scores at 1 week and 4 weeks post-injury compared to sham. *, $p < 0.05$ compared to pre-injury.

Figure 3.

Gap Cross Test does not discriminate between sham and brain-injured animals. Averages of maximum gap crossed for both groups show no significant differences between the groups or longitudinally, with large standard errors demonstrating the inconsistency of animals to cross larger gaps.

Figure 4.

Angle Entrance Task does not discriminate between sham (**A**) and brain-injured (**B**) animals at any of the tested angles. (**A**) Average distances travelled into the corner (as a percentage of total corner length) by the sham animals for each of the angles tested. (**B**) Average distances travelled into the corner (as a percentage of total corner length) by the injured animals for each of the angles tested.

Figure 5.

Whisker Guided Exploration Task discriminates sham from brain-injured animals in several ways. (A) Average time spent moving for both groups at each time point shows consistent amount of movement in sham animals. Brain-injured animals show a significant decrease in time spent moving at 4 weeks compared to pre-injury. *, $p < 0.05$ compared to pre-injury (B) Average time spent scanning with left and right whiskers at each time point for both groups shows a significant increase in total scanning for both groups after craniotomy. Both groups also show a consistent significant increase in left whisker scanning and a less consistent significant increase in right whisker scanning compared to pre-injury. (C) Average time spent in each of three areas of the apparatus: straights, outside turns and inside turns for both groups' at each time point shows a change in predominant location after injury. Rats spent

similar amounts of time in the straights (dark grey) and turns (both lighter greys) at all time points (keeping in a 2:1 ratio). Within the turns, injured rats spent significantly more time on the inside (medium grey) and less on the outside (lighter grey) after injury compared to pre-injury. **(D)** Average number of rears for both groups at each time point shows that sham animals are consistent in the amount of rearing. Brain-injured rats reared significantly fewer times after injury compared to pre-injury. **(E)** Average number of reversals for both groups at each time point shows consistence in the sham animals. Injured animals progressively show a significant decrease in the number of reversals at 1 week compared to pre-injury and at 4 weeks compared to pre-injury and 1 week after injury. †, $p < 0.05$ compared to 1 week brain-injured.