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Neurobehavioral functional deficits following closed head injury in the neonatal pig

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Abstract

Neurobehavioral deficits in higher cortical systems have not been described previously in a large animal model of diffuse brain injury. Anesthetized 3–5 day old piglets were subjected to either mild (142 rad/s) or moderate (188 rad/s) rapid non-impact axial rotations of the head. Multiple domains of cortical function were evaluated 5 times during the 12 day post-injury period using tests of neurobehavioral function devised for piglets. There were no observed differences in neurobehavioral outcomes between mild injury pigs ($N=8$) and instrumented shams ($N=4$). Moderately injured piglets ($N=7$) had significantly lower interest in exploring their environment and had higher failure rates in visual-based problem solving compared to instrumented shams ($N=5$) on days 1 and 4 after injury. Neurobehavioral functional deficits correlated with neuropathologic damage in the neonatal pigs after inertial head injury. Injured axons detected by immunohistochemistry (β-APP) were absent in mild injury and sham piglets, but were observed in moderately injured piglet brains. In summary, we have developed a quantitative battery of neurobehavioral functional assessments for large animals that correlate with neuropathologic axonal damage and may have wide applications in the fields of cardiac resuscitation, stroke, and hypoxic–ischemic brain injury.

Keywords: Head injury; Neurobehavioral assessment; Axonal injury

Introduction

Traumatic brain injury (TBI) is the most common cause of death in childhood. Brain injuries resulting in hospitalization or death occur in at least 150,000 children per year, at a rate of over 200 per 100,000 children (Fisher, 1997). Widespread axonal injury is one of most commonly observed pathologies in pediatric brain injury. The long term morbidity and mortality associated with traumatic brain injury has in part been attributed to axonal injury (Babikian et al., 2005). Outcomes of children with diffuse axonal injury can vary from little or no cognitive deficits to life-long cognitive, behavioral, and motor disabilities.

Acute injury to the developing brain has been the focus of research involving numerous animal models(Adelson et al., 2001; Prins and Hovda, 2003; Prins et al., 1996; Raghupathi et al., 2004). A critical link in successful translational research is the development of animal models of brain injury that can provide a platform to correlate meaningful functional outcome measures with well-characterized histological and molecular substrates. Increasingly, piglets have been used to model acute damage to the developing brain resulting from asphyxic circulatory arrest (Brambrink et al., 1999), cardiopulmonary bypass (Kurth et al., 1999; Schultz

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et al., 2004), and traumatic brain injury (Armstead, 2002; Raghupathi and Margulies, 2002; Raghupathi et al., 2004). Moreover, the histopathology and acute cerebral physiologic responses seen in piglets in these injury paradigms closely resemble that seen in human infants (Hagberg et al., 2002; Martin et al., 1997).

Previous survival studies of acute brain injury in piglets have incorporated relatively simplistic neurological functional outcome scoring measures. Typically a series of 4 or 5 categories of neurological function are used (e.g. mental status, cranial nerves, sensorimotor function, feeding), each of which is assigned a graded score of 1–4 representing the degree of abnormality, yielding a final score ranging from a possible total of 9 for the simplest grading scheme (Midulla et al., 1994), to 150 for a more complicated neurological deficit score (Agnew et al., 2003). These grading systems have shown only limited correlation with histopathologic abnormalities (Priestley et al., 2001), but their sensitivity for assessment of complex behavior or cortical functions is poor, and none have been well validated with long-term survival studies.

In contrast, there are numerous rodent models of acute and chronic degenerative diseases in the young and mature brain, in which standardized and sensitive behavioral outcome measures have been successfully correlated with regional neuropathology due in large part, to an extensive literature in rodent behavioral neuroscience (Almli et al., 2000; Bona et al., 1997; Dixon et al., 2003; Young et al., 1986). Research in swine behavior is historically limited to veterinary science communities, with a focus on neuro-development rather than neuropathology. Results indicate that even newborn piglets are readily amenable to quantitative behavioral assessment, displaying sophisticated social learning skills, highly developed sensory discrimination capabilities for auditory, olfactory, visual and tactile modalities, and the ability to be trained in maze-learning tasks (De Jong et al., 2000; Hammell et al., 1975; Puppe et al., 1999).

Previously we presented our acute (6 h survival) non-impact neonatal (3–5 day old) piglet model of closed head injury, with a markedly lower density of injured axons after a single rapid axial rotation of 160–190 rad/s (moderate) compared to a single severe axial rotation of 240–260 rad/s (Raghupathi et al., 2004). Furthermore, we observed an increase in distribution of injured axons following two consecutive mild axial accelerations of 130–150 rad/s spaced 15 min apart compared to single accelerations at 6 h survival (Raghupathi et al., 2004). Our goal was to develop reliable quantitative functional neurobehavioral assessments for brain injury in piglets. In this communication, we present our double mild (≈140 rad/s) and single moderate (≈190 rad/s) velocity rotations in a survival piglet model and a battery of novel behavior, cognitive, and motor tests for piglets with a sensitivity to detect differences in injury level and correlating with differences in histopathology. Functional data from piglets experiencing mild and moderate non-impact rapid head rotations were compared to instrumented shams over a 12 day period, revealing transient and persistent deficits after moderate but not mild brain injury that correlated with histopathology.

Materials and methods

Acclimation

All protocols were approved by the Institute of Animal Care and Use Committee of the University of Pennsylvania. Neonatal (3–5 day old) farm piglets were studied in 5 littermate groups of 4 or 5 pigs (3–4 females, 1 male). All littermates were acquired from the University of Pennsylvania swine facility to ensure uniformity in birthing, handling, and physical and social environment prior to arrival. All littermate siblings were housed together throughout the 2 week period. Two or three female pigs underwent head rotational acceleration in the axial plane on day 1, and 1 female pig was designated an instrumented sham. The male sibling was utilized only for assessing socialization and was housed with others in the group. Three days prior to injury, piglets were numbered using color coded nail polish on their backs and front hooves to facilitate identification. While piglets were placed together in the empty test space (4′×8′) with a bowl of milk replacer (Littermilk, Land O Lakes, Arden Hills, MN) in the center, they were allowed to explore the space to become acclimated to their environment and the research staff. One and two days before injury, the first day's protocol was repeated with addition of training individual piglets to ambulate a 9″ wide balance beam to a bowl containing 1 ml of milk replacer. The task was repeated until proficiency at the task was displayed. While one piglet was being acclimated to the beam, the remaining litter mates were placed in separate individual pet carriers.

Brain injury and physiologic measurements

On the day of injury (day 0), all female piglets were anesthesized with 4% isoflurane via a snout mask. When a pinch reflex was absent, the piglets were orally intubated with a 3.0-mm endotracheal tube. End tidal CO_2 (Vet/Cap model 2050081) was utilized to confirm endotracheal tube placement and was continually monitored until extubation. Core body temperature (rectal probe), arterial oxygen saturation (oxymeter probe), and mean arterial pressure (pressure cuff on hindlimb) were monitored continuously (MDE Escort II). A peripheral intravenous catheter was inserted into the forelimb and animals were ventilated (Hallowell AWS, 1–2% isoflurane) until return of spontaneous respirations. Animals received 10 ml/kg of normal saline and buprenorphine 0.02 mg/kg IV prior to injury. As a control, uninjured sham piglets were anesthesized, intubated, a peripheral intravenous line was placed, and the bite plate inserted in the mouth. Shams were then taken off isoflurane and removed from the bite plate to simulate the timing of the injury protocol.

For the moderately injured piglets (N=10), 5 littermate groups experienced rapid axial head rotations with the HYGE pneumatic actuator as described previously (Raghupathi and Margulies, 2002; Raghupathi, 2004). The animals' heads were secured to a padded bite plate. Piglets were taken off isoflurane and 1–3 min later received a single rapid non-impact rotational load. Immediately following the rotational load, the snout of the

animal was removed from the bite plate. Two piglets were excluded because they sustained hard palate fractures from the rapid rotation and were sacrificed on day of injury. One piglet was sacrificed on post-injury day 1 due to inability to feed. The seven remaining moderately injured piglets (INJ) in the study underwent average axial angular velocities and accelerations of 188±7 rad/s and 62,896± 10,100 rad/s^2 respectively. No piglets required mechanical ventilation secondary to apnea or respiratory depression following injury. INJ piglets (N=7) had a significantly longer mean latency for return of pinch reflex of approximately 6.8± 2.3 min compared to 2.8±1.0 min for SHAM (N=5).

A second group of injured piglets (MILD), received two consecutive rapid axial head rotations of lower velocity less than 10 min apart. Four groups of 3 littermate or non- littermate 3–5 day old piglets were studied (N=8 MILD, N=4 MSHAM). Eight injured piglets (MILD) received two consecutive axial rotational accelerations 3.1±0.5 min apart. Average axial angular velocities and accelerations were 142±2 rad/s and 34,115±2800 rad/s^2, respectively. MILD piglets had a mean latency for return of pinch reflex of 2.4±0.6 min.

Animals were placed on heating blankets to maintain core body temperature between 36°C and 38°C. Oxygen saturation was maintained at 95%–100% at all times with supplemental oxygen and ventilatory support provided as needed. Return of pinch reflex was recorded in all piglets. Upon return of respiratory effort and airway protective reflexes, the animals were extubated and intravenous catheter removed. The animals were returned to the animal care unit after the following criteria were met: vocalization (without squealing), steady ambulation, no aggression or avoidance behavior, no piloerection, and presence of proper feeding/drinking. We evaluated a broad range of neurobehavioral functions in SHAM and INJ piglets on post-injury days 1, 4, 6, 8, and 11. MILD and MSHAM piglets underwent only open field testing during the 12 day post-injury period. Average weight gain over the 2 week study period was approximately 2 kg for both SHAM and INJ groups.

Behavioral and functional tests

All procedures involved operant conditioning techniques with milk replacer as a positive reward, and no aversive conditioning was performed. During functional testing of one piglet, the remaining four piglets were placed in individual plastic pet carriers in a separate room. Each morning of testing, the animals were fasted for 2 h and weighed to monitor growth. Behavioral and functional assessments occurred in the same order each day at approximately the same time, but piglets were tested in a randomly assigned order for the day. All testing equipment and the test space were washed with Quatricide disinfectant after each pig had completed a test. All assessments were recorded via camera and saved to DVD for subsequent scoring by a blinded evaluator. Comparisons were made between sham (SHAM) and moderately injured (INJ) piglets, and between sham (MSHAM) and mildly injured (MILD) piglets. ANOVA and Tukey tests for multiple comparisons were used to evaluate alterations in behavior between trials, test days, and groups (with significance defined as $p \leq 0.05$). All data are presented as mean±standard error.

Open field testing

Each animal was placed in the 4′×8′ test space with various objects placed in predetermined locations (Fig. 1) for 10 min (designated as the first segment). The presence of both static and interactive objects renders this test more complex, and provides a measure of exploratory interest which involves a high level of sensory processing, which likely depends on an intact prefrontal cortical–striatal–pallidal circuit and concomitant cerebellar function (Pierce and Courchesne, 2001). To assess general activity and exploration, the test space was divided into nine distinct zones (Fig. 1). The number of zone boundary lines crossed (dashed lines, Fig. 1) and the number of different zones entered (≤9) in 1 min intervals during the 10 min testing period was recorded. In addition, specific exploratory behaviors were tracked: sniffing floor, walls, or toys; movement including running, walking, standing still, laying down and sleeping; moving of toys; moving foam object; and attempts to escape the test space. Each behavior was scored as present/absent for every minute-long interval in the 10 min test period, and expressed as percent intervals that the behavior was observed.

For INJ and SHAM groups, after the first 10 min open field test segment, the male littermate was placed in the center of the test space for a 10 min interval to assess the reaction of the test pig to a conspecific stimulus. Specific social behaviors between the 2 piglets were tracked: sniffing, head butting, mounting, fighting, and social sleeping. For the first three behaviors, we noted whether the test piglet was initiating or receiving the behavior. As in the exploratory behavior segment, each social behavior was scored as present/absent for every minute-long interval and expressed as percent intervals observed. In addition, the inter-pig distance (snout to snout) was measured every 15 s during the 10 min social interaction assessment.

Following the 10 min social interaction assessment, the male littermate was removed and a second 10 min open field test segment began, for the INJ and SHAM groups. Behavior,

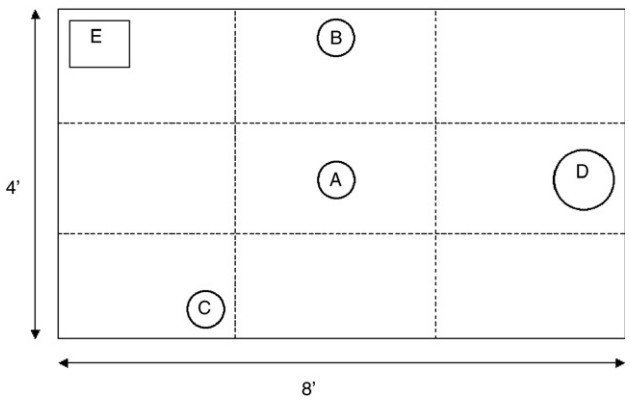

Fig. 1. Open field test space. (A) Piglet starting position. (B) Small blue ball with rattle, (C) small yellow ball with rattle. Both (B) and (C) function as color discrimination stimuli and movement with noise. (D) Large blue ball movement without noise. (E) Foam padding, tactile stimulus.

number of grid lines crossed, and zones entered were again recorded in the same manner as described above for the first 10 min test. After the second 10 min open field test segment, the piglet was removed from the test space and given milk replacer.

Glass barrier task

The glass barrier task assessed problem-solving skills based on visual cues. The test space was sectioned off into a smaller triangular region with the food dish centered along one side approximately 3–5 inches from the pen wall. The test piglet was placed in the opposite corner, and oriented facing the food dish. After being allowed to walk to the dish and eat once, a clear plastic divider was placed in front of the dish and the piglet was brought back to the starting position. After release, the piglet was given 20 s to find its way around the transparent divider to the food dish. If the piglet succeeded, it was allowed to finish the food before starting another trial. If the piglet did not find the food dish within 20 s, the trial was considered a failure and the piglet was brought back to the starting point (without being given a food reward) to begin another trial. Each piglet participated in 5 consecutive trials on each testing day. Time to food reward, errors (e.g. nudges at the glass, walking away from the glass and dish), and failures to reach the food within 20 s were recorded for the five consecutive trials.

Food cover task

The set up of the pen and piglet positioning for the glass barrier were used again for the food cover task, to test *non-visual* object-discrimination learning. In this assessment, the piglet must learn that an opaque cover consistently hides a food reward, and that by lifting the cover she gains access to the food. After the piglet was again allowed to eat briefly from the food dish and returned to the starting position, the food dish was covered with an opaque sheet of plastic. The piglet was released and given 20 s to lift the cover off the dish. If the piglet gained access to the food, the cover was completely removed and the piglet was allowed to continue eating. Once the piglet was finished eating, the next trial was begun. If the piglet did not succeed in lifting the cover off the food dish within 20 s, the trial was considered a failure, and the next trial was begun. Each piglet participated in 6 consecutive trials with the last three trials using medical adhesive tape in 2 places to secure the food cover. Time to food, errors (e.g. failed attempts to removed cover, walking away from food dish), and failures were recorded for the 6 consecutive trials each day.

Balance beam

Motor performance was assessed with balance beam walking, utilizing both a wide (9″) and a narrow (5″) balance beam with a food reward at the end. The piglet was placed in the starting location at one end of the 48″ long beam and given 20 s to cross the beam to the food reward at the other end. Foot slips and falls from the plastic non-slip beam were considered errors and animals were given the opportunity to regain their balance. Failure to reach the food reward within 20 s and an inability to recover from an error to continue towards the reward were both considered failures. Each piglet participated in 6 consecutive trials per beam on each testing day. Time to complete the task, number of failures, and number of errors were recorded.

Histology and immunohistochemistry

The animals were euthanized 12 days after injury to evaluate regional patterns of traumatic axonal injury. Animals were anesthetized with 4% isoflurane. Once pinch reflex was absent, animals were given a lethal dose of intravenous sodium pentobarbital (150 mg/kg). Animals were transcardially perfused through a midline sternotomy with 1 l heparinized saline (5000 units/l) and brains were fixed *in situ* by perfusion with a buffered solution of 10% formalin (3.5 L Sigma Chemical Co., St. Louis, MO). Brains were removed from the cranial vault and post-fixed overnight at 4°C.

Formalin fixed brains were examined for gross pathology by a blinded reviewer. Macroscopic examination included documentation of focal pathology including presence of subdural and subarachnoid hemorrhages and surface contusions. Sixteen serial coronal sections of cerebrum, brain stem, and high cervical spinal cord were examined every 3 mm for tissue tears, intracerebral hemorrhage, and subarachnoid hemorrhage. Following routine processing, tissue was embedded in paraffin wax, and cut in 6 μm thick sections for microscopic examination. Sections were stained with Hematoxylin and Eosin (H&E), or with the immunohistochemical markers for axonal injury beta-amyloid precursor protein (β-APP) and 68 kDa neurofilament protein (NF-68) and counterstained with Meyer's hematoxylin. Slides were examined by a blinded reviewer at scanning power (5–10× magnification). Specific fields were examined at 20–40× magnification. Locations of axonal injury, subarachnoid and parenchymal hemorrhage, and cell death were noted on anatomic schematics of the coronal sections. Apoptotic bodies were used as indicators of DNA fragmentation and nuclear condensation, and were considered to be indicative of cell death. These were assessed in a semi-quantitative fashion by the reviewer.

Statistical analysis

The percent of epochs each behavior was observed in the open field testing was initially analyzed by three-way analysis of variance (ANOVA) to evaluate higher level interaction of group (injured vs. sham), day (post-injury day 1, 4, 6, 8, or 11), and segment (1st vs. 2nd). The number of zones entered and lines crossed in the open field was evaluated in the same manner. The three-way ANOVA revealed that day was not a significant determinant of outcome nor exerted a significant interaction with group or segment. Therefore open field data from post-injury days 1, 4, 6, 8, and 11 were combined for a complete *post hoc* analysis. Consolidated data were examined using a Tukey–Kramer with significance defined at $p<0.05$. Similarly, glass barrier, food cover, and balance beam were initially analyzed by three-way ANOVA to evaluate higher level interaction of group,

day, and trial on latency and errors. A two-way ANOVA to evaluate higher level interaction of group and day on number of failures/day was also performed. Again Tukey–Kramer tests were utilized with significance defined at $p<0.05$.

Results

Open field testing

During open field testing, no significant differences were found in frequency of the observed behaviors between MILD and MSHAM. At the start (0–3 min) of the first solo exploration segment, both INJ and SHAM animals explored the test space avidly (entering 8.1 of 9 zones and crossing 22.3 grid lines) but decreased their exploration significantly by the end (7–10 min) of the first 10 min segment (to 6.1 zones entered and 14.6 grid crossings). While INJ explored the test space during the 10 min period on average less than SHAM (6.5 vs. 7.1 zones entered and 15.9 vs. 17.8 lines crossed), these differences did not reach statistical significance (Power=35%).

For several behaviors (walking, running, standing still, moving foam, and escaping), there were no significant differences observed between INJ and SHAM, or between first and second open field segments (Table 1). When no significance between INJ and SHAM was identified by ANOVA, INJ and SHAM data were combined to examine temporal variations common to all piglets. Because sleeping and laying down were rare behaviors in both INJ and SHAM, observed less than 10% of the epochs, they were not included in further analysis. ANOVA did not reveal significant influence of test day on any of the open field behaviors analyzed. In most non-rare open field behaviors, INJ and SHAM piglets were significantly less active and curious during the second solo segment compared to the first. For example, observations of sniffing toys and moving toys did not vary between INJ and SHAM, but decreased significantly in all piglets between the first and second solo segment, from sniffing toys in 48.6% of the minute-long intervals in the 1st test segment to 27.1% in the 2nd segment and from moving toys in 37.4% to 17.1%, respectively. Similarly, observations of sniffing the floor decreased in all piglets from 80.2% of minute-long intervals in the 1st to 62.1% in the 2nd segment. However, we found significantly greater decreases in floor sniffing between 1st and 2nd segments in INJ (82.0% in 1st segment compared to 58.3% in 2nd segment) than SHAM (77.7% in 1st segment compared to 67.2% in 2nd segment) (Fig. 2). Finally, INJ spent significantly less time sniffing the walls (45.9%) compared to SHAM (63.3%) in the 1st segment, but there was no difference in this activity between groups in the second segment (Fig. 3). In general, we conclude over the 11 day test period, that INJ piglets had significantly lower interest in exploring their test space by sniffing than SHAM.

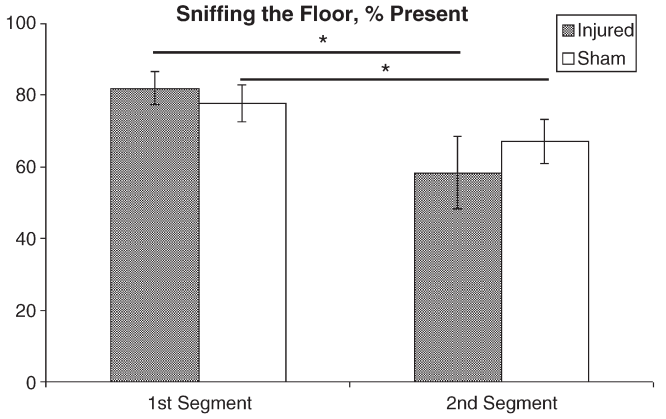

Fig. 2. Open field testing: percent of minute-long intervals that sniffing the floor behavior was observed comparing 1st and 2nd segment and INJ and SHAM. *Denotes statistically significant differences ($p<0.05$).

During the 10 min interval of social interaction with Pig X, the uninjured con-stimulus, there were no significant differences in proximity to Pig X when comparing INJ to SHAM or comparing test days. However, all piglets had significantly closer proximity with Pig X early (0–3 min) in the social interaction segment than late (7–10 min), 3.8±0.8 cm and 9.7±1.8 cm, respectively (Fig. 4). Mounting and social sleeping were rare behaviors in this test segment and were observed in less than 5% of intervals and were not included in further analysis. The only significant difference between SHAM and INJ piglets' observed behaviors with Pig X, was head butting. Over the 10 min interval, INJ piglets were observed to receive head butting from Pig X less than SHAM (18% and 32% of minute-long intervals, respectively). Interestingly, there was no statistical difference between INJ and SHAM for initiating head butting (40% and

Table 1
Percentage of minute-long intervals a behavior was observed

Behavior	INJ (%) across all segments and days	SHAM (%) across all segments and days	1st segment (%) across all groups and days	2nd segment (%) across all groups and days
Sniffing the floor	70.2	72.5	80.2*	62.1*
Sniffing the walls	44.2°	55.1°	52.3	45.1
Sniffing the toys	40.9	33.7	48.6*	27.1*
Running	22.9	24.2	24.8	22.1
Walking	96.8	94.4	96.6	95.0
Standing still	93.4	84.5	89.3	90.8
Laying down	2.2	1.9	1.7	2.4
Moving toys	26.2	26.7	37.4*	17.1*
Escaping	22.8	16.5	18.1	21.8
Sleeping	4.9	6.4	4.4	6.7

°Denotes significant difference ($p<0.05$) between INJ and SHAM. *Denotes significant difference ($p<0.05$) between 1st and 2nd test segment.

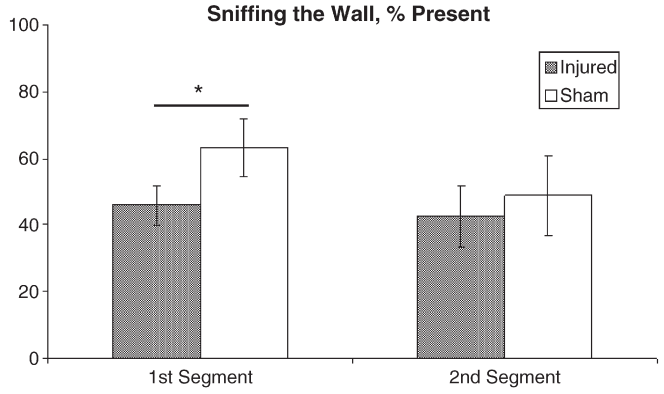

Fig. 3. Open field testing: percent of minute-long intervals that sniffing the wall behavior was observed comparing 1st and 2nd segment and INJ and SHAM. *Denotes statistically significant differences ($p<0.05$).

38% of minute-long epochs respectively). We conclude that INJ and SHAM piglets exhibited similar social behavior but received different attention from the con-stimulus.

Glass barrier task

Latencies for reaching the visible food reward did not vary significantly over the test days, trials, or groups and averaged 3.7±0.4 s across successful trials. SHAM and INJ groups committed the same number of errors per successful trial across all days (0.8±0.1 errors/trial) and each group learned the task well, with a significant decrease in the number of errors committed over the 11 day study period. However, early on, INJ had significantly higher failure rates (inability to complete the task in <20 s) compared to SHAM (1.14±0.71 failures over 5 trials in INJ on day 1 compared to 0 failures in SHAM, and 0.57±0.51 failures over 5 trials in INJ on day 4 compared to 0 in SHAM).

Food cover task

Finding a hidden food reward was more challenging than a visible food reward, associated with longer latencies and failed trials for all animals. All animals learned to find the hidden food reward with similar latencies for successful trials (INJ averaged 8.5±1.4 s while SHAM averaged 8.1±1.0 s) across taped and untaped consecutive trials. Both groups committed the same number of errors per trial across all days (0.3±0.1 errors/trial). Unlike the easier visible food reward task, we observed an increasing facility with practice with the hidden food test, such that both groups had significantly decreased latencies and failures with consecutive trials and on later testing days. INJ piglets had a higher failure rate of 2.1±0.6 failures/day compared to 1.7±0.8 failures/day for SHAM but this measure did not reach statistical significance (Power=35%). Both groups had significantly fewer failures on later testing days. On day 1 comparing untaped (trials 1–3) and taped (trials 4–6) cover trials, INJ had a significantly higher failure rate for untaped cover trials compared to SHAM (0.9±0.1 failures/3 trials compared to 0.3±0.1 failures/3 trials, $p=0.008$), but both groups had similar failure rates for the later taped cover trials conducted each day (0.8±0.1 failures/3 trials). We observed no group differences on later test days. In summary, INJ piglets had significantly higher failure rates for problem solving with visual as well as non-visual cues only on early test days after injury, consistent with transient cognitive deficits.

Balance beam

Both INJ and SHAM successfully learned how to cross the wide and intermediate beams. We observed a decrease in both latency and errors to food reward with subsequent trials for both the intermediate and wide beam tests. For example, averaging across both groups and all days, intermediate beam times were 3.1±0.4 s for Trial 1 compared to 2.2±0.4 s for Trial 6 and intermediate beam errors were 2.1±0.4 errors/trial for Trial 1 compared to 1.0±0.2 errors/trial for Trial 6. Averaging across all trials and days for each piglet, and across piglets in a group, both groups made significantly more errors on the intermediate beam (INJ 1.2 errors/trial; SHAM 1.2 errors/trial) than the wide beam (INJ 0.1 errors/trial; SHAM 0.2 errors/trial) (Fig. 5). INJ piglets were more challenged by the narrower beam than the SHAM, such that INJ had significantly longer latencies to food reward on the intermediate beam compared to wide beam (Fig. 6), but SHAM did not. Interestingly, INJ took significantly shorter time than SHAM to reach reward on both the intermediate and wide beams (Fig. 6). This finding correlates with our observation that INJ had less interest in exploring their surroundings during open field testing.

Histology and immunohistochemistry

Macroscopic and microscopic findings of brains from 5 of the 7 surviving INJ piglets were compared to the 5 uninjured SHAM. Two INJ brains were unavailable for analysis. Gross examination revealed no blood on the surface of any of the INJ or SHAM brains. Microscopic evaluation of coronal sections demonstrated hemosiderin containing macrophages in the leptomeninges in all five INJ brains examined, consistent with resolving subarachnoid hemorrhage. One INJ brain also showed evidence of resolving white matter hemorrhage. One SHAM

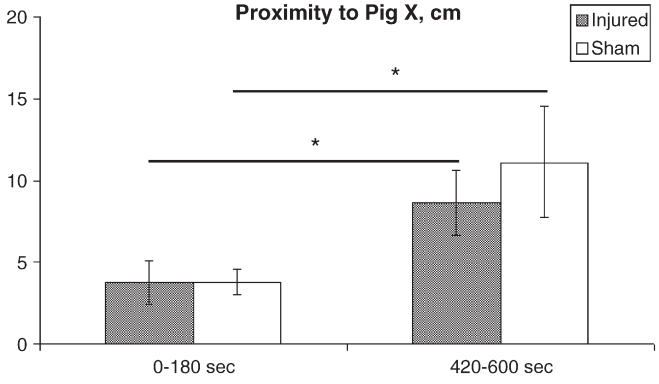

Fig. 4. Proximity of INJ and SHAM to Pig X during the initial 3 min and the last 3 min of the socialization segment of the open field testing. *Denotes statistically significant differences ($p<0.05$).

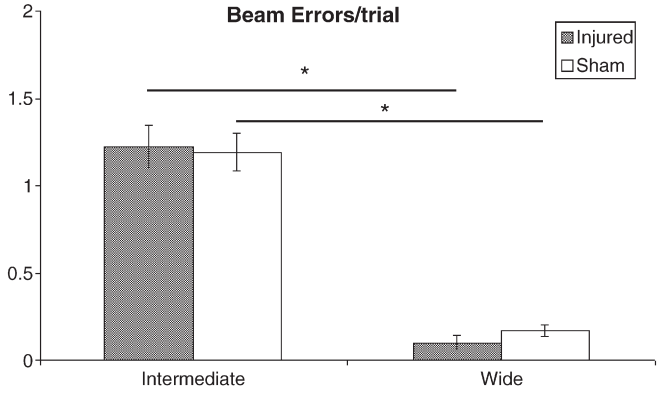

Fig. 5. Balance beam test, average errors for successful trials. *Denotes statistically significant differences ($p<0.05$).

brain was found to have bilateral ischemic lesions in the putamen with neuronal loss and prominent capillaries. Macroscopic and microscopic examination of brains from 8 MILD piglets and 4 uninjured MSHAM demonstrated no evidence of subarachnoid hemorrhage or ischemia.

Immunohistochemistry with Beta-APP did not demonstrate any positive staining in the white matter regions in any of the 5 SHAM piglets. Axonal swelling and retraction bulbs indicative of axonal injury were observed in the olfactory tracts of 1 INJ brain (Fig. 7). In three other INJ brains, axonal injury was observed predominantly in the region of the germinal matrix and internal capsule (3 of 5 animals) in the brains of INJ piglets (Fig. 7). In the fifth INJ brain, we observed only low levels of axonal pathology, located posteriorly. No axonal injury was noted in the MILD or MSHAM groups.

Extension of porcine loading conditions to the human

To relate the neurohistological and functional outcomes with injury scenarios for young infants, Ommaya's relationship (Ommaya et al., 1966) was used to scale the loading conditions from those experienced by a 35 g piglet brain to equivalent loads for a human infant with a 500 g brain mass. In this context, ×'s in Fig. 8 indicate scaled mild double rotations (both loads shown) where there was little or no functional deficits or pathology, and filled squares (Fig. 8) indicate scaled single moderate axial rotations with dysfunctions and axonal pathology. All scaled piglet accelerations are above levels proposed by Ommaya et al. (1966) to be associated with a 50% probability of concussion in a 500 g brain undergoing a non-impact sagittal rotation (dashed line, Fig. 8), based upon sub-human primate studies. In our previous studies, using an instrumented anthropomorphic doll constructed to represent a 6 week old human infant, measurement of head rotational acceleration and velocity were obtained during vigorous shaking in the sagittal direction (Prange et al., 2003). Because actual chest compression properties for infants were unavailable, we used two different configurations to bracket the range of potential responses of the head at the extremes of neck excursion: a padded thorax (filled circles, Fig. 8) and a rubber stopper (open diamonds, Fig. 8) (Prange et al., 2003). Previously we reported that loads measured with the padded chest and rubber stopper were not significantly different (Prange et al., 2003). It is important to note that the newborn neck was represented by a hinge. The hinge and rubber stopper conditions are idealizations that likely *overestimate* the actual rotational acceleration and velocity of the head.

Discussion

We have developed a piglet survival model of non-impact closed head injury and novel reproducible test instruments with sensitivity to detect alterations in neurobehavioral function in neonatal piglets after traumatic brain injury. Cognitive and behavioral deficits were observed in 3–5 day old piglets following a single inertial rotation of the head at moderate levels (188 rad/s, 62,896 rad/s^2) but not after two rotations at mild levels (142 rad/s, 34,115 rad/s^2) spaced 3 min apart. Functional alterations correlated with increased histopathologic injury in piglets experiencing moderate levels of rotation compared to mild levels or shams.

A comparison of the scaled piglet and anthropomorphic doll data in Fig. 8 reveals that the mild and moderate level *axial* loading conditions are well above nearly all the accelerations associated with *sagittal* shaking trials with the more realistic padded torso, but axial angular velocities of the piglet experiments are similar to those of typical sagittal shakes of both doll configurations. The relative importance of angular velocity vs. angular acceleration for producing brain injury remains unknown, but the most violent sagittal shakes (largest acceleration and largest velocities) tended to occur with the rubber stopper. Loads for the rubber stopper, which likely exaggerates head motion by producing a pronounced rebound as the head reversed direction, are in the range of our axial INJ piglet loading conditions associated with transient and sustained functional deficits and axonal injury. Recall that piglet head rotation is axial, which flexes the cervical spine laterally. While we are cautious about comparing *lateral* piglet motions to *sagittal* doll rotations, we are aware that increased lateral bending stiffness and limited lateral angular excursion compared to the sagittal flexion–extension direction, are likely responsible for producing significantly lower angular velocities and accelerations in a child than those shown for the doll in Fig. 8. Furthermore, because we have no piglet neuro-functional data

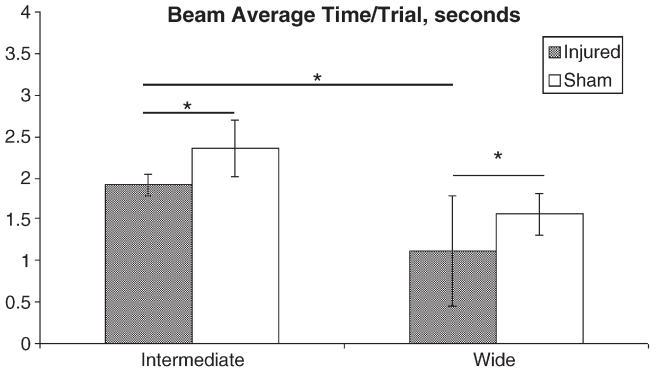

Fig. 6. Balance beam test, average time to food reward for successful trials. *Denotes statistically significant differences ($p<0.05$).

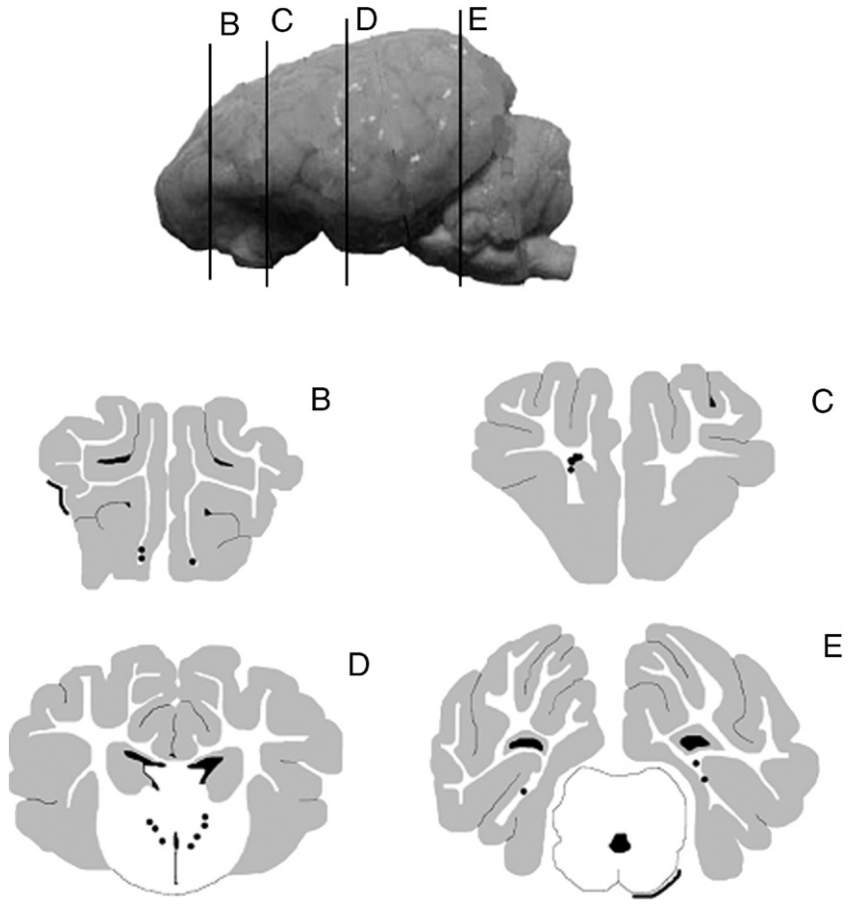

Fig. 7. Schematic depicting a composite of the regional distribution of injured axons (filled circles) and subarachnoid hemorrhage (heavy black lines) for selected slices of the 5 INJ brains examined.

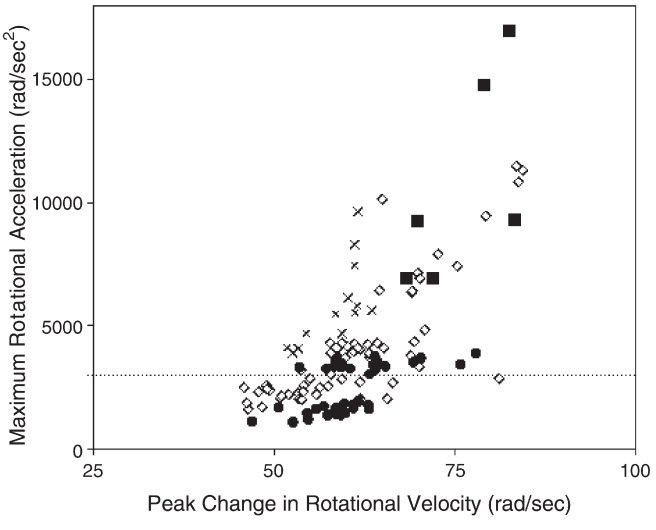

Fig. 8. Maximal rotational accelerations and peak changes in rotational velocity for mild piglets (×'s), moderate piglets (filled squares) both scaled from a 35 g to a 500 g brain, and anthropomorphic doll shakes without impact with padded chest (filled circles) or rubber stopper (open diamonds). The dashed line depicts the sagittal acceleration threshold associated with a 50% probability of concussion for a 500 g brain mass, derived from primate studies by Ommaya et al. (1966).

for rapid *sagittal* rotations, one should be cautious about direct comparisons between our animal data and doll data in Fig. 8. Future animal studies should target sagittal rotations to provide important experimental data that will allow for more relevant comparisons between animal and doll studies.

MILD and INJ piglets had only short periods of unconsciousness and no episodes of apnea, hypotension or hypoxemia, which is reflected in the absence of ischemia or infarct on histopathology. Our loading conditions were mild enough to permit survival and INJ and SHAM piglets had similar weight gain over the 12 day post-injury study period.

It should be noted that both buprenorphine and isoflurane have been reported to have neuroprotective characteristics following brain injury and their use in these studies may have minimized differences in behavioral and pathologic outcomes between INJ and SHAM (Ozden and Isenmann, 2004; Statler et al., 2006).

Previous studies in rodent models have demonstrated improvement in cognitive and motor outcomes after TBI in female vs. male rats and mice (Roof and Hall, 2000). To control for the effects of gender on TBI, we exclusively used female piglets in all our test groups.

During open field testing, INJ piglets were observed to have significantly decreased activity compared to SHAM sustained throughout the 12 day study period. On day 1, INJ piglets had

higher failure rates at problem solving tasks than SHAM. But INJ piglets did not demonstrate any deficits during tests of simple motor function (balance beam latency). These cognitive and behavioral deficits correlated with histologic observations of traumatic axonal injury 12 days after injury in the regions of the olfactory tract, germinal matrix and internal capsule. Abnormalities in open field testing, as well as differences in performance on the glass-barrier task and food cover task, could be explained on the basis of deficits in primary olfactory function, or in higher order cortical functions such as sensory discrimination, attention, motivation and visual–spatial processing. Olfaction is a dominant sensory modality in pigs, and is used concomitantly with tactile sensory functions performed by the snout, as shown by our observation that sniffing behaviors are an integral component of all exploratory and problem-solving tasks we assessed. Neuropathologic injury to the olfactory system in animals with dominant olfactory systems may play an important role in determining cognitive and neurobehavioral functional deficits. Olfactory bulb neurons project directly to widely distributed forebrain and limbic circuits involved in learning, memory, attention, social, and emotional function. These include entorhinal cortex, amygdala, hippocampus, and prefrontal cortex. Recent advances in cognitive neuroscience have shown that activation of these systems by olfactory stimuli in animal species is heavily dependent on olfaction sets in motion complex learning processes (Slotnick, 2001). Rodents rely on olfactory stimuli to support and reinforce visual–spatial learning tasks, and to a greater extent at younger ages before the visual system is fully developed (Rossier and Schenk, 2003). The same ontogeny of olfactory relative to visual systems is likely to apply to swine. Jacob et al. demonstrated in humans that olfactory signals modulate emotional and attentional states through the activation of widely distributed regions of neocortex not specifically dedicated to olfactory function, including prefrontal cortex, amygdala, visual cortex, and cerebellum (Jacob et al., 2001). Thus, our findings on neurobehavioral measures could be explained, at least in part, by the finding of damage to olfactory systems, which may impair performance on tasks involving olfaction directly, or indirectly by affecting motivation, attention, and visual-motor integration, while leaving primary motor systems unaffected. But it must be noted that immunohistochemistry staining demonstrated that only 1 of 5 INJ piglets examined was found to have extensive axonal injury to the olfactory tracts which may contribute to the significant decrease in some sniffing behavior and to the number of failures in seeking a food reward (glass barrier and food cover tasks) in INJ compared to SHAM. It also should be noted that one SHAM piglet had bilateral ischemic lesions in region of the putamen but did not demonstrate any detectable behavior outcome differences in this battery of tests compared to the SHAM piglets. We believe these histologic findings may have been caused by a perinatal insult prior to the piglet arriving at our facility.

Although all piglets decreased their exploration as each session went on, INJ piglets were observed to have larger decreases in some exploration behaviors. This is comparable to observations that cognitively impaired dogs are inactive for longer periods of time and interact with and explore objects less (Head et al., 1997).

Animal models of brain injury that have assessed behavioral, cognitive, and motor outcomes have been generally limited to mice and rodents. In these models, traumatic brain injury is generated by utilizing various focal impact models of brain injury (Prins and Hovda, 2003). After injury, animals are observed to have impairment in both cognitive function (spatial learning tested by Morris water maze) and motor function (beam walking and rotarod apparatus), but no difference in swim speeds was observed in the Morris water maze. While our measures of exploration and problem solving could have been adversely affected by motor deficits, we observed no gross motor function impairment in INJ piglets when compared to instrumented SHAM. Interestingly, injured piglets had shorter latencies to food reward on the beam. We speculate that this may be attributed to injured piglets' lower interest in exploration.

Previous survival studies of acute brain injury in piglets have utilized limited and relatively unsophisticated neurobehavioral assessments (Agnew et al., 2003; Priestley et al., 2001). Priestley et al. utilized a simple neurologic performance scale and a functional disability score that evaluated piglets' ability to feed, ambulate, and explore their environment. These neurobehavioral scores fail to capture more subtle deficits in problem solving or other executive functions. In our studies, we observed that INJ piglets had significantly more difficulty with problem solving based on visual cues (glass barrier test) on post-injury days 1 and 4, but on post-injury day 6 (the next subsequent day of testing) this difference had resolved. Similarly, Agnew et al. reported in their asphyxic cardiac arrest piglet model (5–7 day old piglets) that by day 3 after return of spontaneous circulation, all animals had similar neurobehavioral scores without any measurable deficits. In humans, concussion often results in amnesia and confusion, with headache being the most commonly identified symptom. Guskiewicz et al. (2003) reported the mean duration of symptoms of concussion in college football players of almost 3.5 days. Our data suggest that our neurobehavioral tests maybe sensitive enough to detect these subtle cognitive deficits. INJ piglets had sustained decreases in sniffing activity during open field testing compared to SHAM piglets.

One area of functional outcomes that our tests did not directly assess is memory and learning which may be affected in this model of brain injury. Patients with mild to moderate head injury may have subtle difficulties with memory and learning for years after injury. We have begun utilizing a T-maze test (Bolhius et al., 2004) to evaluate learning, memory, and executive function, that may demonstrate more lasting deficits in cognitive function that we were not able to demonstrate with our current battery of tests. Furthermore, we plan to use this model to investigate repetitive brain injury to determine if there is a period of vulnerability or protection after an initial head injury. Finally, we propose that our reproducible test instruments for neurobehavioral outcomes in neonatal piglets have wide applications in the fields of cardiac resuscitation, stroke, and hypoxic and ischemic brain injury where piglet models are widely utilized.

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