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ORIGINAL ARTICLE

Mice transgenic for reduced folate carrier: an animal model of Down syndrome?

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Abstract In a previous publication we observed aberrant levels of the human reduced folate carrier (hRFC) in cortex from fetal Down syndrome (DS) subjects. Immunoreactivity for hRFC was increased as the only chromosome 21 gene product studied. We, therefore, analyzed mice transgenic for hRFC (TghRFC1) and wild-type (WT) mice for cognitive functions, behavior and in an observational neurological battery (FOB). Cognitive functions were evaluated by the Morris water maze (MWM), the open field (OF) was used for exploratory behavior, locomotor activity and anxiety-related behavior. The elevated plus maze (EPM) was used to confirm findings in the OF testing anxiety-related behavior and the rota rod (RR) to evaluate motor function. In the MWM TghRFC1 mice performed significantly worse ($P < 0.0003$) on the probe trial than WT mice. In the FOB visual placing was significantly reduced inTghRFC1 mice. In the OF TghRFC1 mice crossed twice as often ($P < 0.029$) and in the EPM individuals from this group showed a reduced number of exits from the closed arm ($P < 0.044$) compared to WT mice. TghRFC1 mice showed impaired performance on the RR, spending one-fourth of the time of WT on the revolving rod ($P < 0.0003$). Cognitive impairment is an obligatory symptom of DS and this deficiency corresponds to findings in the MWM of mice transgenic for hRFC. Findings of visual placing and failure on the RR may reflect impaired motor performance including muscular hypotonia in DS subjects. Increased crossings in the OF may indicate modulated anxiety-related behavior observed in patients with DS.

Keywords Down syndrome · Mouse · Reduced folate carrier

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Introduction

Down syndrome (trisomy of chromosome 21) (DS) is the most common genetic cause of mental retardation. A legion of underlying molecular mechanisms has been proposed (Cheon et al. 2007; Engidawork and Lubec 2001, 2003; Ferrando-Miguel et al. 2004; Gulesserian et al. 2007) including involvement of folate metabolism and handling (Boduroglu et al. 2004; Coppede et al. 2006; Eskes 2006; Fountoulakis et al. 2003; Hassold et al. 2001; Hobbs et al. 2000; O'Leary et al. 2002; Rosenblatt 1999; Scala et al. 2006; Takamura et al. 2004). In a previous publication, we observed aberrant levels of the human reduced folate carrier (hRFC) in cortex from fetal DS subjects. As shown by immunoblotting, immunoreactivity for hRFC was significantly increased as the only chromosome 21 gene product studied (Lubec et al. 2003; Ferrando-Miguel et al. 2004/2005). This finding may

support the hypothesis that an aberrant folate system contributes to the pathogenesis of DS or trisomy per se. Folate plays a crucial role during development of the central nervous system (CNS) as well as in adulthood. Children of folate deficient mothers during pregnancy present with increased incidence of neuronal tube defects (Scholl and Johnson 2000). In children suffering from inborn errors of folate metabolism, degeneration of CNS, atrophy of cerebral cortex and mental retardation were described (Erbe 1975). In adults, low serum folate concentration has been related to cerebral atrophy, dementia and to poor cognitive functions, particularly in older adults (Snowdown et al. 2000; Wang et al. 2001a). Furthermore, abnormal folate metabolism has been proposed to be a maternal risk factor for DS (Boduroglu et al. 2004; Coppede et al. 2006; Eskes 2006; Hobbs et al. 2000; Scala et al. 2006; Rosenblatt 1999; Takamura et al. 2004). DS is linked to several impaired metabolic functions including folate and one carbon metabolism related metabolic pathways (Hobbs et al. 2000; Pogribna et al. 2001; Scala et al. 2006). Children with DS often have reduced folate levels but the underlying cause remains elusive (Al-Gazali et al. 2001; Hobbs et al. 2000; Pogribna et al. 2001; Snowdown et al. 2000).

The role of triplicated chromosome 21 for the pathogenesis of DS, proposed as gene dosage effects, has not been fully elucidated yet, however, and thus overexpression of hRFC, encoded on chromosome 21, formed the rationale for the current study.

The reduced folate carrier is a major transport system for folates and classical antifolates in mammalian cells and tissues, since mammalian cells cannot synthetize folate de novo. hRFC, a typical transporter protein with 12 membrane spanning domains, is a bidirectional anion transporter with high affinity for reduced folates and antifolates but low affinity for folic acid (Sirotnak and Tolner 1999). In the CNS, hRFC was detected by immunohistochemistry in the choroid plexuses as well as in axons and dendrites (Wang et al. 2001b) and protein expression of hRFC is regulated developmentally (Sirotnak and Tolner 1999).

Aiming to elucidate the impact of hRFC overexpression on cognitive functions, behavior, locomotor activity and anxiety related behavior, we decided to analyze mice transgenic for hRFC (Patterson et al. 2008) and used wild type (WT) mice as a control group. Results from the current study provide evidence for an in vivo functional role of hRFC for molecular mechanisms leading to the DS phenotype as mice containing an hRFC construct in addition to two mouse copies showed cognitive and behavioral changes similar to human Down syndrome.

Materials and methods

Animals

The inbred strain FVB.129Svev, with ten animals per genotype, male, aged 12–16 weeks was used. All mice were bred and maintained in cages made of Makrolon and filled with autoclaved woodchips in the Core unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna. Mice for breeding were obtained from Dr. David Patterson, Denver, CO, USA.

An autoclaved standard rodent diet (Altromin 1314ff) and water acidified to pH 3 from automatic valves or in bottles were available ad libitum. Room temperature was 22 ± 1°C and relative humidity was 50 ± 10%. Ventilation with 100% fresh air resulted in an air change rate of 15 times per hour. The room was illuminated with artificial light at an intensity of about 200 lx in 2 m from 5 a.m. to 7 p.m. Behavioral tests were performed between 8 a.m. and 1 p.m.

Genetic manipulation to generate transgenic animals

Isolation and initial characterization of the transgenic for hRFC (TghRFC1) mice has been described (Patterson et al. 2008).

Genotyping of mice used in this study

Using an Extract-N AMP tissue PCR kit (Sigma-Aldrich, St Louis, MO, USA), DNA was extracted from the tail tissue. PCR was performed (4 μL tissue extract per 20 μL reaction) using primers specific for the human RFC. The human-specific primers hRFC-R5 5′-TTC TGA ACA CCG TCG CTT GG-3′ (20-mer) and hRFC-F5 5′-AGG CAG CTG AAT TCC TGA GC-3′ (20-mer) amplified the human RFC gene (transgene) with an approximate PCR product size of 169 bp. In this case we could distinguish: WT mRFC/hRFC+ and WT mRFC/hRFC. The following cycling parameters were used: initial denaturation 94°C 2 min; denaturation 94°C 30 s; annealing 55°C 30 s; extension 72°C 30 s; 35 cycles; final extension 72°C 7 min; hold 4°C.

Behavioral and cognitive testing of mice

The TghRFC1 mice used in this study have a single integration site for hRFC, which is not on mouse chromosome 10. When these mice are bred with mice in which the mouse RFC gene has been inactivated by targeted mutagenesis, it is possible to produce mice in which the only active RFC is expressed from the hRFC transgene. These mice show no visible abnormalities, although their behavior has not been

characterized (Patterson et al. 2008). Thus, expression from the hRFC transgene in the TghRFC1 mice is sufficient for apparently normal mouse development.

Functional observational battery

The procedure followed the set-up by Irwin (1968): A battery of tests was applied to reveal defects in gait or posture, changes in muscle tone, grip strength, visual acuity and temperature. To complete the assessment, vitally important reflexes were scored. Throughout the manipulations incidences of abnormal behavior, fear, irritability, aggression, excitability, salivation, lacrimation, urination and defecation were recorded.

Elevated plus maze

This conflict test is based on a natural tendency of mice to actively explore a new environment versus the aversive properties of an elevated open runway and, therefore, a paradigm for the study of anxiety-related behavior in rodents. Mice were observed for 5 min on the plus maze, which had two closed arms, with walls 15 cm high side and end walls and two open arms. The maze was elevated 54 cm from the floor and the arms were 30 cm long. Mice were placed on the center section (5 × 5 cm) and allowed to freely explore the maze. Standard parameters reflecting anxiety-related behavior (i.e., open arm exits, time spent in open arm) were evaluated (Weitzdoerfer et al. 2004).

Open field

Exploratory activity, locomotor activity and consequential anxiety-related behavior were assessed in one 10 min test in an open field (OF) arena (41 × 41 cm long; with 70 cm high walls). Observation was done using an automated video monitoring system consisting of a video camcorder (1/3 in. SSAM HR EX VIEW HAD) coupled to computational tracking system (TiBeSplit). Standard parameters for locomotor activity (i.e., total distance covered, average speed, amount of large movement, amount of local movement, resting time and frequency of spontaneous changes of direction) and exploratory behavior (i.e., frequency of sniffing at the wall, rearing, crossing the center, entries into the center and time spent in the center) were recorded (Weitzdoerfer et al. 2004).

Rota rod performance

Mice were assessed for balance and motor coordination on an accelerating rota rod (RR) (Economex, Columbus Instruments, OH, USA). Revolutions per minute (rpm) were set at an initial value of four, with progressive increase to a maximum of 40 rpm across the 5 min test session. Each animal received a single test session consisting of three training trials. Afterward, each mouse received three more consecutive trials where latency to fall, or to rotate off the top of the revolving barrel was measured by the RR timer. For analysis the longest time spent on the drum was used (Rogers et al. 1999).

Morris water maze

The Morris water maze (MWM) task was used for testing spatial learning and memory. The water maze consisted of a large circular pool (diameter = 122 cm; walls 76 cm depth) partially filled with water in which mice were trained to escape from water by swimming to a hidden platform (1.5 cm beneath water surface) whose location can be only identified using distal extra-maze cues attached to the room walls. The platform was at the same position during the whole experiment (in SW quadrant). The pool was divided into four quadrants by a computerized tracking/image analyzer system (video camcorder Sony CVX-V18NSP coupled to a computational tracking system TiBeSplit, Imagination Computer Services GesmbH, Vienna, Austria).

One pre-training trial was done immediately before the experiments, where each mouse was placed on the hidden platform and was allowed to swim 30 s, afterward was guided back to the platform and allowed to climb onto the platform again. The spatial learning task consisted of four training trials per day and four training days. Mice were released with their heads facing the pool wall from the four compass locations (NE, NW, SW and SE; in this order), and were allowed to swim and search for the platform for 120 s. If the platform was not found, mice were manually placed on the platform and were allowed to remain on it for 30 s. Afterward each animal was returned to its cage for 10 min before its next trial. Measures were taken of latency to find the platform, time, swimming distance and swimming speed via the automated tracking system (Callaerts-Vegh et al. 2006).

Statistical analysis

Data from each group were analyzed separately, using within-group comparison relevant to the parameters of the specific task, after removing the outliers (Dixon 1953). Between-group differences were calculated either by unpaired Student's t test or by non-parametric Mann–Whitney U-test, if data violated a principal assumption of a parametric distribution. In all instances, a probability level of $P < 0.05$ was considered statistically significant.

Results

Characterization of the P1 clone P33A12

End sequencing analysis of P33A12 shows that it is 77,351 nt in length and contains the entire coding sequence of hRFC and no other intact gene. It spans from nt 45,714,910 to 45,792,261 of the NCBI reference sequence of chromosome 21 (NCBI *Homo sapiens* genome view build 36.2). P33A12 contains 9,960 nt of DNA 5′ (telomeric) of the translation start codon of hRFC and over 44 nt 3′ of the hRFC coding region. When P33A12 was used to produce the transgenic mice described here, this was thought to be the entire hRFC gene including its 5′ regulatory sequences, and the hRFC mRNA (gi: 34808709) spans nt 45,759,057–45,792,261. However, subsequent analysis reveals that the hRFC 5′ region is quite complex and contains at least five major 5′UTR promoter regions designated A, A1/A2, B, C, and D (Payton et al. 2007; Flatley et al. 2004; Whetstine et al. 2002a). The 5′ end of UTR E is at nt 45,817,825, and the 5′ end of UTR D is at 45,807,549. Both these promoter regions lie outside of the region spanned by P33A12. The 5′ end of UTR C is at 45,790,636, and thus lies within P33A12. Exon E is very rarely used (<1 in 200 transcripts). Exon D use is primarily restricted to lung and intestine and is also relatively rarely used (Whetstine and Matherly 2001; Whetstine et al. 2002a, b).

Production and characterization of transgenic (Tg) mice expressing hRFC

TghRFC1 have been described previously (Patterson et al. 2008). Briefly, mice breed well, have no obvious physical abnormalities, and express significant levels of hRFC mRNA in a variety of tissues. They have a single chromosomal integration site for P33A12 by fluorescence in situ hybridization (FISH) analysis. Dual color FISH analysis with a BAC containing mRFC demonstrates that the P33A12 integration site is not located on mouse chromosome 10, the site of the mRFC gene. Moreover, it is possible to produce mice diploid for the P33A12 integration site, so integration of P33A12 did not inactivate a critical mouse gene (Patterson et al. 2008).

Behavioral and cognitive testing of mice

The functional observational battery

As shown in Table 1, reduced scores were observed for visual placing in TghRFC1 mice when compared to WT. No significantly different scores were observed between tghRFC1and WT mice for any other finding of the FOB.

Elevated plus maze

As shown in Table 2, TghRFC1 mice showed a reduced number of exits from the closed arm as compared to WT mice.

Open field

The presentation of results is given in Table 3. In the OF the number of crossings was significantly higher in TghRFC1 as compared to WT mice.

Rota Rod performance

As shown in Table 4, TghRFC1 mice performed manifold and significantly worse in the RR performance when maximal time of persistence on the rod was taken as compared to WT mice.

Spatial learning and memory in the water maze task

Results from the MWM are shown in Table 5. The time to reach the platform (latency) was higher at all time points measured in TghRFC1 as compared to WT mice. The number of successful approaches to reach the platform and average velocity was significantly lower in TghRFC1 as compared to WT mice. Distance travelled was significantly higher in TghRFC1 as compared to WT mice on days 2 and 3.

Discussion

Cognitive impairment is an obligatory symptom of DS and this deficiency corresponds to findings in the MWM of mice overexpressing the RFC. This is in agreement with other animal models of DS at the cognitive level of spatial memory. In Ts65Dn decreased performance in MWM, radial arm maze, and passive avoidance were reported (Galdzicki and Siarey 2003; Seregaza et al. 2006) and MWM performance in Ts1Cje is impaired as well (Galdzicki and Siarey 2003).

Spatial memory is also relatively poor in Tc1, an aneuploid mouse strain carrying human chromosome 21 structures and presenting the DS phenotype (Miller 2005; O'Doherty et al. 2005).

In a recent review cognitive functions including MWM, radial maze and T-maze were reported for a series of mice polytransgenic for MMU 16 or HSA21 (Seregaza et al. 2006). Several mouse models overexpressing individual genes encoded on chromosome 21 have been shown to present spatial cognitive decline in addition (Antonarakis and Epstein 2006; Butler et al. 2006; Dierssen et al. 2001).

Table 1 Results of the FOB

Parameter for functions	WT (mean ± SD)	TghRFC1 (mean ± SD)	Intergroup comparison (*P* value) (WT vs. TghRFC1)
Motor			
Body position	1.9 ± 1.4	3.1 ± 1.8	0.0549
Palpebral closure	1.0 ± 1.4	2.0 ± 2.1	0.1575
Spatial locomotion	3.2 ± 1.8	3.5 ± 1.6	0.349
Tremors	0.0	0.6 ± 1.3	
Twitches	0.0	0.2 ± 0.6	
Limb rotation	2.4 ± 1.6	2.6 ± 1.9	0.4559
Limb tone	4.2 ± 1.8	3.8 ± 1.1	0.2894
Abdominal tone	3.2 ± 1.7	3.0 ± 1.1	0.5147
Gait	0.0	0.0	
Righting reflex	0.0	0.0	
Locomotor activity	0.8 ± 1.0	1.2 ± 1.0	0.2406
Wire manoeuvre	3.8 ± 1.1	4.6 ± 1.9	0.1763
Pinna	2.1 ± 206	1.6 ± 1.8	0.3128
Body tone	3.8 ± 1.8	3.4 ± 1.3	0.3421
Pelvic elevation	2.4 ± 1.6	3.0 ± 1.7	0.2644
Visual placing	5.2 ± 1.7	3.6 ± 1.6	*0.0315*
Grip strength	4.8 ± 1.4	4.6 ± 1.3	0.3697
Convulsions	0.0	0.0	
Autonomous			
Piloerection	1.3 ± 1.3	1.8 ± 1.9	0.3421
Diarrhea (N° of animals)	1.0	2.0	0.2656
Skin color	4.8 ± 1.4	5.4 ± 1.0	0.1965
Sensitivity			
Toe pinch	3.4 ± 2.1	3.8 ± 1.8	0.2406
Tail pinch	1.8 ± 0.9	1.6 ± 1.0	0.2644
Aggression			
Provoked biting	2.6 ± 1.9	2.4 ± 1.8	0.398
Exploratory			
Touch escape	2.2 ± 1.1	2.2 ± 2.4	0.4559
Finger approach	1.6 ± 1.8	2.2 ± 1.8	0.2406
Mood			
Tail elevation	1.4 ± 1.3	1.6 ± 1.6	0.4267
Others			
Transfer arousal	3.2 ± 1.8	3.5 ± 1.6	0.349
Bizarre behavior	0.0	0.0	
Exophthalmos	0.0	0.0	
Startle response	3.6 ± 1.6	4.3 ± 1.6	0.1965

$P < 0.05$ significant value for italics

Herein, mice with expressing a transgenic hRFC, clearly presented with pronounced cognitive spatial impairment, a feature of human DS (Vicari and Carlesimo 2006).

The fact that TghRFC1 mice reveal increased time to reach the platform, increased total distance traveled and decreased average velocity along with a reduced number of successful approaches to reach the platform in a given time, points to the possible relevance of hRFC overexpression as a factor in cognitive decline in human DS.

The finding of a diminished visual placing reflex, a proprioceptive function, fits or represents proprioceptive deficits shown in an animal model of DS (Costa et al. 1999) as well as in human DS (Kubo and Ulrich 2006; Wang and Ju 2002). Poor performance with manifold decrease of time remaining on the revolving rod of the RR system may reflect impaired motor performance including muscular hypotonia in DS subjects (Korenberg et al. 1995).

Table 2 Results of the EPM

Parameter	WT (mean ± SD)	TghRFC1 (mean ± SD)	Intergroup comparison (*P* value) (WT vs. TghRFC1)
N of closed arm exits	22.3 ± 4.4	17.1 ± 6.6	*0.0445*
Resting time (%)	55.8 ± 8.3	56.0 ± 5.8	0.4806
Local movement (%)[a,c]	34.3 ± 5.4	35.2 ± 3.3	0.3492
Total distance covered (m)	11.7 ± 2.6	11.4 ± 2.0	0.4022
Distance covered in closed arm (m)	7.7 ± 2.0	6.5 ± 1.1	0.0782
Large movement (%)[b,c]	9.9 ± 3.9	8.8 ± 3.2	0.2834
Time spent in closed arm (s)	159.4 ± 25.3	142.9 ± 31.9	0.2209

[a] Movement speed $0.03 < x < 0.1$ m/s
[b] Movement speed > 0.1 m/s
[c] % of total observation time
$P < 0.05$ significant value for italics

Table 3 Results of the OF

Parameter	WT (mean ± SD)	TghRFC1 (mean ± SD)	Intergroup comparison (*P* value) (WT vs. TghRFC1)
Total distance covered (m)	48.3 ± 10.0	56.0 ± 15.8	0.1034
N of times crossing the center	2.4 ± 1.6	5.1 ± 3.9	*0.0294*
Resting time (s)	26.8 ± 3.9	25.2 ± 3.3	0.1767
Time spent in the margin (s)	77.9 ± 6.2	75.2 ± 5.3	0.1516
Local movement (%)[a,c]	61.7 ± 2.7	60.5 ± 5.6	0.3421
Large movement (%)[b,c]	11.6 ± 3.8	14.3 ± 6.0	0.1264
Spontaneous changes of direction	93.4 ± 65.7	127.2 ± 77.7	0.0827
Average velocity (m/s)	0.08 ± 0.02	0.10 ± 0.03	0.1075

[a] Movement speed $0.03 < x < 0.1$ m/s
[b] Movement speed > 0.1 m/s
[c] % of total observation time
$P < 0.05$ significant value for italics

Table 4 Results of the RR

Parameter	WT (Mean ± SD)	TghRFC1 (Mean ± SD)	Intergroup comparison (*P* value) (WT vs. TghRFC1)
Trial 1[a]	7.9 ± 5.7	1.8 ± 0.5	*0.007*
Trial 2[a]	8.4 ± 5.0	2.3 ± 1.3	*0.0103*
Trial 3[a]	5.6 ± 7.5	1.6 ± 0.7	0.3063
Max[a]	10.6 ± 5.5	2.5 ± 1.1	*0.0003*

[a] Time spent on device (s)
$P < 0.05$ significant value for italics

The RR is a well-standardized and reliable measure for motor deficits (Spano et al. 1999) and the robust data of maximal remaining time of mice on the rod provide evidence for the presence of motor dysfunction in this proposed partial animal model of DS.

In agreement with results from Debû (2004) is the effect shown in the MWM and RR performance, that even after training, the performance remained very variable, both within and between WT and TghRFC1 individuals.

In the OF TghRFC1 mice show significantly more crossings than WT. In the EPM TghRFC1 mice showed less frequent exits of the closed arm, but as no other correlating and supporting evidence was provided in the system the biological role of this finding remains unclear. The finding of "reduced anxiety-related behavior" has to be interpreted with caution. Also in Ts65Dn mice formally reduced anxiety related behavior was detected (Martinez-Cue et al. 2006), however, their and our findings may be influenced or interpreted by cognitive deficits and subsequent lack of behavioral inhibition.

Taken together, we describe a partial DS neurophenotype in terms of spatial cognitive and motor function using robust test systems. The association of cognitive and motor deficits to increased hRFC is shown although we cannot directly link hRFC to impaired function. Further work to be carried out in the laboratory will address brain folate levels to show possible consequences of hRFC upregulation and additional learning and memory studies will be applied to confirm and extend knowledge on the nature of the spatial memory deficit in this proposed animal model of DS. In particular, additional cognitive settings have to be tested to reproduce the different learning and memory impairments observed in DS. The probable involvement of folate handling and metabolism is an attractive hypothesis to explain

Table 5 Results of the MWM

Parameter	WT (Mean ± SD)	TghRFC1 (Mean ± SD)	Intergroup comparison (*P* value) (WT vs. TghRFC1)
Day 1			
Total distance covered (m)	8.4 ± 5.9	12.1 ± 3.5	0.0850
Average velocity (m/s)	0.19 ± 0.03	0.13 ± 0.04	*0.0012*
Successful approaches to reach platform (0.25 means 1/4 trials)	0.8 ± 0.2	0.3 ± 0.3	*0.0029*
Time until animal started movement (s)	0.5 ± 0.0	0.5 ± 0.0	0.3451
Total time covered until platform reached (s)	46.8 ± 30.2	101.5 ± 22.2	*0.0011*
Day 2			
Total distance covered (m)	6.7 ± 4.0	11.0 ± 3.5	*0.0253*
Average velocity (m/s)	0.18 ± 0.03	0.12 ± 0.03	*0.0004*
Successful approaches to reach platform (0.25 means 1/4 trials)	0.9 ± 0.1	0.3 ± 0.2	*0.0006*
Time until animal started movement (s)	0.5 ± 0.0	0.3 ± 0.0	0.3463
Total time covered until platform reached (s)	38.1 ± 22.1	94.8 ± 20.6	*0.0002*
Day 3			
Total distance covered (m)	3.5 ± 2.6	10.0 ± 2.9	*0.0004*
Average velocity (m/s)	0.16 ± 0.02	0.1 ± 0.03	*0.0010*
Successful approaches to reach platform (0.25 means 1/4 trials)	0.9 ± 0.1	0.2 ± 0.2	*0.0003*
Time until animal started movement (s)	0.3 ± 0.0	0.6 ± 0.0	0.3668
Total time covered until platform reached (s)	21.8 ± 16.0	102.5 ± 20.0	*<0.0001*
Day 4			
Total distance covered (m)	7.1 ± 3.1	8.4 ± 2.8	*0.2041*
Average velocity (m/s)	0.19 ± 0.0	0.1 ± 0.03	*<0.0001*
Successful approaches to reach platform (0.25 means 1/4 trials)	0.9 ± 0.1	0.3 ± 0.3	*0.0006*
Time until animal started movement (s)	0.6 ± 0.0	0.6 ± 0.0	0.3438
Total time covered until platform reached (s)	38.0 ± 16.6	97.4 ± 19.6	*<0.0001*

$P < 0.05$ significant value for italics

mental or motor deficits in DS because folate is known to play a major role in cognitive processes (Balk et al. 2007; Durga et al. 2007).

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