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Neurobehavioral Deficits in Progressive Experimental Hydrocephalus in Neonatal Rats

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Summary: Hydrocephalus is usually associated with functional deficits which can be assessed by neurobehavioral tests. This study characterizes the neurobehavioral deficits occurring with increasing duration and severity of ventriculomegaly in an experimental neonatal hydrocephalic rat model. Hydrocephalus was induced in three weeks old albino rats by intracisternal injection of kaolin while controls received sterile water injection. They were sacrificed in batches at one, four and eight weeks post-injection after neurobehavioral tests (forelimb grip strength, open field and Morris water maze tests) were performed. The hydrocephalic rats were also categorized into mild, moderate and severe hydrocephalus based on ventricular size. The indices of muscular strength and vertical movements in severely hydrocephalic rats were 28.05 ± 5.19 seconds and 7.29 ± 2.71 rearings respectively, compared to controls (75.68 ± 8.58 seconds and 17.09 ± 1.25 rearings respectively). At eight weeks, vertical movements were significantly reduced in hydrocephalic rats compared to controls (3.14 ± 1.3 vs 13 ± 4.11 rearings). At one week, indices of learning and memory were significantly reduced in hydrocephalic rats, compared to controls (0.89±0.31 vs 3.88±1.01 crossings), but at 8 weeks, the indices were similar (2.56 ± 0.41 vs 3.33 ± 0.71 crossings). Untreated hydrocephalus is accompanied by decline in motor functions which increase with duration and severity of ventriculomegaly. However, cognitive deficits appear to partially recover.

Keywords*:* Ventriculomegaly, Functional deficits, Learning and memory

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INTRODUCTION

Hydrocephalus is a neurological disorder characterised by an imbalance in the production and elimination of cerebrospinal fluid with consequent enlargement of the cerebral ventricles. The resultant increased pressure in the ventricles, through mechanical stretch, chronic ischaemia and neurochemical dysfunction from the compression of the extracellular space (Del Bigio, 2010) causes considerable damage to the brain, especially to the periventricular structures like the ependymal cells, periventricular axons, and also to neurones in the cerebral cortex.

A number of physical and mental disabilities and co-morbidities have been reported in hydrocephalic children including gait instability, arm and hand dysfunction, visual problems, depression, deterioration of balance and motor function as well as deterioration in learning and memory function (Shokunbi et al., 2002; Del Bigio et al., 2003; Gupta et al., 2007). These neurobehavioural deficits have been correlated with acute alterations in the cerebral white matter diffusion tenor imaging (DTI) properties in structures like the corpus callosum and internal capsule (Yuan et al., 2013). The presentation of these disabilities and their intensity in children is variable, though the severity of brain injury has been said to depend on the age of onset, duration and severity of ventriculomegaly (McAllister, 2012). Demyelination with functional impairment of axons is one of the major mechanisms suspected to contribute to cognitive and motor defects associated with hydrocephalus (Ayannuga et al., 2016).

Neurobehavioural studies provide an estimate of the functional impairment which follows damage to the central nervous system (Tilson and Mitchell, 1984). Neurobehavioural tests have developed rapidly over the years and are increasingly being used in neurological monitoring of CNS functions. They give an insight into the functional consequence of disease processes or conditions and can detect changes in neural function even before morphological changes are seen. However, it is essential to verify that the particular model used for the test is suitable in regard to the behaviour and the disease/condition under investigation (McKinney and Bunney, 1969). These tests include assessments of sensory, motor, cognitive functions and thus mirrors what is seen in human patients.

The study of the development of early reflexes in both congenital and acquired models of hydrocephalus did not reveal significant differences between the hydrocephalic and controls (Khan et al., 2006; Lopez et al, 2009), however, locomotion was found to deteriorate either with increased duration or ventricular enlargement (Del Bigio et al., 2003; Williams et al, 2014). Timely placement of ventricular shunts seemed to restore motor function to a great degree (Del Bigio et al., 1997; Eskandari et al., 2012). Cognitive function, on the other hand, has been observed to be affected early in hydrocephalus (Jones et al., 1995; Khan et al, 2006) and persists with increasing duration (Williams et al., 2014). The degree of memory impairment has been reported to be related to changes in content of hippocampal cholinergic and noradrenergic system (Egawa et al., 2002). We have previously reported that there is memory deficit and that muscular strength and locomotion deteriorate with increasing severity of ventricular enlargement at one time point, that is, 4 weeks post-induction of hydrocephalus (Olopade et al., 2012). There is, however, a paucity of information relating the neurobehavioural deficits observed in hydrocephalus to both the severity and duration of ventricular enlargement. The aim of this study, therefore, is to characterise the neurobehavioural deficits occurring with both increasing ventriculomegaly and duration in an experimental neonatal hydrocephalic rat model.

MATERIALS AND METHODS

A total of forty-five Wistar rats of both sexes, aged three weeks were used for this study. The animals were obtained from the colony established at the central animal facilities of the Faculty of Basic Medical Sciences, University of Ibadan. The experiments were conducted under ethical approval of the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC), following the NIH Guide for the Use and Care of Laboratory Animals, in accordance with the European Communities Council Directives (86/609/EEC) and ARRIVE guidelines, minimizing the number of animals used and avoiding their suffering.

Twenty-four of the rats had induction of hydrocephalus by intracisternal injection of 0.05ml of 25% kaolin (aluminium silicate) solution following an intraperitoneal ketamine/xylazine anaesthesia (90/10 mg/kg), and constituted the hydrocephalic/ experimental group. The pups in the control group (n=21) received an injection of equal volume of sterile water into the cisterna magna. The rats in the control and hydrocephalic groups were further divided into batches 1 week, 4 weeks and 8 weeks post-induction of hydrocephalus. General physical examination and neurobehavioural assessments for motor and cognitive functions were done and recorded. These were forelimb grip strength test, open field test and Morris Water Maze tests, which were done in this order for all the groups.

Forelimb Grip Strength Test: This test involves the forepaws of the rats being placed on a horizontally suspended wire (measuring 2mm in diameter and 1m in length), placed one meter above a soft bedding-filled landing area. The latency to fall (i.e. length of time each rat was able to stay suspended before falling off the wire) was recorded with a stop-watch. A maximum time of 120 seconds was given to each rat after which it was removed and each rat had two trials. This test reflects muscular strength and balance in the animals (Tamashiro et al., 2000; van Wijk et al., 2008).

Open Field Test: The open field is a white painted wooden box measuring 72 by 72cm with black lines drawn on the floor to divide it into 18 by 18cm squares. There is a square in the centre of the open field also measuring 18 by 18cm. The open field test was used to measure locomotion, however the use of different zones within the arena also provides information on anxiety-related behaviours (Turner and Burne, 2014).

The rats were tested separately in the open field for a period of 5 minutes each to assess the following parameters: horizontal movement (measured by the number of lines crossed), vertical movement (measured by rearing, that is, number of times the rat stands on its hind feet alone), grooming (that is, sets of heterogeneous constituents comprising face washing, body licking, paw licking, head and body shaking, scratching and genital licking), length of time spent in the central square, length of time it spent freezing (i.e. staying in one position and not making any movements at all) and number of faecal boluses passed. On completion of the test for each rat, the box was cleaned with 70% alcohol in order to prevent the subsequent rat from bias due to smell.

Morris Water Maze: The Morris water maze is a circular pool of opaque water (120 cm in diameter, 30 cm in height) with a hidden circular escape platform (12 cm in diameter, 1cm below the water level) which the rat must learn its location using contextual and visual cues. This tests hippocampal-dependent spatial learning and memory in rodents (Van Dam et al. 2006; Shabani et al., 2012). The task is based on the principle that rodents are highly motivated to escape from a water environment by the fastest, most direct route.

The pool was marked North, South, East and West and the hidden platform placed in a particular spot. Each rat was dropped into the pool and expected to search for the platform, the length of time it takes to find the platform is recorded. If it did not find the platform after 60 seconds, the rat was guided to the platform and allowed to stay there for 15 seconds. Each rat went through four trials per day for two consecutive days. This test is a measure of the learning ability of the rat. On the third day, a single probe trial was given to test the rats' spatial memory in the water maze while the platform was removed. The rat's memory of the initial location of the escape platform was measured by the time spent as well as its average speed in the target quadrant and the number of times it crossed the island zone where the platform was initially located. This latter record is a test of its memory ability. The Morris water maze test was introduced as an instrument with particular sensitivity to the effects of hippocampal lesions in rats (D'Hooge and Deyn, 2001; Golchin et al, 2013).

At the completion of the behavioural tests, the rats were anaesthetized, sacrificed by transcardial perfusion with 10% neutral buffered formalin and their brains removed and post-fixed in the same solution. Coronal sections of the brains were made at the level of the optic chiasma and the ventricle measured with a digital vernier callipers. The brains were further classified depending on the degree of ventricular enlargement. While the ventricular walls in the control brains were closely apposed, the hydrocephalic brain samples with ventricles measuring less than and more than 1.5mm were designated as mild and moderate hydrocephalus respectively, whereas those with separation of the caudate-putamen from the cortical mantle were designated as severe hydrocephalus, according to our previous classification (Olopade et al, 2012).

Statistical Analysis

The open field and Morris water maze tests were recorded and evaluated using an ANY-Maze behavioural tracking software (Stoeling Instruments, Wood Dale, IL) fitted with a Sony Cyber-shot camera. Means ± standard errors of means (SEM) were generated from the quantitative analyses and statistically evaluated by analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test using the GraphPad Prism 6.0 statistical analysis software package. The various groups were compared with confidence interval set at 95% and level of significance fixed at 5%.

RESULTS

General Observations: From the first week post-induction, the hydrocephalic rats developed dome-shaped heads with hunched back and a lethargic pace or some degree of unsteadiness of gait. They also developed a scruffy fur due to impairment of grooming as well as splaying of the limbs (Figure 1). The body weight of the hydrocephalic rats was less than that of the controls from the first week post-induction. However, the difference only became significant from the third week post-induction and remained so through-out the duration of the study ($p < 0.001$) (Figure 2).

Open Field: The hydrocephalic rats in the 8 weeks post-induction group were found to be slower and less inclined to move about in the open field than controls as well as the other hydrocephalic groups. However, the 1 week post-induction rats were found to be quicker in movements and more active than the other groups. The rate of horizontal exploratory movements measured by the number of transitions within the open field was not statistically significant across the groups ($p = 0.808$). However, there was a statistically significant reduction in the episodes of vertical movements / rearing in the 8 weeks post-induction hydrocephalic rats ($p = 0.01$) and an increase in the rats 1 week post-induction hydrocephalus (Figure 3a). Regrouping of the animals according to the severity of ventriculomegaly (mild, moderate and severe) revealed that the vertical movements were significantly reduced in the severe hydrocephalus group when compared with the controls but there was no significant difference in the horizontal movements

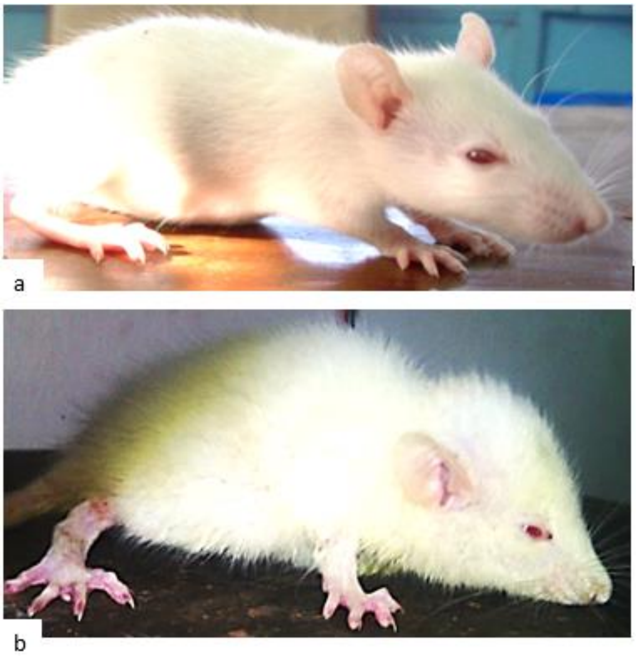

Figure 1: Effects of hydrocephalus on the rat. Compare the control rat in (a) to the hydrocephalic rat in (b). Note the dome shaped head, hunched back, scruffy, ungroomed fur coat and splaying of limbs in the hydrocephalic rat.

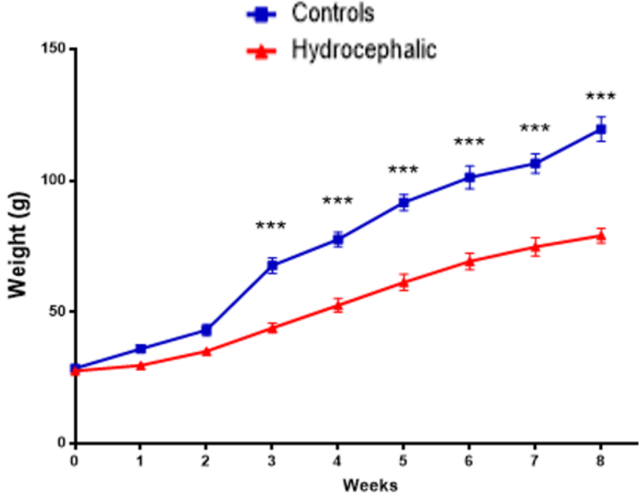

Figure 2: The weekly weights of the control and hydrocephalic rats. The hydrocephalic rats weigh significantly less than the control rats from the third week post-induction until the end of the study (*** p=0.001).

across the groups (p = 0.023, p = 0.162 respectively). However, the vertical and horizontal movements were more in the moderate hydrocephalus group than in the mild, though this difference was not significant (Figure 3b).

Forearm grip strength test: There was no significant difference across the duration-dependent groups in the forearm muscular strength as measured by the latency to fall off the metal wire (p=0.068) (Figure 4a). The forelimb grip test, however, revealed a statistically significant difference across the severity-dependent groups of hydrocephalic rats with the latency to fall decreasing significantly as ventricular dilatation increased among the groups. The moderate and severe varieties of hydrocephalus both exhibited a significantly shorter latency to fall when compared to the controls (p = 0.002) (Figure 4b).

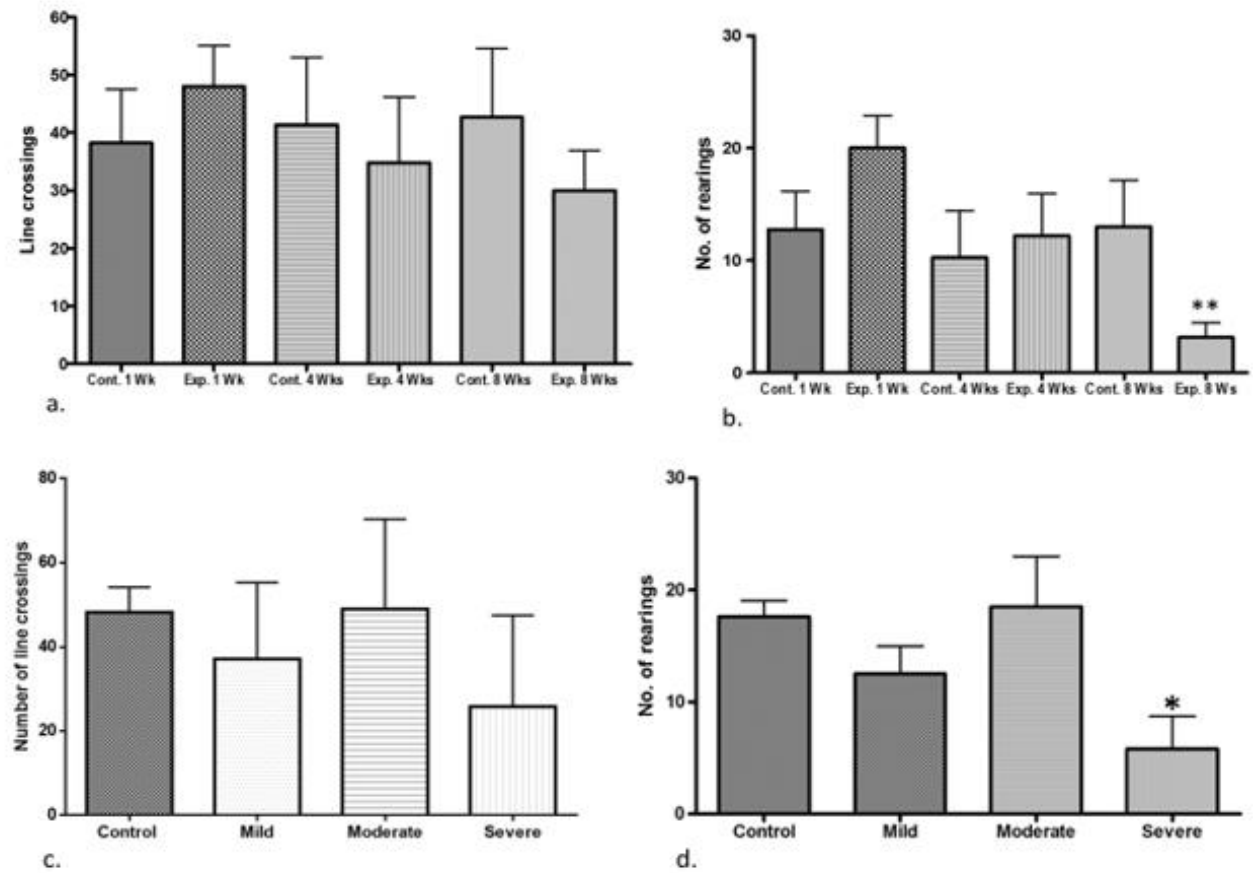

Figure 3: Horizontal movements (line crossings) and vertical movements (rearing) across the duration-dependent (a and b) and severity-dependent (c and d) groups of rats. Vertical movements were significantly reduced in the hydrocephalic rats with the longest duration (i.e. 8 weeks) as well as those with the most severe ventriculomegaly compared to their controls (*p<0.05, **p<0.01).

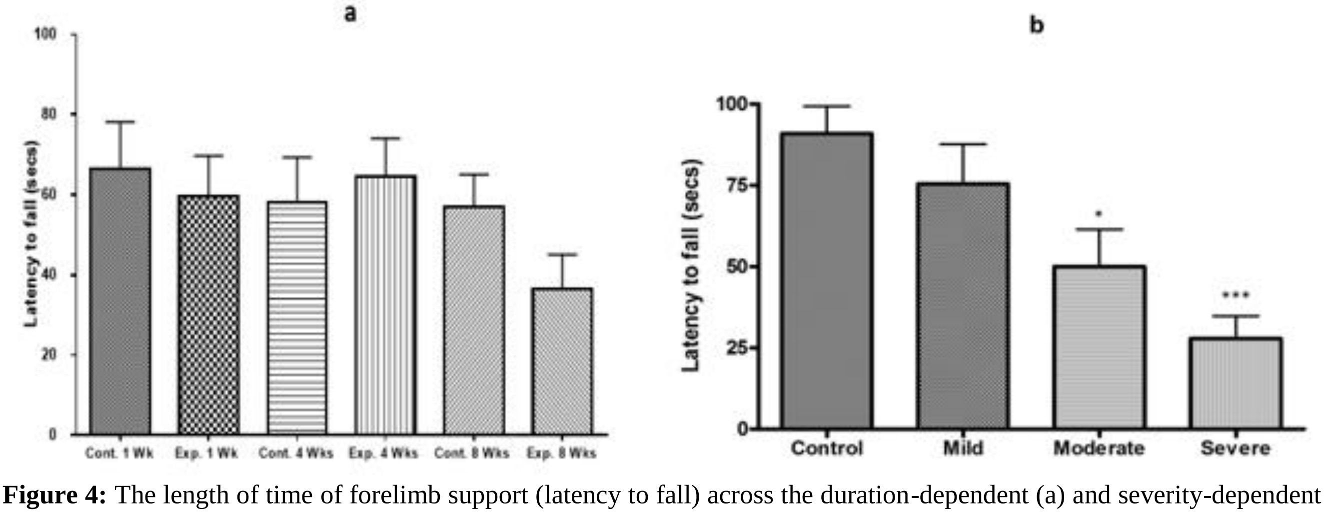

Figure 4: The length of time of forelimb support (latency to fall) across the duration-dependent (a) and severity-dependent (b) groups of rats. The rats with moderate and severe ventriculomegaly had significantly less time of forelimb support than their controls. (* p<0.05, *** p<0.001).

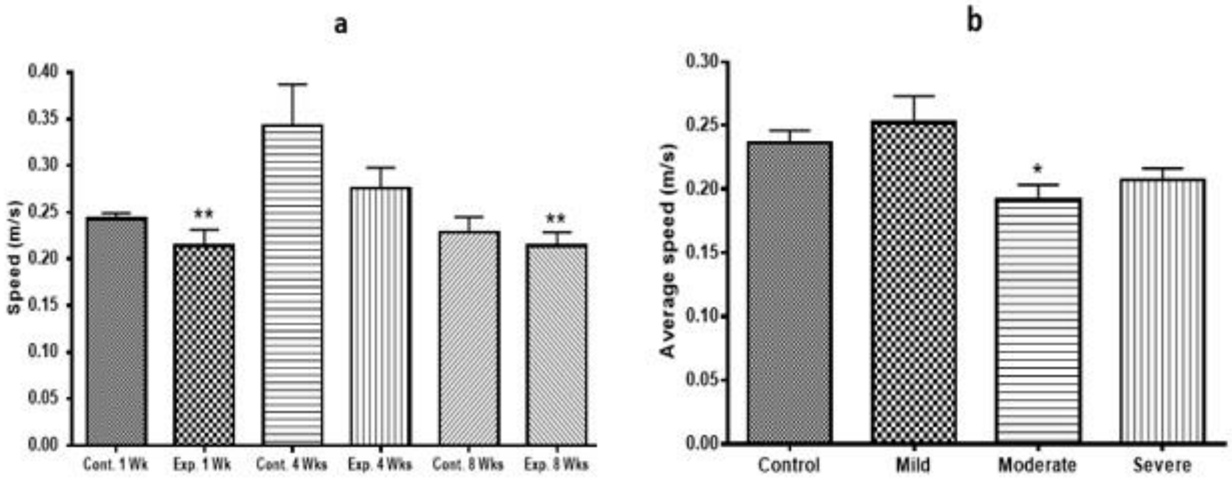

Figure 5: The average swimming speed of the rats across the duration-dependent (a) and severity-dependent (b) groups of rats. The average speed of the hydrocephalic rats was significantly reduced at one and eight weeks post-induction. The moderately hydrocephalic rats also swam significantly slower than the others. (* p<0.05, ** p<0.01).

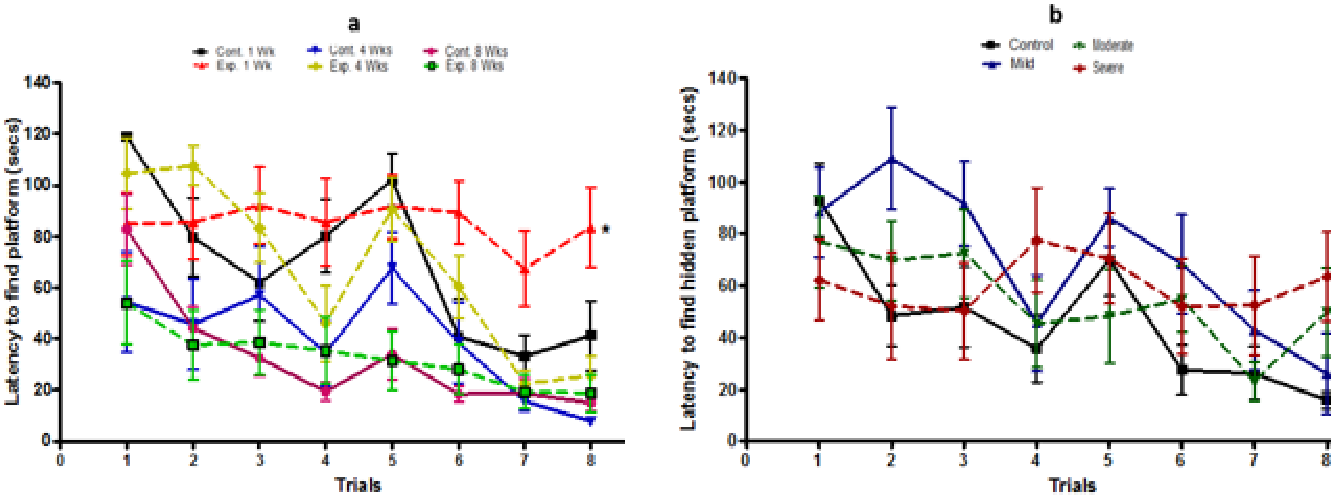

Figure 6: The latency to find platform (i.e. length of time taken to locate the hidden platform) across the duration-dependent (a) and severity-dependent (b) groups of rats. Note that the latency in the hydrocephalic rats 1 week post-induction did not significantly reduce like the other groups.

Morris water maze: There was a significant difference across the duration-dependent groups in average swimming speed in the Morris water maze test, with the 1 and 8 weeks hydrocephalic rats being significantly slower than the controls (p = 0.001) (Figure 5a). The severity-dependent groups revealed that the moderately hydrocephalic rats swam faster than all the other groups while the severely hydrocephalic ones swam slowest (p =0.013) (Figure 5b).

All the rats had a gradually decreasing latency to find the hidden platform with each successive trial, thus signifying that they all were able to learn its location except the hydrocephalic 1 week post-induction group. Thus, there was a statistically significant difference among the groups in the learning ability (p=0.0003) (Figure 6a). The hydrocephalic rats in the 1 week post-induction subgroup learned much slower than their age-matched control as well as the other groups. Both the 4 weeks and 8 weeks post-induction hydrocephalic rats performed as well as their age-matched controls in learning of the location of the hidden platform.

Although comparing the rats' ability to learn versus the severity of ventriculomegaly revealed no significant difference among the groups (p=0.202) (Figure 6b), the severe and moderate hydrocephalic groups seemed to take a longer time. Two severely hydrocephalic rats were found to be unable to swim properly and so were in danger of drowning due to the effects of ventriculomegaly and were thus excluded from the test. It is possible that this could have affected the results.

Retention of memory in the rats was measured by the number of annulus crossings of the platform area (island zone) and length of time spent in the target quadrant. The hydrocephalic rats 1 week post-induction (P.I), had significantly fewer number of

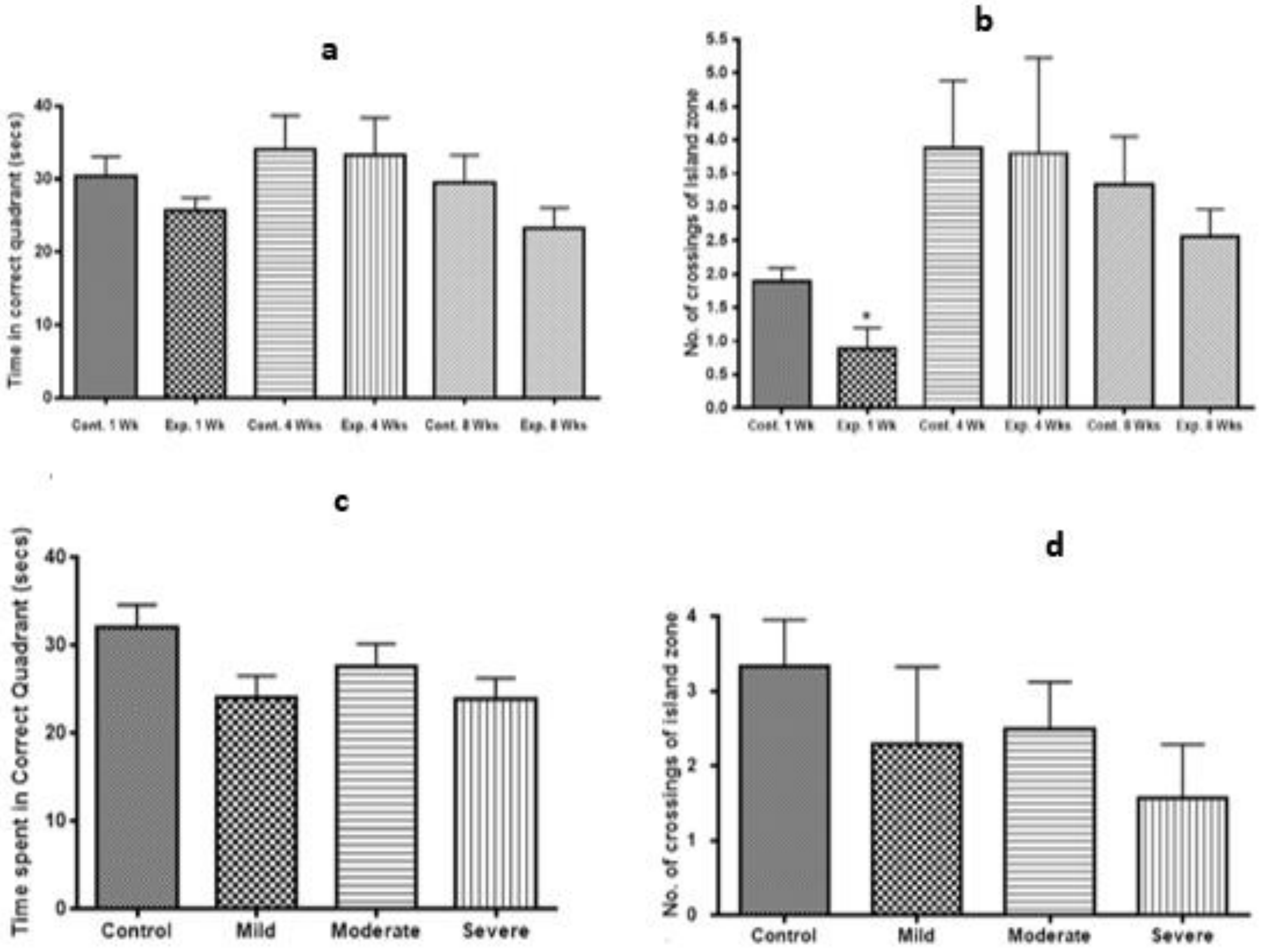

Figure 7: The measures of memory retention during the probe trial, that is, length of time spent in the correct quadrant and number of annulus crossings of the island zone that initially had the platform across the duration-dependent (a and b) and severity-dependent (c and d) groups of rats. Note that the number of annulus crossings in the hydrocephalic rats 1 week post-induction was significantly less than that of the control.

crossings of the platform area but the time spent in the target quadrant was not significantly reduced (Figure 7a). However, there was no statistically significant difference across the severity-dependent groups in the time spent in target quadrant and annulus crossing of platform area, although the severely hydrocephalic rats spent the least amount of time there (Figure 7b).

DISCUSSION

Similar to our previous observations (Olopade et al., 2012), there was a decrease in the weight gained by the hydrocephalic rats from the first week after induction and a persistent reduction in weight of the hydrocephalic rats when compared to the controls. This is also similar to the observations of Lopes et al., (2003) and Shaolin et al. (2015) and who suggested that this may be due to the apathy and loss of appetite observed in the hydrocephalic rats; as reduced weight gain is reported to be one of the first signs of development of hydrocephalus (Del Bigio, 2001). Most studies involving experimental hydrocephalus have reported reduced weight gain following induction of hydrocephalus (Lopes, 2009; Johnston et al., 2013). In a slowly developing Ro1 receptor-acquired model of hydrocephalus however, the body weights of the hydrocephalic and control mice were comparable until advanced stages (McMullen et al., 2012). Studies using congenital forms of hydrocephalus report varied results with some showing no significant differences in weight between hydrocephalic mutants and their controls until terminal stages (Wada, 1988; Edwards et al., 2006) while other studies show that hydrocephalic rats are significantly smaller than their wild-type litter-mate controls from birth (Batiz et al., 2006; Vogel et al., 2012). These findings could be due to species differences though there may be a hypothalamic basis for these weight changes. However, the mechanisms underlying the manner in which hydrocephalus affects weight gain in neonates is not fully understood.

Diffusion tensor imaging (DTI) as well as morphological studies revealed that the periventricular white matter is most susceptible to damage from hydrocephalus, especially the corpus callosum, external capsule (Lopes et al., 2003; Eskandari et al., 2014) and fornix. This is understandably due mainly to the physical stretch and compression from the enlarging ventricles to which they are in close proximity (Del Bigio, 2003). The observed behavioural deficits are most likely, a functional consequence of this damage. However, damage is not limited to the periventricular white matter, as association fibres also show evidence of abnormal development and degeneration in hydrocephalus (Hasan et al, 2008).

The hydrocephalic one week post-induction group had more movements than all the other groups. This is possibly due to increased cholinergic and adrenergic system output which has been reported in acute hydrocephalic as opposed to chronic hydrocephalic rats (Hwang et al, 2011). Thus the increased exploration was probably due to anxiety rather than increased muscular strength. This anxiety is further supported by the fact that the same set of animals did not perform as well in the forearm grip strength test. With increasing duration, however, there was a gradual reduction in spontaneous motor activity, exploration and muscular strength, as the eight weeks post-induction group had significantly less movements than their age-matched controls. The severity of ventriculomegaly, however, remains a pertinent factor in motor function and muscular strength as the severely hydrocephalic rats consistently recorded fewer movements and reduced muscular strength. Del Bigio, (1993) reported that hydrocephalus is associated with damage to axons in the periventricular white matter coursing around the enlarging ventricles, including long tracts that project to the spinal cord. In hydrocephalic children, increased tone and brisk reflexes or spastic paraparesis have been reported in the lower limbs due to stretching, and distortion of the periventricular corticospinal tracts arising from leg area of motor cortex. Impairment of fine motor and visual-motor integration have also been reported (Fletcher et al., 1997; Pant and Cherian, 2012).

The average swimming speeds in the hydrocephalic rats, 1 and 8 weeks post-induction were significantly lower than those of the controls. This further confirms the motor deficits in these groups of rats. Surprisingly, only the moderately hydrocephalic rats had a significantly reduced swimming speed when compared to the controls, despite the fact that the severely hydrocephalic rats showed consistent evidence of motor deficits from the other tests. It is possible for the swimming speeds of the rats to act as a bias in the interpretation of the Morris water maze test.

Among the duration-dependent rat groups, only the hydrocephalic rats 1 week post-induction exhibited learning deficits. Although the swimming speeds of the 8 weeks post-induction group was also reduced, only the 1 week group was affected, showing that the difference is most likely due to learning impairment, not only motor deficits. Although the severe hydrocephalic group did not show a statistically significant learning impairment, it should be noted they had they were the worst in learning. The fact that the very severe rats had to be excluded from the test because of the danger of drowning further confounds the study and we are confident that their inclusion would have tipped the scale significantly.

A significant impairment of memory was observed in the hydrocephalic 1 week post-induction group, compared to the other group. It is noteworthy that the measure of the number of annulus crossings of the island zone that initially had the platform was a more sensitive measure than the total time the rats spent in the target quadrant. This shows that the animal remembers the actual location of the platform as opposed to the general area it was located. The severe hydrocephalic also had a non-significant decrease in the number of crossings, possibly also due to the fact that two severely hydrocephalic rats were excluded from the test.

The time-based trend of neurobehavioural deficits observed in this study was an initial functional deficit observed early in the condition (i.e. 1 week post-induction). With time, there seemed to be an attempt at recovery (as seen in the test scores 4 weeks post-induction). However, if the ventricular enlargement persisted and/or progressed, the functional reserves of the brain become overwhelmed, and then the deficits appear again, this time with greater magnitude (as seen 8 weeks post-induction).

The clinical manifestations of hydrocephalus depend especially on the time of appearance and form of onset, if acute/subacute or chronic (de Oliveira et al., 2012). Hydrocephalus, represents a model of brain resilience, as the deficits in cerebral perfusion generate structural and functional injuries which are partially or completely compensated by the remaining cortical areas (Ewing-Cobbs et al., 2003). Following acute as well as chronic insutls cognitive and motor skills can be considerably modulated in the brain (Price and Friston, 2002; Oliveira et al., 2011), although this has limits. The deficits seen in the hydrocephalic child sometimes stabilizes and may be thought to be arrested, but sometimes it just reappears, suggesting that there was actually an overwhelming of the brain's functional reserves. That is why it is advocated that a continuous monitoring be done on all that have been diagnosed hydrocephalic, even if there seems to be a relief of symptoms Del Bigio, 2003).

Considering the level of severity of ventriculomegaly, both motor and cognitive deficits observed increase with increasing severity. Thus, the animals with the most severe ventriculomegaly have been reported to have the worst behavioural outcomes (Williams et al., 2014) except when it progresses very slowly (McMullen et al., 2012), in which case functional recovery is possible. The increasing ventricular diameter erodes more and more periventricular structures making it more difficult for the brain's reserves to cope and thus necessitating a quick intervention.

Combining the effects of duration and severity of hydrocephalus, we conclude that the worst functional outcomes would be seen in those combining severe ventriculomegaly with the longest duration. However, comparing them, we propose severity of ventriculomegaly to be a better index for measuring the functional effect of hydrocephalic injury than its duration. This is to refine the previous assertion from McAllister (2012) stating age of onset, duration and severity of ventriculomegaly as determinants of the severity of brain injury in hydrocephalus.

A possible limitation to our study was that we were unable to control for ventricular size while testing for effect of duration on neurobehaviours, as we measured ventricular size post hoc.

In conclusion, our results suggest that hydrocephalus produces significant injury in the rat brain with decline in motor, learning and memory functions. The motor deficits increase with the duration and severity of ventriculomegaly, but learning and memory appear to recover partially over time. The mechanisms for recovery of memory deficits deserve further study.

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