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Chronic brain injury and behavioral impairments in a mouse model of term neonatal strokes

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Abstract

Stroke in term neonates remains a significant cause of long-term neurological morbidity. This study was designed to assess the relationships between ischemic stroke induced by permanent unilateral carotid ligation in P12 CD1 mice and the structural and functional outcomes in the young mice as a consequence.

After P12 ischemic strokes, mice were behaviorally tested using accelerated rotarod, spontaneous alternation on a T-maze, open-field, and cylinder tests between P33 and P39. Brain injury was scored by histology at P40 with cresyl violet-stained coronal sections and computerized quantification of the ischemic injury.

The ligation-injured mice were not different from controls on cylinder testing for asymmetric use of their forelimb, or on rotarod measures. In the spontaneous alternation task, however, injured mice demonstrated significantly lower rates of alternation indicating a deficit in working memory. Open-field testing repeated on two consecutive days revealed that the ligated mice were less active than the controls and that they failed to habituate to the open field environment between sessions indicating a learning deficit. Overall, our results demonstrate that ischemia induced by our neonatal stroke model produces behavioral deficits that are consistent with the brain injury.

Keywords

Neonatal; Mice; Stroke; Rotarod; Cylinder test; T-maze; Open-field behavior

1. Introduction

Neonatal stroke occurs in approximately 1 per 4000 term births and along with pediatric strokes is an important cause of neurologic morbidity [21]. Although the majority of children survive their stroke, about 75% have sequelae including cerebral palsy, epilepsy, and a range of cognitive impairments, including learning and memory problems [9,17]. These sequelae are the result of injury to a range of brain structures including the hippocampus. Recently, we developed and described a new model of ischemic strokes in neonatal mice, which is a modification of the Levine [19], and Rice et al. [26] model. This model uses unilateral permanent carotid ligation alone to cause ischemic infarcts in 60-80% of P12 CD1 mice [6]. The advantages of this model over other hypoxic-ischemic procedures are that (1) it only requires a single ischemic insult to produce stroke and seizures that are correlated with brain injury, (2) hypoxia is not needed to produce the stroke, thus, there is no hypoxic exposure of the contralateral side, and (3) it is induced in mouse brain at age P12 and it has been reported that rodent brains at this age correspond to the brain of a full term infant [28]. Furthermore, because it has been reported that there are mouse strain specific differences in ischemic models [24,31-33], a model such as ours has the potential to take advantage of these strain related differences, and to use mice with genetic mutations in critical genes. To establish the relevance of this mouse model to the human condition we previously demonstrated that a high percentage of the ligated mice have acute behavioral and electrographic seizures following the stroke. The acute seizure severity correlates with the severity of the injury when quantified at the age of P40 [7]. We also previously reported that this model consistently produced injury in the hippocampus and cerebral cortex, with less severe injury observed in the striatum and thalamus [6,16].

The hippocampus is critical for learning and memory and is particularly susceptible to injury during neonatal ischemic episodes, yet only a few studies have evaluated the behavioral impact of the hippocampal damage produced by such insults, and the majority of these were carried out in adult rats and mice. Some studies have reported learning impairments in the Morris water maze after neonatal hypoxic-ischemia in mice and rats [5,25], however, these studies raise concern regarding the validity of water maze testing in an animal with a subtle hemiparesis; it may struggle to complete the task for reasons unrelated to its hippocampal function. Other tests used to assess hippocampal functions do not require complex motor coordination to perform. Spontaneous alternation, for example, is a simple exploratory working memory task sensitive to hippocampal function [8,18]. Rats have been reported to show deficits on this task following hypoxic-ischemia and subsequent hippocampal injury [12,13,22]. More recently, Carloni et al. reported impaired performance of neonatal rats on T-maze spontaneous alternation following hypoxic-ischemic brain injury [4].

Given that we have previously established that our model of ischemic stroke in the neonatal mouse produces consistent hippocampal injury, the goal of the present study was to behaviorally characterize these mice while still at a young age. For the model we aimed to establish the chronic behavioral deficits at ~3 weeks after the injury so that future studies with pharmacological interventions could be assessed for their rescue of behavioral symptoms along with their ability to ameliorate the injury. We chose to evaluate these mice on a T-maze spontaneous alternation task because it is sensitive to hippocampal function, simple to perform (does not require complex motor coordination) and is sensitive to ischemic damage. In addition, the open-field test was administered to examine overall gross locomotor activity, habituation (a simple form of learning that relies on hippocampal integrity), and to determine any bias in spatial distribution and rotational behavior as it is possible that hemiplegia may develop as a result of the injury. Since the injury also involves the unilateral sensorimotor cortex, and to some extent the striatum, we also conducted cylinder and rotarod tests in order to evaluate the voluntary use of forelimbs and gross motor learning. Our results show that our unilateral carotid

ligation model of neonatal stroke in P12 CD1 mice produces deficits in spontaneous alternation and open field measures but spares overall motor coordination.

2. Materials and methods

2.1. Subjects

All research was conducted according to a protocol approved by the Johns Hopkins University School of Medicine Animal Care and Use Committee (IACUC). A total of 30 CD-1 mice (15 males and 15 females from 3 litters) were used for the behavioral tests. Of the 30 mice, 15 were ligated (7 males, 8 females, 5 from each litter) and 15 served as sham controls (8 males, 7 females; littermate controls, 5 from each litter). Of the 7 male pups that were ligated one died a few days after surgery. Of the ligated mice only those that showed injury at P40 were included in the analysis ($n/n = 11/14$; 5 males, 6 females, i.e., 78.6%). We have previously reported the incidence of stroke-injury in this model to be 60-80%. Unlike the widely used hypoxia-ischemia model (i.e., Rice-Vannucci model) this model does not utilize the global hypoxia exposure following ligation to induce the stroke. Therefore the 30% animals that remain uninjured by the carotid ligation alone are comparatively more similar to controls since their brains are not exposed to the global hypoxia in addition to the unilateral ischemia. These animals therefore would not be expected to show any behavioral deficits. Since the percent injured and therefore also uninjured animals remain consistent in our model we expect the same number of ligated animals to remain uninjured in our future therapeutic drug trials within the drug and placebo groups. The strategy of analyzing only the animal with some evidence of injury has been successfully applied in a previously published study looking for the neuroprotective effects of Gabapentin on this neonatal stroke model [34]. During behavioral testing mice were housed in a vivarium maintained at 25°C on a 12:12 h light:dark cycle with lights on at 07:00 h. Food and water was available ad libitum. All behavioral testing took place during the light cycle between 10:00 and 15:00 h. The cylinder and corner tests were conducted on a separate cohort of mice (19 males and 13 females from 4 litters), 17 (9 males, 8 females) of which were ligation-injured and 15 served as sham controls (10 males, 5 females).

2.2. Surgery

All litters of CD-1 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Newly born litters of pups arrived at postnatal 5 days old (P5) and were allowed to acclimate for 7 days. Animals were housed in polycarbonate cages on a 12 h light dark cycle and food provided ad libitum. On P12, animals were subjected to permanent unilateral double ligation of the carotid artery. Briefly, animals were anesthetized with isoflurane carried by a 50-50 mixture of O₂ and N₂O. The right common carotid artery was double ligated with 6-0 surgisilk and the outer skin closed with 6-0 monofilament nylon. Sham control animals were treated identically except for the carotid ligation. Prior evaluation of perioperative temperatures with this protocol found that rectal temperatures remained at 34±2 °C and did not vary significantly between ligated and sham groups [33]. Perioperative respiratory rate and PCO₂ have not been measured in this model. Duration of anesthesia in this study was 9.3±0.4 min for the ligates and 6±0 min for the sham control group of mice. 50% O₂ compensated for the expected reduction in respiratory rate with deep anesthesia and prevent the possibility of procedure related systemic hypoxia during the ~10 min periods of surgery in this model of unilateral brain ischemia. Additionally since the O₂ delivery was passive (i.e., non-invasive, no intubation) therefore the lungs saw a fraction of O₂% content. Sham controls underwent same amount of surgical invasive injury. Duration of anesthesia was kept as close as possible. Central nervous system effects of isoflurane anesthesia have been described on the scale of many hours of exposure [29]. The goal here was to keep the period of anesthesia as short as possible. Difference of few minutes with short protocols has not shown to be a relevant confounding factor either for cell damage or behavior effects.

2.3. Acute seizure scoring

Seizure activity was scored according to a seizure rating scale as previously reported [23]. Every 5 min, in the 4 h following the surgical protocol the score corresponding to the highest level of seizure activity observed during that time period was recorded. Briefly, seizure behavior was scored as follows: 0=normal behavior; 1=immobility; 2=rigid posture; 3=repetitive scratching, circling, or head bobbing; 4=forelimb clonus, rearing, and falling; 5=mice that exhibited level four behaviors repeatedly; and 6=severe tonic-clonic behavior. After 4 h, the mice were returned to the dam and each of their seizure scores was individually summed to produce a total seizure score.

2.4. Histology and computerized atrophy measurement

Brain atrophy measurements were done as previously described [7,16]. Briefly at P40, a day after completion of the behavior tests, mice were anesthetized with 90 mg/kg chloral hydrate, perfused transcardially with ice-cold 4% paraformaldehyde, post-fixed for 12 h in the same fixative, cryoprotected, and snap frozen. Using MCID 7.0 Elite (InterFocus Imaging Ltd., Cambridge, UK) hemispheric areas of 50 μ m-thick, Nissl-stained coronal sections equally spaced and spanning rostral striatum to caudal hippocampus were measured ($n=10$ -12 sections per animal). The hippocampi and hemispheres of each analyzed section were outlined separately, and the areas were calculated based on a pixel threshold value that differentiates between brain and background. Hippocampal and hemispheric atrophy was calculated for each section as $(1 - (\text{area injured side} / \text{area uninjured side})) \times 100$. The values from each section were then averaged to calculate the hippocampal and hemispheric brain atrophy for each brain.

2.5. Behavioral procedures

2.5.1. Cylinder test—The cylinder test adapted for use in mice [20] was used to assess forelimb use and rotation asymmetry. The mouse was placed in a transparent cylinder 9 cm diameter and 15 cm in height. After the mouse was put into the cylinder, forelimb use of the first contact against the wall after rearing and during lateral exploration was recorded by the following criteria: (1) The first forelimb to contact the wall during a full rear was recorded as an independent wall placement for that limb. (2) Simultaneous use of both the left and right forelimb by contacting the wall of the cylinder during a full rear and for lateral movements along the wall was recorded as “both” movement. (3) After the first forelimb (for example, right forelimb) contacted the wall and then the other forelimb was placed on the wall, but the right forelimb was not removed from the wall, a “right forelimb independent” movement and a “both” movement were recorded. However, if the other (left forelimb) made several contacting movements on the wall, a “right forelimb independent” movement and only one “both” movement was recorded. (4) When the mouse explored the wall laterally, alternating both forelimbs, it was recorded as a “both” movement. A total of 20 movements were recorded during the 10-min test by two raters positioned on either side of the cylinder. The final SCORE= (Right forelimb movement-Left forelimb movement)/(Right forelimb movement + Left forelimb movement + both movement) as previously described in the rat [30].

2.5.2. Rotorod—The Rotorod (Accuscan, Columbus, OH) consists of a semi-enclosed chamber which contains a beam ($\varnothing=3$ cm, length=5cm) made of ribbed plastic and flanked by round plates on either side to prevent any escape. The rod is suspended at a height of 35 cm above the floor. To begin a trial the mouse was placed on top of the beam facing away from the experimenter's view, in the orientation opposite to that of its rotation, so that forward locomotion was necessary for fall avoidance. The Rotorod accelerated gradually without jerks from 0 to 35 rpm over a 2-min trial. Latencies for the mice to fall from the rod were recorded automatically by computer. Each mouse was given 4 trials with a 10-min inter-trial interval.

2.5.3. T-maze spontaneous alternation—This procedure was carried out in an enclosed “T” shaped maze (Med Associated, St. Albans, VT) in which long arm of the T (47 cm × 10 cm) serves as a start arm and the short arms of the T (35 cm × 10 cm) serve as the goal arms. In this task the mouse was placed in the start arm and after 5 s the door was opened and the mouse was allowed to choose and explore one of the goal arms. When the mouse had fully entered the choice arm (tail tip all the way in) a guillotine door was closed and the mouse was confined to the choice arm for 30 s. The mouse was then removed, the guillotine door lifted and the next trial initiated. This was repeated for a total of 15 trials. If the mouse did not make a choice within 2 min the trial was ended and advanced to the next. At the conclusion of each trial the maze was cleaned of urine and feces.

2.5.4. Open field habituation—All procedures were carried out in a square open field chamber (40.6 cm × 40.6 cm, Accuscan, Columbus, OH) mounted within sound attenuating shells. Behavior was monitored via a grid of invisible infrared light beams mounted on the sides of the walls of the arena. Data was collected and analyzed via VersaMax Analyzer software (Accuscan, Columbus, OH). To examine activity levels and habituation, mice were exposed to the test chambers for 30 min on each of two consecutive days. To begin a session, each mouse was placed in the center of the chamber and allowed to move about freely. The arena was cleaned with 70% ethanol after each mouse completed a session.

2.6. Analysis

All data was analyzed by independent sample *t*-tests and ANOVAs. Of the behavioral data, spontaneous alternation on the T-maze was analyzed with an independent sample *T*-test (one-tailed) because prior pilot data showed the spontaneous alternation on the T-maze was decreased in injured animals at P40. Rotorod and Open field data was analyzed by ANOVA. Rotorod data involved Group (sham vs. ligated) as a between subjects factor and Trial as a within subjects factor; Open field measures analyzed across sessions involved Group (sham vs. ligated) as a between subjects factor and day (day 1 session vs. day 2 session) as a within subjects factor; Open field measures analyzed within a session included Group (sham vs. ligated) as a between subjects factor and Time Block (5 min blocks of time) as a within subjects factor. Open field spatial distribution analysis included Group (sham vs. ligated) as a between subjects factor and either Quadrant (left front, left rear, right front, and right rear of open field) or Zone (margin and center of open field) as within subjects factors. There were no performance differences between male and females on any of the tests; therefore, analysis was carried out collapsed across gender. Of the ligated mice only those that showed injury were included in the analysis. Ligation-injured mice were determined by the presence of a visually discernable cystic infarct lesion in the ipsilateral hemisphere. In all cases where the injury was not obvious (i.e., associated with no atrophy score; Fig. 1A), microscopic examination of cresyl violet-stained sections was done looking for focal atrophy, gliosis and cell loss in the hippocampus. One ligate-injured mouse was excluded from analysis of the open field data as an extreme outlier due to a 15-fold increase in locomotion, and a predominant clockwise wild running behavior demonstrated by the mouse on both days of testing. Such behavior may possibly be a form of pre/post-ictal automatism associated with the post-stroke epilepsy in the model (unpublished observations). A probability below 0.05 was considered significant.

3. Results

3.1. Histology vs. acute seizure score

Ischemic injury quantification found that the ligated mice showed an average $61 \pm 10.8\%$ hippocampal atrophy and an overall $34 \pm 5\%$ hemispherical atrophy of the right (ipsilateral) hemisphere at P40. Paired sample correlation was found between percent hemispherical atrophy and the corresponding hippocampal atrophy in the brains of ligated mice ($r^2 = 0.85$, p

< 0.05; Fig. 1A). Average acute seizure score in the ligation-injured mice was 6 ± 2.3 with a range of a minimum score of 0 and a maximum score of 19. Percent atrophy scores significantly correlated with acute seizure scores for individual mice that underwent behavioral testing (Fig. 1B; hippocampal atrophy $r^2=0.67$, $p < 0.05$; hemispheric atrophy $r^2=0.86$, $p=0.001$) as has been previously reported [7]. Thus higher scores for acute seizures occurring in the 4 h following the ligation were associated with more severe strokes.

3.2. Cylinder test

We found no significant differences between performance scores for the two groups with their cylinder test scores (Fig. 2). This indicates that ligated mice do not have a forelimb use asymmetry for weight shifting during vertical exploration at P40 and the test could not distinguish between stroke and sham animals.

3.3. Rotorod

We found no differences in motor coordination and motor learning skills between the sham and ligated mice on the rotorod task. All mice improved performance over subsequent trials at a comparable rate as evidenced by the increased fall latencies (see Fig. 3). A repeated measures ANOVA revealed a significant main effect of Trial ($F_{3,72}=12.694$, $p<0.0001$) in the absence of a main effect of Group or a Group $\times$ Trial interaction. This Trial effect reflects the increase in latency to fall over subsequent trials observed in both groups of animals indicating ligated mice learned the motor skill as well as the controls did. Therefore the rotorod test detected no motor learning deficits for the injured mice at 3 weeks after the ligation.

3.4. T-maze spontaneous alternation

To measure exploratory behavior as well as memory, we assessed performance on a T-maze spontaneous alternation task. Results from the T-maze spontaneous alternation task can be seen in Fig. 4 which shows that the ligation-injured mice alternated at a lower rate than sham mice ($p < 0.05$). No correlation was found between the severity of the brain injury and the corresponding alternation rate in the ligated mice. Evaluation of number of trials completed (out of 15 trials total) by each group showed that there were no significant differences between the two treatment groups. Ligation-injured mice completed on average 11.5 ± 2.7 of the 15 trials compared to the 10 ± 2.8 trials by the control group of mice. This indicates that the lower alternation rate in ligation-injured mice was not due to higher counts of failed trials in ligation-injured mice. We also examined the percent of right turns and found no significant differences between the two groups (52.9% for control mice and 50.1% for ligation-injured mice); therefore the decreased percent alternation in the ligated injured mice can not be accounted for by a visual, sensory or motor preference to turn in one direction or the other. T-maze alternation rate for the ligated mice with seizures was less than in controls (68.2 ± 7.3 ($n=5$) and 77.3 ± 4.1 ($n=15$) respectively, $p=0.2$) although this did not reach significance. Meanwhile T-maze for ligated with seizures was very similar to ligated without seizures [68.2 ± 7.3 ; $n = 5$ and 68.7 ± 7.6 ; $n=9$ (ligated injured = 6 and ligated uninjured= 3) respectively, $p = 0.4$].

3.5. Open field activity and habituation

To examine overall locomotor activity and habituation, spatial distribution and rotational bias mice were observed in an open field over 2 consecutive daily sessions. Unless otherwise stated analysis of all between session data was performed on only the first half of each session (first 15 min) because all of the mice were the most active during this period and activity declined substantially by the second half.

The distance traveled in the first 15 min of each daily session served as an index of locomotor activity. Fig. 5A depicts a decrease in the overall distance traveled from day 1 to day 2. This

decrement across days reflects habituation across the two sessions as evidenced by a significant main effect of Day ($F_{1,23} = 36.618, p < 0.0001$). There was also a significant Day $\times$ Group interaction ($F_{1,23} = 5.331, p = 0.03$) reflecting the fact that the sham mice exhibited habituation from day 1 to day 2 while the ligated mice did not. There was no main effect of Group indicating that the 2 groups did not differ in their overall level of locomotor activity.

To examine habituation and locomotor activity within each of the sessions, the total distance traveled during the entire 30 min session was analyzed in 5 min blocks. Analysis of the day 1 session (Fig. 5B) revealed significant main effects of Group ($F_{1,23} = 5.360, p = 0.0299$) due to the longer overall distance traveled by the sham mice, and Time Block ($F_{5,115} = 120.279, p < 0.0001$) reflecting the decrease in distance traveled over the 30 min session. There was also a Time Block $\times$ Group interaction ($F_{5,115} = 2.414, p = 0.0403$) which follow up analysis showed was due to the significantly higher activity of the sham mice during the first 5 min block ($p = 0.026$) and their marginally increased activity during the last five minute block ($p < .058$). Analysis of the day 2 session yielded only a significant main effect of Time Block ($F_{5,115} = 135.839, p < 0.0001$) reflecting a decrease in locomotion over the course of the session (Fig. 5C). Thus, the ligated mice had lower overall activity on the day 1 session only, but both the shams and the ligated mice showed habituation within each session.

Open-field testing group by day interaction indicated that habituation over 2 days for the ligated with seizures was less than in controls, although this difference did not reach significance ($F(1, 17) = 1.9, p = 0.18$). There was no group $\times$ day interaction comparing the ligated mice with seizures ($n = 5$) to ligated mice without seizures ($n = 9$) ($F(1, 8) = 0.0036, p = 0.85$) indicating that between day habituation was similar in these two groups.

In addition to habituation, we determined if the spatial distribution of activity differed between the two groups during the first 15 min of each session. The time mice spent in the four quadrants of the open field (left front, left rear, right front, and right rear) as well as the time mice spent along the peripheral and the center zones of the field were examined. Analysis of the quadrant data from day 1 yielded only a main effect of Quadrant ($F_{3,69} = 4.040, p = 0.010$) due to all mice spending more time in the left front quadrant relative to all other quadrants (data not shown). Quadrant data from day 2 did not yield any significant effects. When we examined the time mice spent in the peripheral and central zones of the field during the two daily sessions analysis yielded only a significant effect of Zone (day 1: $F_{1,23} = 975.23, p < 0.001$ and day 2: $F_{1,23} = 344.198, p < 0.001$) due to all mice spending more time along the walls of the field than in the center of the field (data not shown).

Lastly, we examined the number of clockwise and counter clockwise revolutions made by the mice in the open field during each of the two daily sessions. Paired comparisons of the data from day 1 revealed that the sham mice exhibited similar numbers of clockwise and counterclockwise rotations (20.1 ± 2.6 vs. 19.3 ± 1.6 , respectively) while the ligated mice exhibited fewer numbers of clockwise rotations compared to the number of counterclockwise rotations (12.3 ± 2.3 vs. 19.9 ± 3 respectively), a difference that was only marginally significant ($p = 0.08$). On day 2 there were no differences between the numbers of clockwise and counterclockwise rotations exhibited by either the sham (13.1 ± 2.3 vs. 12.9 ± 1.3 respectively) or ligated (10.0 ± 1.8 vs. 14.6 ± 3 , respectively) mice. However, ligated mice exhibited significantly fewer counts of clockwise movements compared to controls on both days 1 and 2 ($p < 0.05$), while their counterclockwise movements remained similar to control. No correlation was found between the severity of brain injury and the counts of clockwise circling in the ligation-injured mice.

4. Discussion

Previously we demonstrated that in P12 CD1 mice our unilateral carotid ligation model of neonatal stroke produces features similar to that seen in neonatal stroke including acute seizures [6]. In the present study we confirmed that a high percentage of the injured mice have acute seizures that have been characterized in the model [7], the severity of which correlates significantly with the severity of the brain damage quantified weeks after the insult. The further aim of the current study was to behaviorally characterize mice to determine the functional outcomes after stroke in this animal model, which is essential for assessment of treatments aimed at post-stroke recovery. Our results show (1) that the acute seizure scores of injured mice have a strong correlation with the severity of the stroke-injury similar to previous reports [1], (2) no gross motor deficits could be detected when tested 3 weeks after injury on rotarod and cylinder tests, and (3) neonatal stroke alters the ability of the injured mice to alternate efficiently on a T-maze and to habituate to an open field at P40.

Our findings that the ligated mice show deficits on the spontaneous alternation task clearly demonstrate that our model of neonatal stroke can produce deficits in cognitive behaviors that are hippocampal dependent such as working memory. This is consistent with the findings of other studies that have examined the effects of neonatal stroke on spontaneous alternation tasks in adult rats and mice [15,22]. A recent study that examined spontaneous alternation after neonatal stroke in neonatal rats (PD28) also reported a deficit in alternation rate, but in addition reported that the ischemic rats chose the arm ipsilateral to the damaged side significantly more than the arm contralateral to the damage [4]. This is in contrast to the current study in which no arm preference was apparent; mice chose the left and right arms equally. One possible reason for the difference in these two studies may be that in the previous study rats were tested and in the current study mice were tested. We did not find a significant correlation between severity of the stroke-injury and the extent of the behavioral deficit. This is perhaps not surprising since the behavioral deficits likely result from multiple issues, some of which are the impact of the stroke on hippocampal sub-granular zone neurogenesis, extent of cortical involvement, plasticity, and individual genetic backgrounds in the outbred strain.

In the open field we observed that the ligated mice were less active overall on day 1 compared to the sham controls, but that on day 2 activity levels were similar between the two groups. This would seem to be an inconsistent result, however, several studies have examined activity in stroke models of rats and reported that compared to controls ischemic rats are hypoactive [36], hyperactive [2], and either no different or hyperactive depending on the age [12]. The discrepancies between these reports may reflect the differences in the various methods used to induce ischemia, the difference in the age of the subjects at the time of stroke or assessment, or the time of day and length of the activity session. An advantage of the current study is that activity was observed for 30 min during the same time of day over two consecutive days; therefore, we were able to assess habituation over this time frame. Habituation is a simple form of learning that is manifest as a decrease in activity after prolonged exposure or repeated exposure to the same environment [10,11]. Our data show that both groups of mice had similar activity levels on day 2 but not on day 1 because the shams were less active on day 2 compared to day 1. In contrast, the ligated mice exhibited similar levels of activity on both days. Thus, the difference in the activity levels on the 2 days was due to the sham mice habituating to the environment while the ligated mice did not.

Habituation is a hippocampal dependent task that reflects learning when assessed over a prolonged period of time, but can reflect memory when assessed over repeated exposures. We examined habituation in the open field both across sessions (repeated exposure) and within each session (prolonged exposure) and found that only the sham controls habituated across session but both groups showed habituation within each of the two sessions. Habituation is

usually seen in the first 5 min of the open-field test. If novel environments induced stress or a novelty response we would expect to see an effect on control mice as well. If stroke mice have a higher level of anxiety/stress to novel environments compared to control then we would expect them to have also showed other anxiety or stress related behavior like spending more time in the periphery of the open field rather than the center. This however was not the case. Therefore it is less likely that the activity level on the second day in ligated mice was stress related, than as a result of impaired habituation. The failure of the ligated mice to habituate across sessions is further evidence that our model of neonatal stroke can have a negative impact on cognitive behavior that is hippocampal dependent. It could be argued that the failure of the ligated mice to show habituation across sessions is due to an inability to reduce activity, however, our data also show that the ligated mice habituated within each 30 min session. The findings that the ligated mice do not habituate across session, but that they habituate within a session indicates that these mice can learn about the new environment (seen as within session habituation), but that they can not retain the information from one session to the next (seen as a lack of across session habituation). Taken together our data suggests that the deficit in habituation seen in injured mice is the result of a deficit in memory consolidation and/or retrieval.

We also examined the spatial distribution of the open field activity in ligation-injured and control mice and did not find any differences. In contrast Zhang et al. [37] reported altered spatial activity after focal stroke in an adult model of ischemia induced by middle cerebral artery occlusion in male Kunming mice [37]. The discrepancy between the Zhang study and the current study may be due to the fact that Zhang and colleagues monitored activity over longer durations (22 h) of time, or the difference in the ischemic induction methods. Similar to the current findings long-term open field tests conducted in C57/BL6J mice with stroke insults at P7 did not yield differences in exploratory behaviors [35]. Quantification of circling movements revealed that the ligated mice had significantly fewer circling movements ipsilateral to the damage (i.e., clockwise circling) compared to controls during exploratory movements in the open-field test. Since the injured mice did not exhibit asymmetric motor deficits these results indicate a persistent alteration in the pattern of exploratory behavior after neonatal stroke.

Because of potential damage to motor and sensorimotor areas a large number of studies have been conducted investigating functional motor changes after the induction of stroke in adult rodents. Clear motor differences exist in the behavioral deficits seen in rat vs. those seen in mice after stroke [14]. For example, in rats, rotorod testing has been used extensively to distinguish infarcted animals from sham [27], whereas in mice, the rotorod test was unable to distinguish stroke and sham animals within a few days after stroke [3]. Similarly, in the current study there were no differences in performance between ligated and sham mice on the rotorod. We also conducted the cylinder test which has been proposed to measure a more pure motor deficit, and found no deficits in the ligated mice indicating that alternative pathways may compensate for damage from stroke. Thus, in the current study, neither the rotorod nor the cylinder test detected motor coordination deficits in the injured mice 3 weeks after the stroke. It is possible that high neuronal plasticity of the mouse neonatal brain is responsible for shifting motor coordination from injured sensorimotor cortex to undamaged brain structures. It is worth noting that we attempted to assess P40 CD1 mice on the corner test, which evaluates neglect and vibrissae stimulation and taps into both cortical and subcortical functions, however, all mice failed to perform this task.

Lack of a stroke-injury (i.e., obvious infarct or cell loss on microscopic examination) indicates that the ligated uninjured mice ($n = 3$) may have been exposed to blood flow changes that were not pathogenic to begin with and/or may additionally have been compensated by collateral circulation. We removed these three animals from our main analyses because histology, rather

than imaging (which is not feasible in live mouse pups due to limits in resolution), is the gold standard for assessing the presence of a stroke in chronic outcome drug studies. Including animals that were never injured (i.e., ligated uninjured) introduces a great deal of variability to the results and makes more likely that an agent, which does in fact reduce the injury and improve outcome, will be discarded as ineffective [34].

Experiments are in progress to better characterize working memory deficits in this animal model of ischemia and its relationship with the damage of the hippocampal formation and its effect on post-stroke neurogenesis. Although the behavioral differences at P40 are small they are significant, consistent and reproducible. In this study, although the dam and mice pups had 1 week after being shipped to acclimate before undergoing the ligation surgery, we cannot rule out neonatal stress interacting with the ligation injury and having an additive effect on the behavioral deficits. In conclusion, our results of the behavioral characterization of our model of ischemia demonstrate that the spontaneous alternation T-maze and open-field test are sensitive to stroke produced by this model which may make such tasks useful in assessing potential therapeutic strategies that could be carried out during or immediately after the ischemic insult.

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Fig. 1.

(A) Strong correlation of hippocampal injury to corresponding hemispherical injury. (B) With acute seizure scores ranging from 0 to 19, ligation-injured mice showed a significant correlation of seizures with severity of hippocampal (●) and hemispheric (□) injury as quantified by percent atrophy.

Fig. 2. Cylinder test failed to differentiate the stroke injured mice from controls. The Stroke injured mice showed no discernable asymmetry of forelimb motor coordinated movements and were similar to controls.

Fig. 3.

No deficits in motor performance on rotorod by stroke injured mice. Stroke injured mice showed latencies of fall from the accelerated rotorod at intervals similar to controls. They also showed similar motor learning skills to the novel activity as seen by increasing latencies to fall over repeated trials that were similar to the control mice.

Fig. 4. Altered performance on T-maze spontaneous alternation task after neonatal stroke. Stroke injured mice showed deficits in spontaneous alternation tasks on the T-maze that were significantly less efficient than controls.

Fig. 5. Performance in the open-field test. (A) Total locomotor activity in both groups across the 2 days of testing. Habituation of locomotor activity between the 2 days of testing was significant only for controls. (B and C) Habituation activity within the day 1 (B) and day 2 (C) daily sessions. Both groups of mice habituated within each session, however the sham mice were more active during the day 1 session.