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Cognitive and behavior deficits in sickle cell mice are associated with profound neuropathologic changes in hippocampus and cerebellum

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Abstract

Strokes are perhaps the most serious complications of sickle cell disease (SCD) and by the fifth decade occur in approximately 25% of patients. While most patients do not develop strokes, mounting evidence indicates that even without brain abnormalities on imaging studies, SCD patients can present profound neurocognitive dysfunction. We sought to evaluate the neurocognitive behavior profile of humanized SCD mice (Townes, BERK) and to identify hematologic and neuropathologic abnormalities associated with the behavioral alterations

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Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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observed in these mice. Heterozygous and homozygous Townes mice displayed severe cognitive deficits shown by significant delays in spatial learning compared to controls. Homozygous Townes also had increased depression- and anxiety-like behaviors as well as reduced performance on voluntary wheel running compared to controls. Behavior deficits observed in Townes were also seen in BERKs. Interestingly, most deficits in homozygotes were observed in older mice and were associated with worsening anemia. Further, neuropathologic abnormalities including the presence of large bands of dark/pyknotic (shrunken) neurons in CA1 and CA3 fields of hippocampus and evidence of neuronal dropout in cerebellum were present in homozygotes but not control Townes. These observations suggest that cognitive and behavioral deficits in SCD mice mirror those described in SCD patients and that aging, anemia, and profound neuropathologic changes in hippocampus and cerebellum are possible biologic correlates of those deficits. These findings support using SCD mice for studies of cognitive deficits in SCD and point to vulnerable brain areas with susceptibility to neuronal injury in SCD and to mechanisms that potentially underlie those deficits.

Keywords

Learning; memory; hippocampus; anemia; cerebellum; depression; anxiety; pain; neuronal injury

Introduction

Cerebrovascular accidents are serious complications in sickle cell disease (SCD) patients and occur in 5 to 10% of children and approximately 25% of adults by the fifth decade of life (Helton et al., 2014; Ohene-Frempong et al., 1998; Vichinsky et al., 2010). Moreover, approximately 22% of SCD children also develop silent strokes, which are evident on magnetic resonance imaging but are not associated with overt clinical neurologic deficits (DeBaun et al., 1998). As a consequence of overt or silent strokes, SCD patients can have global neurocognitive impairments as well as sensory and motor deficits (DeBaun et al., 1998). However, there is mounting evidence to suggest that even in the absence of overt cerebrovascular disease and/or parenchymal cerebral abnormalities on imaging studies, children and adults with SCD who are neurologically intact can still have profound neurocognitive dysfunction (Vichinsky et al., 2010; Wang et al., 2001). Researchers showed that nearly 20% of infants and toddlers with SCD score more than two standard deviations below the normal control mean on cognitive and motor neurodevelopmental evaluations (Glass et al., 2013). Further, some SCD patients without cerebrovascular accidents also show reduced performance in standard global cognitive function, working memory, processing speed and executive function tests (Steen et al., 2003; Vichinsky et al., 2010; Wang et al., 2001). Therefore, a great deal of evidence indicates that central nervous system dysfunction is highly prevalent among SCD patients and can present as significant neurocognitive deficits.

In addition to cognitive deficits, researchers have shown that SCD patients can also present alterations in other behavior domains. While large epidemiologic studies are lacking, several investigations show that attention-deficit/hyperactivity disorder, clinical depression, and anxiety are common among children and adult SCD patients (Benton et al., 2011; Levenson

et al., 2008; Lukoo et al., 2015; Wallen et al., 2014). Among children and adolescents with SCD admitted with vaso-occlusive episodes (VOEs), nearly 8% have some mental health diagnosis including mood and anxiety disorders and patients with those diagnoses have higher number of hospitalizations and prolonged length-of-stay (Myrvik et al., 2013). Therefore behavior alterations are prevalent in SCD, especially among patients who have frequent VOEs.

The mechanisms underlying cognitive deficits and behavior alterations in SCD are incompletely understood. In SCD patients who have had strokes, neurocognitive dysfunction can be partially explained by those cerebrovascular events. However, the mechanisms leading to cognitive dysfunction in SCD patients without strokes remain poorly understood. Few studies have examined the mechanism of brain dysfunction in SCD patients and researchers have shown that recurrent pain episodes, chronic hypoxia, worsening anemia, and aging correlate with the degree of cognitive deficits (Glass et al., 2013; Hogan et al., 2006; Steen et al., 2003; Vichinsky et al., 2010). However, these deficits and associations have not been consistently reported (Armstrong et al., 2013; Schatz and McClellan, 2006; Schatz and Roberts, 2007; Thompson et al., 2002; Wang et al., 1993). Therefore, continued investigations to elucidate mechanisms of brain dysfunction and behavioral deficits in SCD are needed to identify modifiable variables that contribute to those abnormalities.

Humanized SCD mice display hematologic abnormalities (leukocytosis, hemolytic anemia), pathologic features (kidney and liver dysfunction), and somatosensory alterations (sensitization of sensory nerve fibers) observed in humans with SCD (Cataldo et al., 2015; Garrison et al., 2012; Hanna; Kenyon et al., 2015; Kohli et al., 2010; Mancini et al., et al., 2006; Paszty et al., 1997; Wu et al., 2006). We hypothesized that SCD mice would have cognitive deficits and alteration in mood and emotionality. We tested these hypotheses by examining several behavior domains (cognitive function, anxiety, motivation, and depression) and seeking possible hematologic and neuropathologic correlates of the behavior changes observed in humanized SCD mouse strains.

Material and Methods

We conducted this study after approval from the Animal Care and Use Committee from Children's National Health System, Washington, DC and in accordance with recommendations from the Guide for the Care and Use of Laboratory Animals.

Animals

We examined several behavior domain in two strains of humanized SCD mice, the B6;129-*Hba^{tm1}(HBA)^{Tow} Hbb^{tm2}(HBG1,HBB*)^{Tow}/Hbb^{tm3}(HBG1,HBB)^{Tow}/J* strain, here referred to as the Townes strain (Hanna et al., 2007; Wu et al., 2006), and the *Hba^{tm1Paz} Hbb^{tm1}^{Tow} Tg(HBA-HBBs) 41Paz/J*, the BERK strain (Paszty et al., 1997). Townes mice (homozygous, heterozygous, and controls) do not express mouse hemoglobin. Homozygous Townes mice express more than 96% and heterozygous approximately 30% of human sickle and 70% of human hemoglobin A (Kenyon et al., 2015; Wu et al., 2006). Details about breeding and genotyping (Supplemental Figure 1) of Townes and BERK SCD mouse strains are provided in the supplemental files. Throughout experiments, animals had unrestricted access to mouse

chow and water, and were housed in ventilated cages in a temperature- and humidity-controlled (21°C) facility under a 12-h light–dark cycle. Female mice were housed together in order to synchronize estrous cycles and thus control for variability. Behavior experiments were not conducted on days when cages were changed in the animal facility as to avoid the effect of stress on behavior tasks.

Study Design and Experimental Protocol

We evaluated behavior in SCD and respective control mice in a cross-sectional fashion using several cohorts of naïve BERK and Townes mice animals that had not been previously enrolled in other studies. All Townes cohorts included balanced numbers of age-matched male and female mice of each genotype. As BERK mice have high spontaneous mortality (Jackson Laboratory and personal observation in our animal facility), are of limited availability, and most homozygous males are used for breeding and maintenance of the colony, only female mice were available in sufficient numbers for a few of the behavior tests performed.

Behavioral testing was performed between 9:00 AM and 3:00 PM in a quiet and dedicated behavior laboratory room. During experiments, mice of all genotypes (heterozygotes, homozygotes, and controls) were included in each cohort in order to control for the effect of time and possible investigator variability. In order to avoid confounding effects of repeated handling and multiple behavior assays, each animal underwent only one behavior test, with one exception, i.e., animals undergoing voluntary wheel running test also underwent grip strength measurements.

Behavioral Studies

Water T-maze—In order to examine the effect of SCD on cognitive learning and memory, we conducted the water T-maze test as previously described (Tanimura et al., 2008; Wang et al., 2011). A T-maze (San Diego Instruments, San Diego, CA) apparatus made of beige plastic (7.5 cm wide, 32 cm long, and 17 cm tall) was placed in a predetermined location in a dedicated behavior laboratory and spatial cues were provided by the furniture, additional equipment, and overhead lighting. The test included a pre-testing session aimed at identifying whether a mouse had turning biases [defined as five entries or more into the same arm (right or left) out of eight trials]. If a mouse showed turning bias during the pre-testing session, the scape platform was placed on the non-preferred arm during the testing sessions. Each trial started with placement of the mouse in the starting arm of the T-maze and was completed when the mouse either, reached and stayed on the platform for two seconds, or when 60 seconds had elapsed. When the mouse did not reach the platform within the allotted time, it was gently guided to it after 60s. Once each trial was complete, the mouse was left on the platform for 15 s and subsequently returned to its cages. During each trial, a mouse showed a correct response if it navigated directly from the start arm to the platform without entering the arm without the platform. Spatial learning criterion was reached when a mouse showed correct responses during at least seven out of eight trials for three consecutive days. We also examined cognitive flexibility by testing reversal learning. On the day after each mouse reached spatial learning criterion, the reversal learning test was initiated. Testing procedures for reversal learning were similar except that the position of the

platform was reversed. Criterion for reversal learning was reached when a mouse displayed at least seven correct responses in the eight trials for three consecutive days. During the T-maze test, we also measured latency to reach the platform in each trial during the first three days of spatial and reversal learning tests.

Elevated plus maze—In order to evaluate the effect of SCD emotionality, we conducted the elevated plus maze test as previously described (Lister, 1987). Briefly, the elevated plus maze (San Diego Instruments, San Diego, CA) has a "+" shape and is composed of two open and two identical enclosed arms elevated 40 cm above the floor. During a single video-recorded testing session, one mouse was placed in the center of the plus-shaped maze facing one of the open arms and was allowed to freely explore the maze for five minutes.

Subsequently, a trained observer, who was unaware of the animal's genotype, counted the number of entries into each of the open and enclosed arms and measured time spent inside the enclosed and open arms and center area of the maze using a stop watch. Rodents have a highly exploratory nature and yet possess a fear of open spaces and brightly-lit areas (Albani et al., 2015). In the elevated plus-maze, greater time spent in closed arms is suggestive of anxiety-like behavior, an interpretation that is validated by findings that time spent in open arms is increased by anxiolytic drugs (Lister, 1987).

Forced swim test—In order to examine the effect of SCD on mood, we performed the forced swim test as previously described (Bogdanova et al., 2013; Castagne et al., 2010; Wang et al., 2011). The forced swim test is also known as the behavior despair test and is used as a model of depression and to determine clinical efficacy of antidepressant drugs (Bogdanova et al., 2013; Cryan and Mombereau, 2004; Porsolt et al., 1978). Briefly, the forced swimming test included a 6-min pre-test session followed by a 6-min test session 24 hours later. The pre-test session is meant to serve as a stressor which is believed to produce a state of behavior despair or passive stress coping behavior (Porsolt et al., 1978). For each session, mice were placed individually into a water (23–25°C) filled, 22-cm deep plastic bucket that measured 21 cm in diameter. The 6-min test sessions were video-recorded and a trained observer, unaware of the animal's genotype, scored every five seconds of the last 4-min of the test session using a time sampling technique to determine immobility, climbing, or swimming times (Bogdanova et al., 2013; Wang et al., 2011). In the forced swim test, greater time spent immobile is suggestive of depression-like behavior, an interpretation validated by findings that immobility behavior is decreased by effective antidepressant drugs (Barkus, 2013; Cryan and Mombereau, 2004; Cryan et al., 2005; Lucki et al., 2001).

Voluntary wheel running—In order to evaluate overall physical activity and motivation in SCD mice, we measured daily distance ran on the voluntary wheel in young and old mice (Cobos et al., 2012; Meijer and Robbers, 2014; Novak et al., 2012). Mice were individually placed in transparent polycarbonate cages (34 cm long, 20 cm wide and 13 cm tall) that contained a stainless steel activity wheel (5 cm wide and 11.5 cm in diameter), which could turn in both directions (Mini-Mitter Company, Inc., Bend, OR). Multiple cages were placed side by side such that mice were able to see each other. A computer program was connected to all wheels and automatically recorded individual wheel revolutions as well as the distance traveled by each animal in the wheel over 24-h intervals. Mice were allowed to

acclimate to individual cages with free access to the running wheel for three days before any data was collected. Starting on the fourth day, measurements of total daily distance ran were recorded for four to five consecutive days. Animals remained in the individual cages for the duration of the experiment (seven to eight days).

Grip Force—In order to determine the effect of SCD on motor strength, we performed the grip force test in SCD mice using the Grip Strength Meter (GSM, San Diego, Inc., San Diego, CA) as described (Kehl et al., 2000). In addition to assessing motor strength, the grip force is interpreted as a surrogate measure of deep tissue hyperalgesia in SCD mice (Kohli et al., 2010). Before testing, animals were trained on grip force measurement over five sessions. During measurements, animals were gently held by the tail and were allowed to grasp a steel grip gauge with their forepaws. The mice were gently pulled away from the grip gauges in a steady fashion until the grip was released. The force exerted at the time of grip release was recorded as the grip force. An identical procedure was used to measure overall total grip force except that all four limbs were allowed to grasp two steel grip gauges at the same time. Grip force was measured for three consecutive days and at each time point, grip force was calculated as the average of five consecutive measurements. Grip force measurements were controlled for body weight by dividing the measured grip force (g) by the mouse body weight (g).

Hematologic parameters

Blood was collected from anesthetized (isoflurane in 100% oxygen) mice via cardiac puncture into heparin-coated syringes. Female and male mice of all genotypes at young and old age were included in this experiment. Samples were left at room temperature for 10min and complete blood cell counts were obtained using the Hemavet blood counter (Drew Scientific, Dallas, TX). The instrument was calibrated weekly and adjustments on cell detection counts were performed according to manufacturer's instructions every time reagents were replaced.

Histopathology

Mice were anesthetized (3% isoflurane in 100% fraction of inspired oxygen) and perfused (intracardially) first with phosphate buffered saline (pH 7.4) until clear perfusate was observed out of the right atrium, and subsequently with 4% paraformaldehyde. The brains were removed and postfixed with 4% paraformaldehyde at 4°C for 24 hours. All brains were sectioned coronally, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin (H&E). At least two sections per region were examined by a neuropathologist (M.Q.) who was unaware of the animal's genotype. Areas examined included the frontal and temporal cortical and subcortical areas as well as hippocampus, and cerebellum. Micrographs were obtained using an Olympus BX40 microscope, Olympus DP71 camera, and DP Manager DP-BSW acquisition software.

Brain iron levels

A group of Townes siblings were anesthetized with isoflurane and euthanized by cervical dislocation. Cortex, cerebellum and hippocampus were harvested, immediately frozen, and stored at -80 C. Tissue samples were prepared by cutting 25–40 mg of the frozen tissues,

homogenized in 1 mL of distilled water by rapid agitation with silica beads in a FastPrep 24 (MP Biomedical, Santa Ana, CA), and centrifuged (15,000 rpm, 10 minutes, 4 C). The supernatant was used for iron assays. Iron levels were measured colorimetrically following manufacturer's instructions (BioAssay Systems, Hayward, CA). Absorbance values of the two trials of each standard and samples were averaged in all analyses. Values for the known concentrations were used to create standard curves, and iron values for the samples were calculated by interpolation.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) with SigmaPlot 11.0 (Chicago, IL). One-way to multi-factor factorial ANOVAs were used to analyze fixed effect models. Repeated measures ANOVAs were used to model longitudinal data (voluntary wheel running and grip strength). For all ANOVAs, residuals were examined for normality and homogeneity. Log transformation of the data was performed when applicable. The Holm-Sidak test was used for post hoc pairwise comparisons when appropriate once main effects of genotype, sex, and age were determined. For the analysis of the water T-maze test (days to criterion for spatial and reversal learning), we used Kruskal-Wallis ANOVA on ranks. In addition, we performed a Kaplan-Meier survival analysis with Logrank (Mantel-Cox) as post hoc tests. F test statistic for each factor and between factors (interactions) entered in the ANOVA analysis are reported and least square means (model based means) and their standard errors are reported unless otherwise indicated. P-values ≤ 0.05 were considered significant.

Results

Table 1 lists number of mice and median age and interquartile range in weeks for each genotype and strain according to behavior testing and hematologic and pathologic evaluation.

SCD mice have cognitive learning and memory deficits

Male and females from all genotypes reached criterion for spatial and reversal learning at similar times ($p > 0.5$). However, in the water T-maze task, SCD mice showed significant learning and memory impairment (Figure 1) as homozygous and heterozygous Townes took significant longer time to reach spatial learning criterion compared to controls ($p < 0.001$, Figure 1A, Kruskal-Wallis ANOVA on Ranks). Kaplan-Meier survival analysis confirmed the presence of spatial learning impairment as both homozygous and heterozygous Townes took significantly longer to reach criterion compared to controls (both $p < 0.001$, Figure 1B). Contrary to the observed spatial learning impairment, homozygous and heterozygous Townes reached reversal learning criterion at similar times compared to control mice ($p > 0.2$, Figures 1D, 1E). These increases in time to reach criterion for spatial learning do suggest that SCD mice have impaired cognitive learning and memory deficits. In contrast, during the reverse learning task, these deficits are not present, which suggests learning flexibility.

We also examined the latency to reach the platform in the water T-maze during the first three days of spatial and reversal learning testing. As shown in Figures 1C and F, on average

mice were able to reach the platform within five to 25 seconds over the first three days of spatial and reversal learning testing. During spatial learning, the effect of genotype on latency to reach the platform varied according to day of testing as there were day*genotype interactions ($F_{4, 110} = 4.7$, $p=0.002$). Specifically, while animals of all genotypes were able to reach the platform faster on the third compared to the first (all $p<0.001$), homozygotes took significantly longer times to reach the platform on the second day of spatial learning testing compared to controls ($p=0.011$, Figure 1C). In contrast, there were no significant differences among genotypes in time to reach the platform on days one ($p = 0.408$) and three ($p = 0.092$).

During reversal learning, animals of all genotypes reached the platform in significantly shorter times on the third compared to the first day ($F_{2, 110} = 237$, $p<0.001$ for main effect of day, Figure 1F). Over the three days of reversal learning testing, there was also an overall effect of genotype ($F_{2,110} = 4.5$, $p=0.015$, Figure 1F) as homozygotes, but not heterozygotes ($p=0.121$) took longer times to reach the platform than did controls. Post-hoc analysis indicated that homozygotes took significantly longer times to reach the platform on the second and third days compared to controls ($p = 0.022$, Figure 1F).

SCD mice have increased depression-like behavior

Regardless of genotype, male and female mice had similar immobility, swimming, and climbing times during the forced swim test (all $p>0.38$). However, homozygous Townes spent significantly greater time immobile during the forced swim test compared with controls and heterozygotes, thus suggesting the presence of depression-like behavior ($F_{2,72} = 18.66$, $p<0.001$, for main effect of genotype, Figure 2A). In addition, there was an effect of genotype on time spent climbing ($F_{2,72} = 11.57$, $p<0.001$, main genotype effect, Figure 2B). Specifically, homozygous and heterozygous Townes spent significantly less time climbing during the forced swim test compared to control mice ($p<0.001$ and $p=0.002$ homozygotes and heterozygotes respectively, Figure 2B). There was also an effect of genotype on time spent swimming ($F_{2,72} = 5.99$, $p=0.004$) and interestingly, heterozygous spent significantly longer time swimming compared to homozygous mice ($p=0.005$, Figure 2C) and to controls ($p=0.017$). The increased immobility and decreased climbing times suggests the presence of depression-like behavior in homozygous SCD mice.

SCD mice have increased anxiety-like behavior

Among Townes mice, regardless of genotype, there was no effect of sex on times spent inside arms or on number of entries to open or closed arms ($p = 0.269$). In contrast, homozygous Townes mice spent significantly greater time (percentage) in closed arms ($F_{2,96} = 9.54$, $p<0.001$) and lower time in the center and open arms ($F_{2,96} = 9.6$, $p<0.001$) of the plus-maze compared with controls and heterozygotes (Figure 3A and 3B). There was also an effect of genotype in number of entries to closed arms ($F_{2,96} = 9.54$, $p=0.001$, main effect of genotype, Figure 3C) as homozygous Townes had significantly less entries onto closed arms compared to heterozygotes and controls. The findings of increased time spent in the enclosed arms of the elevated plus maze suggests that homozygous Townes mice have increased anxiety-like behavior.

We also examined whether increased anxiety-like behavior was present in BERK mice (Supplemental Figure 2). Akin to observations in Townes, homozygous BERKs displayed increased anxiety-like behavior. There was a significant effect of genotype in time spent in closed arms and in center and open arms ($F_{2,39} = 12.35$, $p < 0.001$). Specifically, homozygous BERKs spent significantly greater percentage of time in closed arms and lower time in the center and open arms of the plus-maze compared with controls and heterozygotes (all $p < 0.003$, Supplemental Figure 2). Additionally, there was a significant effect of genotype in number of entries onto open arms ($F_{2,39} = 9.68$, $p < 0.001$) as homozygous and heterozygous BERKs had significantly less entries onto open arms compared to controls (Supplemental Figure 2D).

Older SCD mice have voluntary wheel running performance impairment

We conducted the voluntary wheel running test to examine the effect of SCD in a complex behavior that involves several domains including defensive, motivational, and depression- and anxiety-like behaviors, and motor function (Meijer and Robbers, 2014; Novak et al., 2012) in one cohort of young and another of old mice in a cross sectional fashion. Among young and old Townes mice, there was a significant effect of sex on distances ran on the voluntary wheel as both young and old female mice ran significantly longer distances compared to males (main effect of sex, $F_{1,70} = 49.89$, $p < 0.001$, Figure 4). Conversely, the effect of genotype on distances ran on voluntary wheel varied according to mouse age as there were age*genotype interactions ($F_{2,70} = 4.56$, $p = 0.01$). Noticeably, in control mice, distances ran on the voluntary wheel did not vary with age as both young and old control mice ran similar distances on the wheel ($p = 0.387$). Conversely, older homozygous and heterozygous Townes ran significantly shorter distances on the voluntary wheel compared to younger animals [homozygous ($p < 0.001$) and heterozygous ($p = 0.003$), Figure 5]. At young age, Townes controls, heterozygotes and homozygotes ran similar daily distances on the voluntary wheel ($p > 0.5$, Figures 4A, 4B). In contrast, over days, older female homozygous and heterozygous Townes ran significantly shorter distances overall on the voluntary wheel compared to controls ($p < 0.001$ and $p = 0.005$ for homozygotes and heterozygotes compared to controls respectively, Figure 4C). Further, over days, older male homozygous and heterozygous Townes also ran significantly shorter distances overall compared to controls ($p = 0.001$ and $p = 0.014$ for homozygotes and heterozygotes compared to controls respectively, Figure 4D).

Older BERK female mice also had decreased voluntary wheel performance overall as homozygous and heterozygous BERKs combined ran significantly shorter distances compared to control mice ($F_{1,14} = 7.52$, $p = 0.016$, Supplemental Figure 3).

SCD mice have decreased muscle strength

We then measured muscle strength and hypothesized that Townes mice would have decreased muscle strength, which could have contributed to decreased voluntary wheel performance in older mice. Among Townes, females, regardless of genotype, had greater forelimb ($F_{1,36} = 48.33$, $p < 0.001$, Figure 5) and total grip strength ($F_{1,36} = 56.82$, $p < 0.001$, Figure 5) compared to males over days tested. In addition, there was an effect of genotype in forelimb ($F_{1,36} = 30.33$, $p < 0.001$, Figure 5) and total grip strength ($F_{1,36} = 26.21$, $p < 0.001$,

Figure 5). Specifically, among Townes, homozygotes (male and female) had significantly lower forelimb and total grip strength compared to controls (all, $p < 0.001$, Figure 5) and heterozygotes (all, $p = 0.008$, Figure 5). Heterozygous Townes had similar forelimb and total grip strength compared to controls (all, $p = 0.198$, Figure 5).

In concert with previous studies, homozygous BERKs had significantly lower overall forelimb ($F_{1,27} = 41.75$, $p < 0.001$) and total ($F_{1,27} = 8.07$, $p = 0.002$, overall effect of genotype) grip strength compared to controls, Supplemental Figure 4. Heterozygous BERKs had significantly lower forelimb grip strength compared to controls ($p < 0.001$) overall. In contrast, heterozygous BERKs had similar total grip strength compared to controls ($p = 0.074$, Supplemental Figure 4).

Effect of age, sex and genotype on hematologic parameters

After we observed cognitive and voluntary wheel performance deficits, we sought to identify biologic correlates of those deficits. We then obtained complete blood counts in young and older mice and hypothesized that anemia, which occurs in sickling mice (homozygotes), could worsen with aging and that decreases in hemoglobin could possibly contribute to and correlate with those deficits. There were significant main effects of genotype for hemoglobin ($F_{2,59} = 49.62$, $p < 0.001$), hematocrit ($F_{2,59} = 41.30$, $p < 0.001$), and red blood cell counts ($F_{2,59} = 138.64$, $p < 0.001$). However, while homozygous Townes were anemic compared to controls ($p < 0.001$, Figure 6A, 6B, and 6C), there was an effect of age in homozygous, but not heterozygous and control Townes [age*genotype interactions for hemoglobin ($F_{2,59} = 5.16$, $p = 0.009$) and hematocrit ($F_{2,59} = 3.66$, $p = 0.032$)]. Specifically, among homozygous Townes, older mice had significantly lower hemoglobin ($p < 0.001$) and hematocrit ($p = 0.004$) compared to younger homozygotes (Figure 6A, 6B). Conversely, heterozygous and control mice were not anemic and among them there were no significant age-related changes in hemoglobin and hematocrit (all, $p > 0.49$, Figure 6).

Homozygous Townes had significantly higher white blood cell counts compared to controls and heterozygotes ($F_{2,59} = 172.65$, $p < 0.001$, $p < 0.001$, Figure 6D). However, there were significant sex*genotype interactions ($F_{2,59} = 11.35$, $p < 0.001$) indicating that among homozygotes, males had higher white blood cell count compared to females ($p < 0.001$). Among female, but not male mice, heterozygous Townes had higher platelet counts compared to controls and homozygotes ($p < 0.001$, Figure 6E).

SCD mice have abnormal neuronal morphology in hippocampus and cerebellum

We then sought to identify neuropathologic correlates of cognitive and behavior deficits observed in SCD mice and analyzed H&E slides from brains of young and older Townes mice. In concert with previous reports of brain pathology in BERK mice (Manci et al., 2006), on brain H&E slides there was no evidence of strokes or overt morphological differences in cortical or subcortical areas comparing homozygous, heterozygous, and control Townes. Conversely, among older heterozygous and homozygous Townes, dark/pyknotic neurons, suggestive of hypoxic injury, were observed both in hippocampus and cerebellum. Further, among older animals, based on number and distribution of these abnormal neurons, three distinct groups corresponding to the animal's genotype—Townes

controls (healthy neurons), heterozygotes (mild neuronal injury) and homozygotes (severe neuronal injury) could be identified by the neuropathologist (MQ) who was unaware of animals' genotype. Old homozygotes exhibited large bands of dark/pyknotic (shrunken) neurons, mostly in the CA1 and CA3 fields of the hippocampus (Figure 7). Heterozygous Townes also had dark/pyknotic neurons in CA1 and CA3 fields; however, compared to homozygotes, those shrunken neurons were present in significantly lower numbers and were interspersed with normal neurons. Contrary to old homozygotes, bands of dark/pyknotic neurons were not observed in heterozygous Townes. We also examined a cohort of young Townes mice and found no significant differences in hippocampus histopathology comparing young and old Townes controls and young and old heterozygous mice (data not shown). In contrast, while young homozygous Townes had numerous dark/pyknotic neurons in CA1 and CA3 fields, the severity of neuronal morphologic changes in the hippocampus was significantly milder than that observed in older homozygotes, thus suggesting that neuronal injury in hippocampus was significantly more severe in older mice (Figure 7).

With regards to the cerebellum, Townes controls at young and older age had normally appearing Purkinje. The neuronal morphology observed in cerebellum of young homozygotes and young and old heterozygotes appeared similar and revealed slight increases in neuronal acidophilia compared to controls. Conversely, old homozygous Townes had increased Purkinje cell acidophilia and focal areas of decreased neuronal density (neuronal drop-out, Figure 7).

SCD mice have altered iron homeostasis in the brain

As iron dyshomeostasis has been implicated in behavior and cognitive deficits in mice (Brunette et al., 2010; Carlson et al., 2010), we measured total iron levels in several brain regions in Townes mice. Among controls, heterozygotes and homozygotes, cerebellum iron levels were significantly higher than those measured in cortex and hippocampus ($F_{2,34} = 31.93$, $p < 0.001$, Figure 8). Further, heterozygous and homozygous Townes combined had significantly lower hippocampus iron tissue levels compared to controls ($p = 0.03$, Figure 8), thus suggesting iron deficiency in hippocampus of SCD mice.

Discussion

This study is the first to demonstrate that humanized SCD mice (Townes and BERK strains), which are known to display several phenotypes of human SCD (homozygous) and sickle cell trait (heterozygous), also display cognitive deficits, depression- and anxiety-like behaviors, and have decreased voluntary exercise capacity. These behavior deficits in homozygous SCD mice were associated with profound alterations in hippocampus and cerebellum neuronal morphology, which are suggestive of hypoxic injury. Additionally, some behavior alterations, hematologic abnormalities, and the neuropathologic changes in hippocampus and cerebellum in homozygous mice appeared to be more severe in older than in younger animals. These results point to vulnerable brain regions which are susceptible to neuronal injury in SCD mice. Further, these findings suggest that humanized SCD mice could be valuable for investigations of the mechanisms that underlie cognitive deficits and emotionality and mood alterations associated with SCD.

Here we showed that in the water T-maze, older homozygous and heterozygous Townes took significant longer time to reaching spatial learning criterion, which suggests that learning and memory processes are impaired in SCD mice. Interestingly, those deficits were not present during the reversal learning task, which suggests the presence of cognitive flexibility in SCD mice. Animal and human studies indicate that intact hippocampal and cerebellar functions are required for normal cognitive learning and memory and that both spatial and reversal learning in the water T-maze are impaired after hippocampal lesions (Burgess et al., 2002; Gandhi et al., 2000; Hunt et al., 2007; Wang et al., 2011). However, during reversal learning, the striatum, frontal cortex, and nucleus accumbens neurons are engaged (Clarke et al., 2008). Thus it is conceivable that the engagement of brain regions other than hippocampus, which did not appear abnormal on histopathology, explains the findings of unaltered reversal learning. Taken together, these findings suggest that mice with SCD show cognitive flexibility, recapitulates the cognitive deficit phenotype observed in humans with SCD, and could be valuable for studies of therapies aimed at improving cognitive deficits in SCD.

On the basis of clinical magnetic resonance imaging, researchers have shown that the volumes of hippocampus, amygdala, cerebellar cortex, thalamus, and basal ganglia of SCD patients are significantly decreased compared to those of controls subjects (Kawadler et al., 2013). In addition, in SCD patients, these reductions in basal ganglia and thalamus volumes correlate with cognitive dysfunction as shown by lower Working memory, Performance IQ, and Perceptual Organization scores (Mackin et al., 2014). Here we found that the cognitive deficits observed in older Townes mice were associated with significant neuronal changes in hippocampus and cerebellum that are suggestive of hypoxic injury. Importantly, these neuronal changes were more severe in hippocampus and more pronounced in older homozygotes than in young homozygotes and heterozygous mice. These findings raise the possibility that in Townes mice, there may be a gene dose effect and that the differing degree of sickle hemoglobin expression (Kenyon et al., 2015; Khaibullina et al., 2015) and/or disease severity and duration affect cognitive function. While the neuropathologic changes identified in SCD mice warrant further characterization, it is conceivable that the neuronal changes in hippocampus and cerebellum could at least in part explain the cognitive deficits in SCD mice. These results also suggest that SCD poses a risk for cognitive deficits and neuronal damage in hippocampus that possibly increases with aging and anemia severity.

The voluntary wheel test is often used to examine physical activity and muscle function in rodents in studies of obesity, energy homeostasis, and muscle dystrophy (Grounds et al., 2008; Novak et al., 2012). However, voluntary wheel running performance also reflects a complex behavior in and of itself and involves several behavior realms including defensive, motivational, and depression- and anxiety-like behaviors and motor function (Meijer and Robbers, 2014; Novak et al., 2012). Here we observed that older homozygous and heterozygous SCD mice had impairment in voluntary wheel running performance as well as depression-like behavior. Further, voluntary wheel running performance declined with aging as older homozygous and heterozygous mice ran shorter distances than did younger animals. It is conceivable that voluntary wheel performance deficits in older homozygotes was related to worsening anemia as old homozygous mice had lower hemoglobin and hematocrit

compared to younger animals. However, while worsening anemia could partially explain the declining voluntary wheel running performance in homozygous mice, it does not explain the decreased performance in heterozygous Townes as these mice are not anemic. Therefore, anemia might contribute to but does not entirely explain the voluntary wheel running deficits in SCD mice.

Our findings of reduced voluntary exercise capacity in two strains of SCD mice are in concert with several studies showing that children and adults with SCD have reduced exercise capacity and decreased muscle force(Callahan et al., 2002; Chaudry et al., 2013; Dougherty et al., 2011; Ong et al., 2013; van Beers et al., 2014). In SCD, mechanisms believed to underlie these limitations include pulmonary and peripheral vascular diseases and anemia(Callahan et al., 2002; Chaudry et al., 2013; Dougherty et al., 2011; Ong et al., 2013; van Beers et al., 2014). We posit that yet another possible contributing factor is muscle hyperalgesia, which in rodents can be associated with decreased muscle strength and reduced running distances on the voluntary wheel(Cobos et al., 2012; Grace et al., 2014). Specifically, in inflammatory pain models, distances ran on the voluntary wheel are significantly reduced by hind paw inflammation, a deficit that is dose-dependently reversed by anti-inflammatory and analgesic drugs(Cobos et al., 2012). We and others have shown that humanized SCD mice have decreased grip force, which is believed to reflect muscle hyperalgesia/pain as after administration of opioids, cannabinoid, or α_2 -adrenoreceptor agonists, grip force increases(Calhoun et al., 2015; Kohli et al., 2010). Therefore, it is conceivable that in SCD mice, muscle hyperalgesia also contributes to reduced muscle strength and decreased voluntary wheel running performance.

A number of studies show that mood and emotionality disturbances are prevalent in SCD patients(Sogutlu et al., 2011). In SCD children hospitalized for VOEs nearly 10% have mental, health illnesses including anxiety, mood, disruptive behavior, and substance use disorders(Myrvik et al., 2013). Importantly, children who carry mental health diagnoses are admitted more frequently and stay in the hospital longer than those who do not(Myrvik et al., 2013). Further, in large studies of adult SCD patients, depression is reported in approximately 30% and anxiety in 7% of patients and increased somatic pain/symptom burden is significantly associated with both depression and anxiety(Sogutlu et al., 2011). In that same study, depression and anxiety were predictors of and associated with increased prevalence of daily pain(Levenson et al., 2008). Therefore, in SCD patients, recurrent episodes of pain appear to be associated with alterations in emotionality and mood disorders. In rodent chronic pain models, there is a correlation between nociception alterations and emotionality and mood changes(Tajerian et al., 2014; Zhang et al., 2014). We and others have shown that SCD mice have increased sensitization of sensory nerve fibers, altered nocifensive behavior, and hypersensitivity to noxious stimuli(Calhoun et al., 2015; Kenyon et al., 2015; Kohli et al., 2010). Here we showed that, SCD mice also have cognitive and emotionality and mood disturbances. While we did not establish a causal relationship between nociception alterations and behavioral changes observed in SCD mice, future studies will define such relationship.

One must entertain the possibility that age dependent limitations in voluntary exercise capacity observed in SCD mice could have affected animals' performance and confounded

the results of the behavior tests. We posit that while certainly possible, a number of results do not support this hypothesis. For example, in the water T-maze, older Townes mice from all genotypes (control, heterozygotes, and homozygotes) were able to reach the platform in less than 15 seconds on the third day of testing, thus suggesting those deficits in exercise capacity were unlikely to impede an animal from reaching the target and learning criterion. In the forced swim test, while older homozygous Townes spent more time immobile than did controls, both controls and homozygous mice spent similar time swimming, suggesting that the depression-like phenotype (increased immobility time) in homozygous mice was unrelated to impairment in swimming or exercise capacities. Further, heterozygous Townes, which showed impaired voluntary wheel running performance (similar to that of homozygotes), spent significantly longer times swimming than did controls or homozygous mice. In the elevated plus maze, while we did not evaluate older Townes mice, which is a limitation in this study, middle-aged homozygous but not heterozygous mice displayed depression-like behavior compared to controls. However, older BERK female homozygotes did show increased depression-like behavior, whereas older heterozygous BERKs, which also had impaired exercise capacity, did not. Further, females of all Townes genotypes ran significantly shorter distances than males on the voluntary wheel. We posit that if abnormalities in voluntary exercise capacity were to affect performance on other behavior tasks examined, differences between male and female performances on those other tasks would have been observed. In all but voluntary wheel running and grip force tasks, male and female Townes mice had similar performance. Nevertheless, while the possibility that deficits in voluntary exercise could have affected cognitive performance emotionality and mood behavior exists, the observations noted above suggest otherwise.

Among Townes, control, heterozygous, and homozygous have similar genetic backgrounds (supplemental files). A limitation of this investigation is that among BERKs, while heterozygous and homozygous have similar mixed genetic background, the ideal control strain (HbAA-BERK), which has similar mixed genetic background and expresses normal human hemoglobin, is commercially unavailable. For this reason, we used C57BL/6J as controls, which albeit not the best control group and only partially matches the mixed genetic background of heterozygotes and homozygotes, it represents the predominant background strain for BERK SCD mice but. Others have shown that, while cold sensitivity and grip force are similar comparing C57BL/6J and HbAA-BERK, these strains display differences in nocifensive behavior (heat and mechanical sensitivity)(Cain et al., 2012). Here we found that the results observed in most behavior tasks examined in BERK mice were in concert with those seen in Townes strain with one exception. In the elevated plus maze task, homozygous BERKs spent more time in the closed arms than did controls, indicating depression-like phenotype similar to Townes. In contrast, homozygous and control BERKs had similar number of entries to closed arms, unlike Townes. These discrepancies could possibly be related to differences in genetic background of BERK mice genotypes. Therefore, while not an issue for the Townes mice, it is conceivable that the results observed for the BERK strain were confounded by differences in genetic background among different BERK genotypes and should interpreted with caution.

Given ongoing hemolysis in SCD mice, we hypothesized that iron content would be higher in brains of SCD mice. Our hypothesis was proven incorrect as we found that there were

differential iron levels among brain regions and that SCD mice actually had lower iron levels in hippocampus. These findings were surprising and are worthy of further exploration. Others have shown that in many organs in SCD mice including liver, lung, and kidneys, but not heart or bone marrow have higher iron content than in controls (Manci et al., 2006). However, animal studies indicate that iron content in peripheral organs such as the liver, poorly correlates with brain iron levels thus suggesting that the regulatory systems for central and peripheral iron are independent (Jones et al., 2003). Interestingly, there is some evidence from animal studies to suggest that mesencephalic and basal ganglia iron homeostasis are linked to serotonin signaling, which can have implication for a number of behavior domains including mood and emotionality (Carneiro et al., 2009). Nevertheless, while iron dyshomeostasis is known to occur in SCD, it is unknown whether iron brain content is related to any of the behavior phenotypes observed in SCD mice. Future studies will explore the relationship between brain iron content and metabolism and behavior in SCD.

In conclusion, several pathologic underpinnings of SCD, such as iron dyshomeostasis, chronic anemia, inflammation, recurrent episodes of tissue hypoxia and ischemia and reperfusion due to vaso-occlusion, acute and chronic pain, and sensitization of sensory nerve fibers, can potentially impact cerebral function (Andreotti et al., 2014; Iampietro et al., 2014; van Beers et al., 2015; Vichinsky et al., 2010). Here we described cognitive deficits, emotional behavior and mood changes, and reduced voluntary exercise capacity in two humanized SCD mouse strains. We also showed that these deficits are accompanied by anemia, decreased iron levels in hippocampus, and profound neuronal abnormalities in hippocampus and cerebellum, some of which worsened with aging. Taken together, these findings support the notion that humanized SCD mice are suitable models for studies of the pathobiology of cognitive, mood and emotional deficits of SCD. Future studies will provide novel insights on the molecular and cellular mechanisms underlying brain dysfunction in SCD and allow for the design of targeted therapies to improve the neuropathologic and cognitive changes associated with the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Sick cell disease (SCD) mice have cognitive deficits as described in SCD patients
2. SCD mice also have increased anxiety- and depression-like behavioral changes
3. Two strains of SCD mice show poor performance on voluntary wheel running task
4. The deficits are associated with severe neuronal injury in hippocampus and cerebellum
5. Some behavioral deficits and neuropathologic changes in SCD are worse in older mice

Figure 1.

Cognitive learning and memory in Townes SCD mice. Homozygous and heterozygous Townes mice had cognitive deficits as they took significantly longer to reach criterion for spatial learning compared to control mice (A). Kaplan-Meier survival analysis of times to reaching criterion for spatial learning also indicates the presence of cognitive deficits in homozygous and heterozygous Townes mice as both took significantly longer time to reach criterion of spatial learning compared to control mice (both $P < 0.001$, B). During the water T-maze spatial learning test, homozygous mice took significantly longer times to reach the platform on the second day of testing compared to control mice (C). SCD genotype had no effect on reversal learning as homozygous and heterozygous Townes reached criterion for reversal learning at similar times compared to control mice (both, $P = 0.2$, Figure 1D and 1E). During the reversal learning test, homozygous mice took longer times to reach the platform on the second and third days of testing compared to control mice (F). P values reflect post

hoc analysis comparing the groups indicated by the brackets. Fifty-eight mice underwent the water T-maze test, N=17–24 per genotype including balanced numbers of age-matched male and females. * reflects $p<0.05$, † reflects $p<0.01$, and ‡, $p<0.001$.

Figure 2.

Depression-like behavior in Townes SCD mice. Homozygous Townes mice spent significantly greater time immobile compared to controls and heterozygous Townes suggesting that homozygous animals have significant depression-like behavior compared with controls and heterozygous mice (A). Homozygous and heterozygous Townes mice spent significantly less time climbing during the forced swim test compared to control mice (B). Homozygous mice spent less time swimming compared to heterozygous mice (C). P values reflect post hoc analysis comparing the groups indicated by the brackets. Seventy-eight mice underwent the forced swim test, N=23–29 per genotype including balanced numbers of age-matched male and females. * reflects $p < 0.05$, † reflects $p < 0.01$, and ‡, $p < 0.001$.

Figure 3.

Anxiety-like behavior in Townes SCD mice. Homozygous mice spent a significantly greater percentage of time in closed arms and lower percentage of time in the center and open arms of the elevated plus maze compared with controls and heterozygotes, thus suggesting that homozygous Townes mice displayed significant anxiety-like behavior compared with controls and heterozygotes (A and B). In addition, homozygous mice had significantly lower number of entries onto the closed arms (C) but similar number of entries to the closed arms compared to heterozygotes and controls Townes mice (D). P values reflect post hoc analysis comparing the groups indicated by the brackets. One hundred and two mice underwent the elevated plus maze test, N=26–48 per genotype including balanced numbers of age-matched male and females. * reflects $p < 0.05$, † reflects $p < 0.01$, and ‡, $p < 0.001$.

Figure 4.

Effect of genotype, age, and sex on voluntary wheel running distances in Townes SCD mice. Controlling for genotype, young and older females ran significantly longer distances compared with male mice ($p < 0.001$, A, B, C, and D). The effect of genotype on distances ran on the voluntary wheel varied according to mouse age ($P = 0.008$ for age*genotype interactions). At young age (A and B), Townes controls, heterozygous and homozygous mice ran similar daily distances on the voluntary wheel ($P > 0.5$). In contrast, at older age, female homozygous and heterozygous Townes mice overall (C), ran significantly shorter distances on the voluntary wheel compared to control mice ($p < 0.001$ for homozygotes and $p = 0.005$ heterozygotes compared to controls respectively). Among older Townes, male homozygous (D) mice overall, also ran significantly shorter distances compared to controls ($p = 0.012$). Symbols reflect P values from post hoc analysis by day comparing homozygous and heterozygous with control. * reflects $p < 0.05$, † reflects $p < 0.01$, and ‡, $p < 0.001$. Eighty-two mice participated in the voluntary wheel running test, $N = 12-15$ per genotype including balanced numbers of age-matched male and females.

Figure 5.

Effect of genotype and sex of Townes SCD mice on muscle strength. Female Townes mice (A and C), regardless of genotype, had greater forelimb and total grip strength compared to male (B and D) mice over all three days tested ($P < 0.001$). Homozygous mice (male and female) had significantly lower forelimb and total grip strength compared to control Townes (all, $p < 0.001$) and to heterozygous Townes (all, $p = 0.008$). Over the three days tested, heterozygous Townes had similar forelimb and total grip strength compared to control mice (all, $P = 0.198$). Symbols reflect P values from post hoc analysis by day comparing homozygous with control. * reflects $p < 0.05$, † reflects $p < 0.01$, and ‡, $p < 0.001$. Forty-two mice participated in the grip force test, $N = 13-16$ per genotype including balanced numbers of age-matched male and females. g force/ g b.w. indicates g force/ g of body weight.

Figure 6.

Effect of age, sex, and genotype of Townes SCD mice on hematologic parameters. Overall, homozygous Townes mice had significantly lower hemoglobin, hematocrit, and red blood cell counts compared to control Townes (A, B, and C). In addition, among homozygous Townes, older mice had significantly lower hemoglobin ($p < 0.001$) and hematocrit ($p = 0.004$) compared to younger homozygous mice. In contrast, among control and heterozygous Townes, young and older mice had similar hemoglobin and hematocrit (all, $p > 0.49$). Homozygous Townes mice also had significantly higher white blood cell counts compared to control and heterozygous mice (D). Among homozygous mice, males had higher white blood cell count compared to females ($p < 0.001$). Among female, but not male mice, heterozygous Townes had higher platelet counts compared to controls and homozygous mice (E). Older homozygous male mice had higher platelet counts compared to male control mice. Symbols reflect P values from post hoc analysis comparing homozygous with control mice for each sex and age group. * reflects $p < 0.05$, † reflects $p < 0.01$, and ‡, $p < 0.001$. Seventy-one mice participated in this experiment, $N = 9-13$ per genotype including balanced numbers of age-matched male and females.

Figure 7.

Neuropathology in Townes SCD mice. Representative Hematoxylin and Eosin (H&E) stained slides for each genotype of Townes mice (N=10–13 per genotype). Unlike control Townes, old homozygous mice had long bands of dark/pyknotic (shrunken) neurons seen mostly in the CA 1 and CA 3 fields of the hippocampus. In heterozygous Townes mice, similar dark/pyknotic neurons were present in CA1 and CA3 fields of the hippocampus in significantly lower numbers, than in that seen in homozygotes, and those dark/pyknotic neurons were interspersed with morphologically normal neurons. In young homozygotes, the degree of hippocampus injury was milder than that observed in old homozygous mice. In the cerebellum of old homozygous mice, there were decreases in neuronal density (neuronal drop-out) compared to control Townes mice. All hippocampus panels are shown in original magnification x40 and CA1, CA3, and cerebellum panels are shown in original magnification x200.

Figure 8.

Iron tissue levels in cortex, hippocampus and cerebellum in Townes mice. Cerebellum iron tissue levels were significantly higher than those in cortex and hippocampus in controls, heterozygous and homozygous (all $p < 0.0015$). In hippocampus, heterozygous and homozygous Townes combined had significantly lower total iron tissue levels compared to control mice ($p = 0.03$). Fourteen mice were enrolled $N = 4-5$ per genotype. * reflects $p < 0.05$

Number of animals enrolled in this cross sectional study of behavioral domains and hematology and histopathology changes in sickle cell mice *

Table 1

Behavior test and Laboratory evaluation	Strain	Genotype			
		Control (N) Age (IQR)	Heterozygotes (N) Age (IQR)	Homozygotes (N) Age (IQR)	
Cognitive learning Water T-Maze	Townes	17	24	17	
		15.8 (15.6–16.7)	14 (13–15.7)	13.6 (13–15)	
Mood evaluation Forced swim test	Townes	26	23	29	
		21.2 (20–21.7)	18.3 (16.1–21.2)	19.6 (16.2–23.4)	
Emotionality Elevated plus maze	Townes	48	26	28	
		8.5 (7.6–8.8)	10 (9.5–10.3)	10.1 (10–11.5)	
	BERK	10	17	15	
		22 (21.5–23.2)	24.2 (22.4–24.3)	23 (22.6–23.4)	
Exercise capacity Voluntary wheel running	Townes (young)	12	13	14	
		6.2 (4.6–6.6)	6.3 (5–6.3)	5.9 (4.5–6.3)	
	Townes (older)	14	14	15	
		16.5 (16.5–16.8)	16.7 (15.4–17.7)	16.7 (16–17.5)	
	BERK	5	6	6	
		27 (22.8–30)	27.2 (27.2–28.7)	28 (27.7–30.4)	
Muscle strength Grip force	Townes	16	13	13	
		17.5 (17.6–18)	18 (16.4–19)	17.8 (17–19)	
	BERK	11	8	11	
		28 (24–31)	28.2 (28.2–28.7)	29 (28–31)	
Hematology Complete blood count	Townes (young)	13	13	12	
		6.3 (5.2–7.2)	6.4 (5.7–6.9)	6.6 (5.2–6.9)	
	Townes (older)	9	12	12	
		16 (14.7–16.5)	18 (16–20.2)	17 (16–19.6)	
Brain pathology Brain H&E	Townes (young)	6	3	4	
		10.7 (10.7–11)	7.4 (7.4–7.4)	7.2 (6.9–7.4)	
	Townes (older)	4	10	9	
		17 (15–18)	20.1 (20–20.3)	19.3 (17.5–19.7)	
Brain iron Total iron levels	Townes	4	4	5	

Behavior test and Laboratory evaluation	Strain	Genotype		
		Control (N) Age (IQR)	Heterozygotes (N) Age (IQR)	Homozygotes (N) Age (IQR)
		17 (15–19)	18.1 (17.8–18.1)	17.6 (17.4–17.7)

* Age is that at the start of respective behavior test and is shown as median and interquartile range in weeks. N indicates number of animals, IQR interquartile range, and H&E hematoxylin and eosin stain. All genotypes had balanced number of male and female mice except in BERK groups, which included only female mice