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Supplementary Online Data

DNA DAMAGE AND SYNAPTIC AND BEHAVIOURAL DISORDERS IN GLUCOSE-6-PHOSPHATE DEHYDROGENASE-DEFICIENT MICE.

Running title: G6PD deficiency in aging

Margaret M. Loniewska[1], Anmol P. Gupta[1], Shama Bhatia[1], Isabel Mackay-Clackett[1], Zhengping Jia[2] and Peter G. Wells[1,3]

[1]Faculty of Pharmacy, University of Toronto;
[2]Neurosciences and Mental Health, The Hospital for Sick Children & Department of Physiology, University of Toronto; and
[3]Department of Pharmacology and Toxicology, University of Toronto

Toronto, Ontario, Canada

Methods

Activity of G6PD in young and aging mice

For the measurement of G6PD activity, different brain regions dissected from young (2-5 months) and aging (12-18 months) mice were homogenized with a hand homogenizer in 500 µL of 50 mM Tris-HCl pH 7.4, and the homogenate was sonicated with a hand sonicator (Fisher Scientific 60 Sonic Dismembrator) for 10 sec. The homogenates were centrifuged for 30 min at 15,000 x g in a refrigerated microcentrifuge. The pellet was discarded and the protein content was determined in the supernatant using the bicinchoninic acid assay (SMITH *et al.* 1985). The G6PD activities in WT and G6PD-deficient mice were compared using a modified protocol (NINFALI *et al.* 1997; NICOL *et al.* 2000). Reaction rates for G6PD were determined by UV spectrophotometry using a plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA) at 340 nm at 15 sec intervals over 5 min. The final activity of G6PD was determined using the following equation: Net optical density x 40/6200 = µmol/min/ml of clear homogenate (IU/ml).

For the analysis of erythrocyte G6PD activity, blood samples were taken from the tail vein without sacrificing the animals using EDTA-coated capillary tubes to avoid blood clotting (Microvette 100, Sarstedt, Montreal, PQ). The red blood cells were separated by centrifugation at 600 x g and washed twice with PBS, each time followed by centrifugation at 600 x g. A 500 µL aliquot of PBS was added after the final wash. The red blood cell count was obtained using a hemocytometer (Brightline, Sigma-Aldrich, Oakville, ON). The blood sample was mixed with cold ddH2O in a 1:4 (blood:water) ratio to burst the cells and release the cytoplasmic enzymes, and G6PD activity was determined using the protocol above.

Comet assay: measurement of DNA single strand breaks in aging mice

DNA damage was measured by alkaline single-cell gel electrophoresis (SCGE), also known as the comet assay, as previously described (SINGH *et al.* 1988) with minor modifications. The general protocol includes dissociation of tissue into individual cells, mounting the cells in agarose onto microscopes slides, lysis of the cells with detergent and high salt to release the DNA, unwinding of the DNA, separating the damaged DNA fragments using electrophoresis, and finally staining and visualizing/scoring the resulting DNA "comet tails". The technique is based on the principle that any strand break in the DNA will cause the supercoiling to relax, allowing negatively charged loops of DNA to freely extend and migrate in the electric field, toward the anode creating a "tail". For the comet assay, animals were anesthetized by isofluorane and killed by cervical dislocation. Separate brain tissue specimens from the two brain areas of interest (hippocampus and cerebellum) were washed in cold mincing solution (Hanks' Balanced Salt Solution [HBSS], Ca++ and Mg++ free, 20 mM Na_2EDTA, 10% DMSO, pH 7.5) and placed in 1 ml of chilled mincing solution (1.5 ml for cerebellum). The tissue was cut into smaller pieces and the mixture was pushed through a 26 ¾ gauge needle to obtain dissociated cells. The sample was flash frozen until embedding. For embedding, the sample was mixed (1:1 v/v) with 0.5% low melting point agarose (prepared in Dulbecco's phosphate-buffered saline, Ca++, Mg++, and phenol free, pH 7.4) and layered on conventional slides that were pre-dipped in 1% normal melting point agarose. Slides were immersed in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100 and DMSO 10%, pH 10) for 3 days at 4°C to lyse the cells and allow DNA unfolding.

After lysis, the slides were rinsed with and placed into alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH>13) for 1 hr for DNA unwinding. The slides were positioned in a 20-slide COMET assay tank (Cleaver Scientific Ltd., Rugby, UK) and covered with fresh electrophoresis buffer. Electrophoresis was performed at 0.6 mV/cm and 300 mA for 24 min. Slides were then neutralized with 0.4 M Tris for 5 min, immersed in 100% cold ethanol for 5 min and air dried. Slides were then stained with SYBR Gold (Invitrogen, Molecular Probes, Eugene, OR) and visualized with a fluorescence microscope (Zeiss Axioplan 2 Imaging upright fluorescence) and a CCD camera. For each sample a total of at least 100 comets were scored using the TriTek CometScore™ Freeware v1.5. The olive tail moment is the length of the tail multiplied by the fraction of DNA in the tail.

γH2AX in individual brain regions: measurement of DNA double strand breaks in young and aging mice

Western blotting was performed to detect the formation of DNA double strand breaks by the presence of H2AX phosphorylated at Ser139 (**γH2AX**) (BURMA *et al.* 2001; KINNER *et al.* 2008). Nuclear protein was purified from brain samples using a modified protocol (DIMAURO *et al.* 2012). Briefly, each brain sample was homogenized using a hand homogenizer in 300-500 µL of STM buffer (250 mM sucrose, 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, complete mini protease inhibitor cocktail [Roche Diagnostics, Indianapolis, IN], and 1 mM Na_3VO_4). The homogenate was vortexed at high speed after 30 min of incubation on ice. Homogenates were centrifuged at 800 x g for 15 min at 4^o C. The supernatant was discarded and the pellet was re-suspended in 200-300 µL of nuclear extraction buffer (NET) buffer (200-500 µl NET buffer comprising: 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-X-100, complete mini protease inhibitor

cocktail, and 1 mM Na_3VO_4). The resuspended solution was vortexed at high speed, followed by 30 min of incubation on ice, and sonicated with a hand sonicator (Fisher Scientific 60 Sonic Dismembrator) for three 15-sec intervals separated by incubations in ice for an equivalent amount of time. The resulting samples were centrifuged at 9000 x g for 30 min and the pellets were discarded. The protein concentration of the supernatant was determined using the bicinchoninic acid (**BCA**) assay using Pierce BCA Protein Assay Kit (ThermoScientific, Rockford, IL) (SMITH *et al.* 1985). Protein samples (50 µg) were mixed with Pierce Lane Marker Reducing Sample Buffer (ThermoScientific, Rockford, IL) and separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) run at 150 V for 75 min, in running buffer (25 mM Tris, 192 mM glycine, and 0.1% sodium dodecyl sulfate, pH 8.3). Protein fractions were then transferred to a nitrocellulose membrane (0.2 µM, BioRad) at 100 V for 55 min in Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) at 4 °C. Membranes were then washed twice in Tris-buffered saline tween-20 (**TBST**) wash solution (50 mM Tris, 150 mM NaCl and 0.1% tween-20, pH 7.6) for 5 min and then blocked in 3% bovine serum albumin (**BSA**) blocking solution in TBST for 60 min at room temperature. Membranes were washed with three changes of TBST for 5 min each and incubated with 0.4 µg/ml of primary rabbit anti-γH2AX polyclonal antibody (#07-164; EMD Millipore, Etobicoke, ON) and 3% BSA in TBST solution overnight at 4 ºC. The next morning, the membranes were washed with three changes of TBST for 5 min each followed by a 60 min incubation in TBST (1:30 000 dilution) containing a secondary goat anti-rabbit horseradish peroxidase conjugated polyclonal antibody (#A0545; Sigma-Aldrich, Oakville, ON). Membranes were washed with three changes of TBST for 5 min each. All blots were incubated with Pierce SuperSignal West Dura Extended Duration Substrate (#34075; ThermoScientific, Rockford, IL) for 5 min and

analyzed with a FluorChem8800 imager. To probe for the Histone H3 loading control, membranes were stripped using Pierce Restore Plus buffer (#46430; ThermoScientific, Rockford, IL) for 15 min at room temperature and washed with three changes of TBST for 5 min each. This was followed by a 1 hr blocking step in 5% skim milk blocking solution in TBST for 60 min at room temperature. Membranes were incubated in 0.02 µg/mL primary rabbit anti-H3 polyclonal antibody (#07-690; EMD Millipore, Etobicoke, ON) in 5% skim milk blocking solution in TBST for 60 min at room temperature and processed with a secondary antibody as above.

Purkinje cell analysis and calbindin immunoblotting in aging mice

For H&E staining (FISHCHER *et al.* 2008), the brains of aging mice (12-18 months) were perfused via cardiac puncture with PBS, followed by 10% neutral buffered formalin, isolated and further fixed overnight in 10% neutral buffered formalin before being embedded in paraffin. Paraffin-embedded brains were sliced into 5 µm-thick sections, which were mounted onto glass microscope slides for H&E staining. Embedding and staining were carried out at the Centre for Modeling Human Disease (CMHD) Pathology Core at the University of Toronto. H&E-stained slides were used for the assessment of PC numbers. Each G6PD genotype and sex contained samples from 3 animals. For each slide, the mean number of PCs was determined from 10 randomly selected fields. For immunoblotting, a homogenate was prepared with half of a mouse cerebellum mixed with 500 µL of homogenization buffer (NP-40, 1 mM PMSF, 6 mM KF) and homogenized using a hand homogenizer. The homogenate was centrifuged at 16,000 x g and the supernatant was analyzed for protein concentration using the Bradford assay (BRADFORD 1976). A 10 µg sample of cellular protein was separated using a 10% SDS-PAGE gel under reducing and

denaturing conditions and transferred onto a PVDF membrane (Amersham Hybond-P, GE Healthcare Life Sciences, Little Chalfont, UK). The PC marker calbindin-D-28K was detected with a mouse monoclonal anti-Calbindin-D-28K primary antibody (1:3000, Sigma-Aldrich, Oakville, ON) and a donkey anti-mouse IgG (H+L) HRP-conjugated secondary antibody (1:25000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Each sample was standardized by probing the same membrane for β-actin. Peroxidase activity was visualized by an enhanced chemiluminescence (ECL) detection system (Amersham ECL Plus, GE Healthcare Life Sciences, Little Chalfont, UK).

Hippocampal function

The procedural details for electrophysiological recordings were described previously (MENG *et al.* 2002; MENG *et al.* 2003; ZHOU *et al.* 2011). Briefly, hippocampal slices (400 μm) were prepared from the aging wild-type and G6PD-deficient mice (age-matched) and allowed to stabilize in a holding chamber for at least 2 hr. A single slice was then transferred to the recording chamber and submerged and superfused with 95% O_2-5% CO_2-saturated artificial cerebrospinal fluid (**ACSF**, 2 ml/min). The ACSF contained 120 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM D-glucose. The recording pipette (3 megaohm [MΩ]) was filled with ACSF solution. Synaptic responses in CA1 pyramidal neurons were evoked by bipolar tungsten electrodes placed on the Schaffer collaterals 50-100 μm from the cell body layer for the activation of glutaminergic neurons. Evoked field excitatory postsynaptic potentials (**fEPSPs**) were measured by taking the slope of the rising phase between 5% and 60% of the peak response. Long-term potentiation (**LTP**) was induced with the theta burst stimulation (**TBS**) protocol consisting of 5 bursts of 4 pulses of 100 Hz stimulation at 200 ms intervals. All

data acquisition and analysis were done using pCLAMP 8 software (Axon instruments, Molecular Devices, Sunnyvale, CA). In LTP studies, the data were normalized to the average of the baseline responses.

Tube Test for Social Dominance

Social dominance was assessed in both the young and aging mice using the tube test (MICZEK AND BARRY 1975). Each match involved two age-matched (within 20 days) and sex-matched mice of different genotypes (G6PD-normal and G6PD-deficient) that were not housed together. The experimenter was blinded to the genotype of the mice being tested. Each mouse of a pair was simultaneously placed in opposite ends of a transparent, acrylic tube (3.175 cm inside diameter by 30 cm length; Laird Plastics, Brampton, ON). The match ended when one mouse placed all four paws outside the tube, and the mouse remaining inside the tube was deemed the 'winner'. Each animal was tested three to five times, depending on pairing availability, against animals of opposing genotype but never against the same mouse more than once. Mice were separated into individual cages and habituated to the testing room for approximately 1 hr prior to testing. Each mouse was first trained by being passed through the tube once completely. The tube was cleaned in between each test with Virox and distilled water.

Puzzle box test

Executive function in young mice (2-5 months) and aging mice (12-18 months) was assessed using the puzzle box test. The puzzle box consisted of two compartments made of Plexiglas, a start box (58 × 28 × 27.5 cm^3, bright light) and a goal box (14 × 28 × 27.5 cm^3, dark; covered on 5 of 6 sides with black Plexiglas), separated by a removable,

interchangeable divider made of black Plexiglas. In addition, there is an underpass in the floor under the divider at the intersection of the two boxes, which allows the mouse to enter into the goal box when the door has been closed. Mice were tested over a 3-day period (three trials per day) with increasingly difficult obstacles between the start box and goal box, based on a protocol adapted from (BEN ABDALLAH *et al.* 2011; MILENKOVIC *et al.* 2014) and described in table 3. In each trial, the mouse is introduced facing the wall opposite of the interchangeable divider and is allowed a maximum of 5 minutes to enter the dark chamber, with the latency to enter being manually timed and recorded. Animals that were successful in reaching the goal box were allowed to remain there for approximately 20 sec before being returned to their home cage for a 2 min recess in between trials. While multiple mice were tested as a set on a given day, each mouse was tested in all three trials sequentially before testing was begun for the next mouse. The puzzle box was cleaned with Virox (Virox Technologies, Oakville, ON) in between testing for mice from different cages. See **Table S1** in the online supplementary data file for an outline of trials conducted as part of the puzzle box test.

Marble Burying Test

Stereotypic behaviour was assessed in both the young and aging mice using the marble burying test (ANGOA-PÉREZ *et al.* 2013). Mice were habituated to the testing room for approximately 5 min prior to testing. Each mouse was left undisturbed for 30 min inside an enclosed cage filled 7.5 cm deep with corn cob bedding and 20 marbles (blue, 1.5 cm diameter) were arranged on the surface in a 4 x 5 grid which covered two-thirds of the surface. The mouse was placed on the side without marbles. The number of marbles buried were manually counted at the end of the study time; a marble was considered buried if 50%

or more of its surface was covered by the bedding. The experimenter was blinded to the genotype of the mice being tested.

Rotarod Performance

Motor coordination was measured in aging mice using an accelerating rotarod apparatus (SHIOTSUKI *et al.* 2010; DEACON 2013). After a 1 hr acclimatization time, mice were required to perch on a stationary rod for 60 sec, after which the rod began rotating at 5 rpm for 90 sec. This was repeated once, and these trials were used to accustom the animals to the test. The actual test consisted of the same 60 sec stationary period, followed by the 90 sec period at 5 rpm and then by an acceleration period gradually increasing speed to 25 rpm for up to a total of 5.5 min. The latency to fall during the test was recorded. The test was repeated once and the longest time from the two tests was used as an animal's measure of motor coordination.

Ledge Balance Test

The ledge balance test (WANG *et al.* 2002) involved placing an aging mouse in the middle of a narrow (2 mm) cardboard edge 31 cm in length and allowing it to move towards either side to find a platform. The mice were left on the edge for a total of 2 min. If the mouse reached the platform in under 2 min, it was placed in the middle of the ledge again and the test was repeated. A repeated test was also allowed for mice that fell off the ledge. The test was repeated for each mouse at least 2 times unless the mouse did not move from the middle and could not walk towards the platform in 2 min. Each test was video-recorded, and the videos were scored in a blinded fashion on a scale of 1 to 4. The scores were defined as: 1 – made the platform with no trouble within the time allowed, 2 – reached

the platform with some difficulty, 3 – did not reach the platform but remained on the ledge for the test, 4 – mouse fell off the ledge and could not balance. The final results were grouped according to age divided into 200-day intervals.

Hindlimb Clasp Test

The hindlimb clasp test (LIEU *et al.* 2013) was performed in aging mice by holding a mouse up in the air by the tail and monitoring the reflex of the hindlimbs. An abnormal test is observed when the animals holds its hindlimbs close to the body and/or clasps the hindlimbs together. This test was scored on a scale of 1 – 4 defined as follows: 1 – normal hindlimb splay and movement, 2 – hindlimbs slightly held to the body for a part of the test, 3 – hindlimbs held close to the body with no movement, 4 – hindlimbs close to the body with clasping in front. The final results were grouped according to ages divided into 200-day intervals.

Passive Avoidance Test

Passive avoidance represents a form of single-pass learning in which mice experience a brief unpleasant stimulus (mild foot shock) upon exposure to a one set of environmental cues (dark versus light chamber) (CRAWLEY 2000). Aging mice were placed in a two-chamber cage with one dark (safe) chamber and one light (unsafe) chamber. The mice were allowed to explore the light chamber for 20 sec with a closed door to the dark chamber. The door was opened and the time was recorded for the mouse to enter the dark chamber, where it received a mild shock (1 mV for 4 sec). The following day the animal was placed back into the light chamber and the latency to enter the dark chamber was recorded after the door to the dark chamber was opened. The maximum time allowed was

5 min. If the animal entered the dark chamber in under 5 min, another shock was administered. The test was repeated for one more day in cases where the shock was not administered upon entry, and again 1 week later.

Taste Aversion Test

Taste aversion is a cognitive behavioural test that measures the ability of the animal to learn and later recall an association of a specific taste (sweetness) with feelings of malaise (WELZL *et al.* 2001). This test has been used in a model of aging disease (JANUS *et al.* 2004). Conditioned taste aversion was measured in aging G6PD-deficient and wild-type control mice up to 25 days after conditioning. Aging mice, 1 mouse per cage, were acclimatized for 7 days to a 7-hr drinking cycle (the animals received water for only 7 hr during a 24 hr period), which trained the mice to drink at least 1 mL of water within the first 30 min of access to water. This was confirmed by measuring how much water was consumed in the first 30 min. On day 0, the mice were given a 0.5% saccharin solution for the first 30 min of the drinking cycle, and this was paired with a 4% body weight dose of 0.14 M LiCl i.p. to induce a feeling of malaise. At 2, 3, 4, 5, 6, 10, 14 and 25 days later the saccharin was presented again for the first 30 min of the drinking cycle and the amount of saccharin solution consumed was measured.

Table S1

Day	Trial	Door	Underpass Obstruction
1	A	Open	Open (Easy)
	B	Closed	
	C		
2	A		
	B		Bedding (Increase in difficulty)
	C		
3	A		
	B		Cardboard Flap (Difficult)
	C		

Table S1: Outline of trials conducted in the puzzle box test for executive function. Mice were tested over a 3-day period (three trials per day) with increasingly difficult obstacles in moving from a white start box to a dark goal box, based on published protocols (BEN ABDALLAH *et al.* 2011; MILENKOVIC *et al.* 2014). The two boxes are separated by a divider with an underpass and a door, the latter of which is closed for all trials except for the first trial (trial A) on day 1. On the second day, ground corncob bedding is introduced into the underpass to further obstruct the path for a mouse to enter the dark box, requiring it to burrow its way through the bedding in the underpass to reach the dark goal box. On the third day, in the last two trials of the experiment, the bedding is removed, and a cardboard flap is placed over the underpass, physically obstructing the underpass entrance into the goal box. This requires the mouse to remove the flap or lift the flap using its head before it can access the dark goal box. In each trial, the mouse is allowed a maximum of 5 minutes to enter the dark chamber, with the latency to enter being manually timed and recorded.

Table S2

Comparisons	1 Young Females +/+ vs def/def	2 Young Males +/y vs def/y	3 Aging Females +/+ vs def/def	4 Aging Males +/y vs def/y	5 Young Female +/+ vs Young Males (+/y and def/y)	6 Young vs Aging +/+ Females	7 Young vs Aging def/def Females
Trial 1	0.0011	N.S.	0.0011	N.S.	<0.0001	0.0010	0.0370
Trial 2	0.0397	N.S.	N.S.	0.0942	0.0002	0.0694	N.S.
Trial 3	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
Trial 4	N.S.	N.S.	N.S.	0.0123	0.0584	N.S.	N.S.
Trial 5	N.S.	N.S.	N.S.	N.S.	0.0042	N.S.	N.S.
Trial 6	N.S.	N.S.	N.S.	N.S.	0.0008	N.S.	N.S.
Trial 7	N.S.	N.S.	N.S.	N.S.	0.0002	N.S.	N.S.
Trial 8	N.S.	N.S.	N.S.	N.S.	0.0438	N.S.	N.S.
Trial 9	N.S.	N.S.	N.S.	N.S.	0.0860	N.S.	N.S.

Table S2: Summary of the statistical results from puzzle box testing in G6PD wild-type and deficient mice. The puzzle box test was performed to assess executive function and problem solving. The test consists of nine trials, split evenly over three consecutive days. Performance was modeled using Kaplan-Meier curves, which combine the latencies to enter the dark box with the rate of incomplete trials at a given point in time. The probability values are given for the difference between the two groups being compared. The groups included young (2-5 months) and aging (12-18 months) G6PD-normal wild-type females (+/+) and males (+/y), and young and aging G6PD-deficient females (def/def) and males (def/y). Results for young +/+ vs. def/y males were not different for any trial (see column 2), so these data were combined for comparison with young +/+ females (see column 5). Dark green-coloured boxes indicate that the former group performed better than the latter (e.g. column 3, where +/+ mice performed better than def/def mice). Dark red-coloured boxes indicate that the former group performed worse than the latter (e.g. column 4, def/y aging males performed better than +/y aging males). Lightly shaded green and red boxes indicate a marginal level of significance ($0.05 < p < 0.10$). N.S. indicates comparisons that were not significantly different. Statistical analysis was performed using Kaplan-Meier curve analysis followed by the Mantel-Cox log-rank test. The minimum significance level used throughout was $p<0.05$.

Figure S3

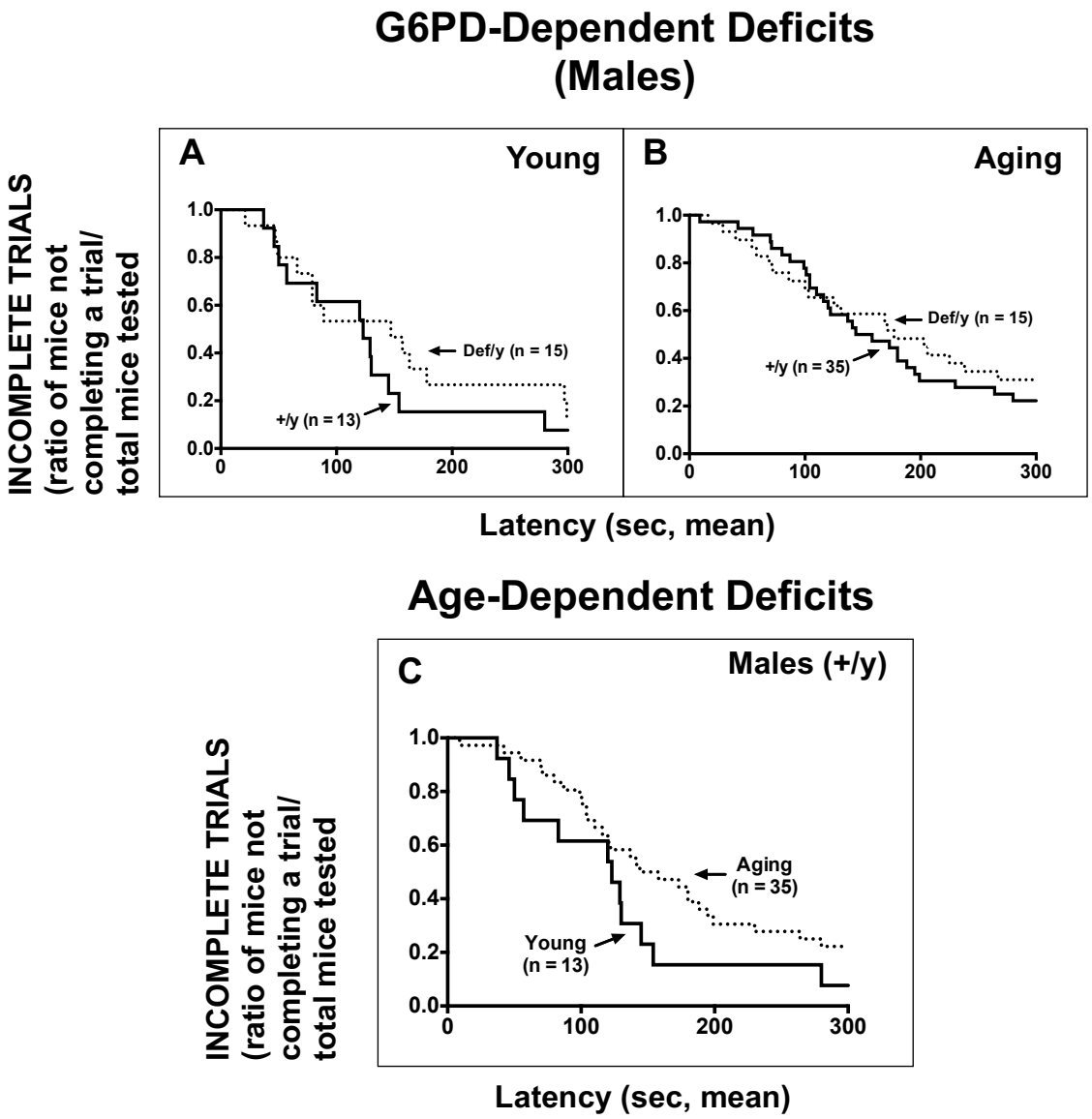

Figure S3: Effect of G6PD deficiency on executive function in males. The puzzle box test was performed to assess executive function and problem solving in G6PD-deficient mice. Lower latency values indicate higher executive function. Performance was analyzed using Kaplan-Meier curves, in this case relating the latencies for entering the dark box to the rate of incomplete trials at a given point in time. The number of mice tested for each genotype is shown in parentheses beside the respective survival curve. **Panel A:** Young G6PD wild-type (+/y) normal males compared with G6PD-deficient (def/y) counterparts. **Panel B:** Aging G6PD wild-type (+/y) normal males compared with mutant G6PD-deficient (def/y) counterparts. **Panel C:** Young G6PD wild-type (+/y) normal males compared with aging counterparts. Each figure compares the performance of the two groups in trial 1 of the puzzle box test. None of these comparisons showed a significant difference, although there was a consistent trend for higher executive function in male young vs. their G6PD-deficient (def/y) counterparts, and for male G6PD-normal young vs. aging mice. Statistical analysis was performed using survival curve analysis followed by the Mantel-Cox log-rank test. The minimum significance level used throughout was $p<0.05$.

Figure S4

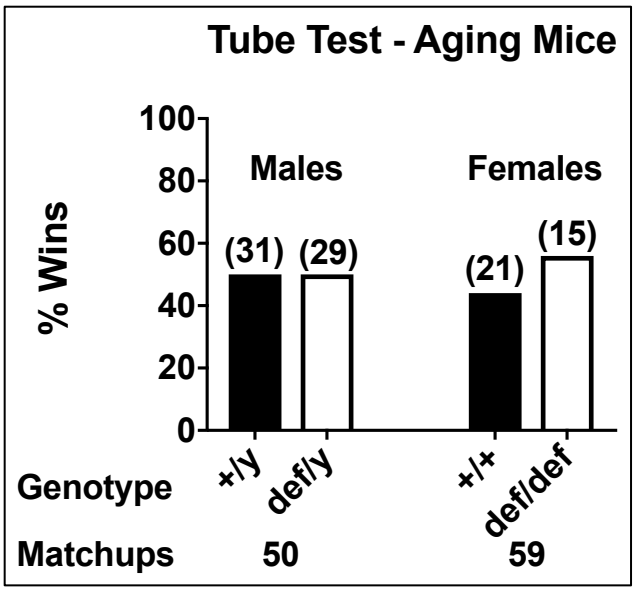

Figure S4. Effect of G6PD deficiency on social dominance in aging mice. The tube test was performed to assess dysfunction in social aggression. Bars indicate the % wins of unique matchups for each group and the X axis represents the *G6pd* genotype along with the number of matchups performed for each group. G6PD wild-type (+/y and +/+) normal mice were paired with sex and age-matched (within 20 days) G6PD-deficient (def/y and def/def, respectively) counterparts. The number of wins for each group was counted and a Fisher's exact test was used to determine whether the scores were significantly different from the 50:50 win/loss outcome expected by chance. The number of mice for each genotype is shown in parentheses above the respective bars in the figure. The minimum significance level used throughout was $p<0.05$. No significant differences were observed.

Figure S5

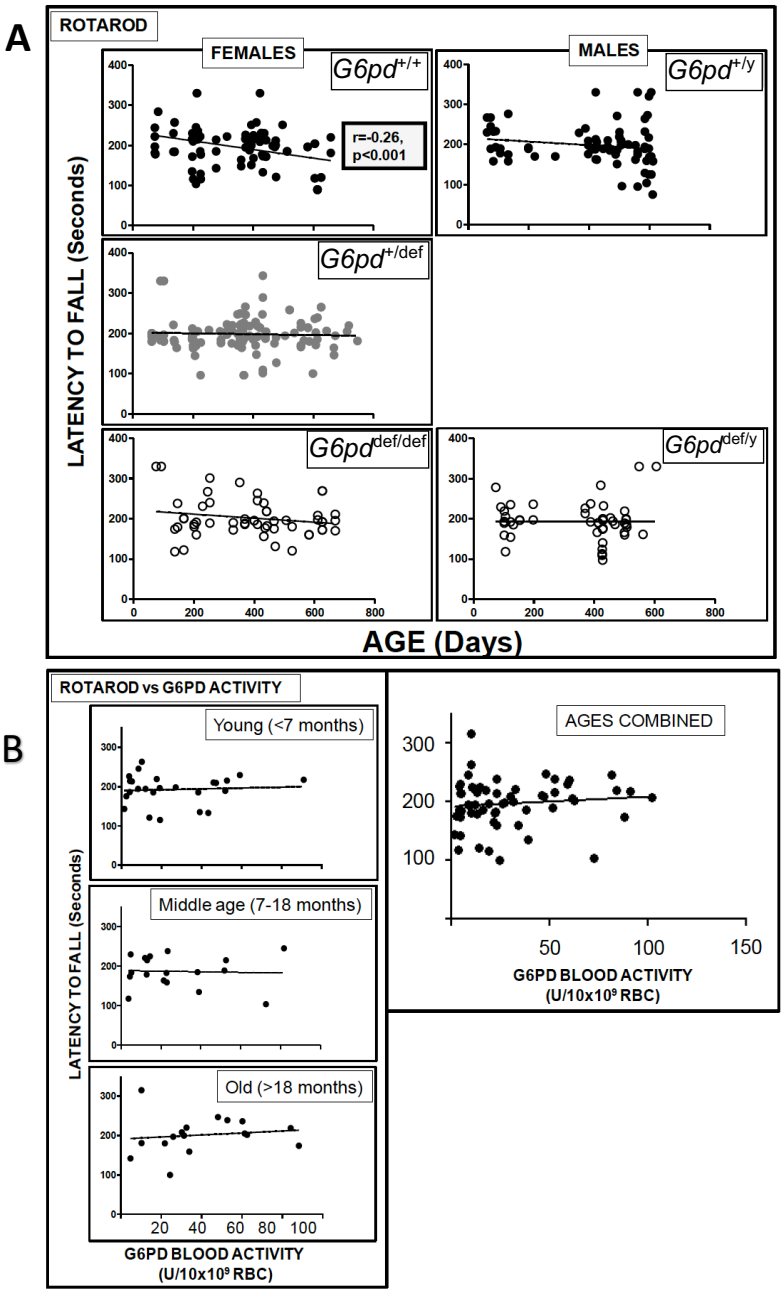

Figure S5: Lifespan analysis of motor coordination in G6PD-deficient mice measured by rotarod performance and its correlation with G6PD activity during aging. Mice of different ages and all G6PD genotypes were tested using the rotarod apparatus, and the latency to fall was recorded. Blood G6PD activity was measured in the same mice. The relationship between the two factors in each panel was assessed by Pearson's correlation analysis. The Pearson r value is shown for significantly correlated groups. Aging was correlated with decrease in rotarod performance in only G6PD-normal (+/+) females. G6PD activity was not correlated with rotarod motor coordination performance during aging. The minimum significance level used throughout was $p<0.05$.

Figure S6

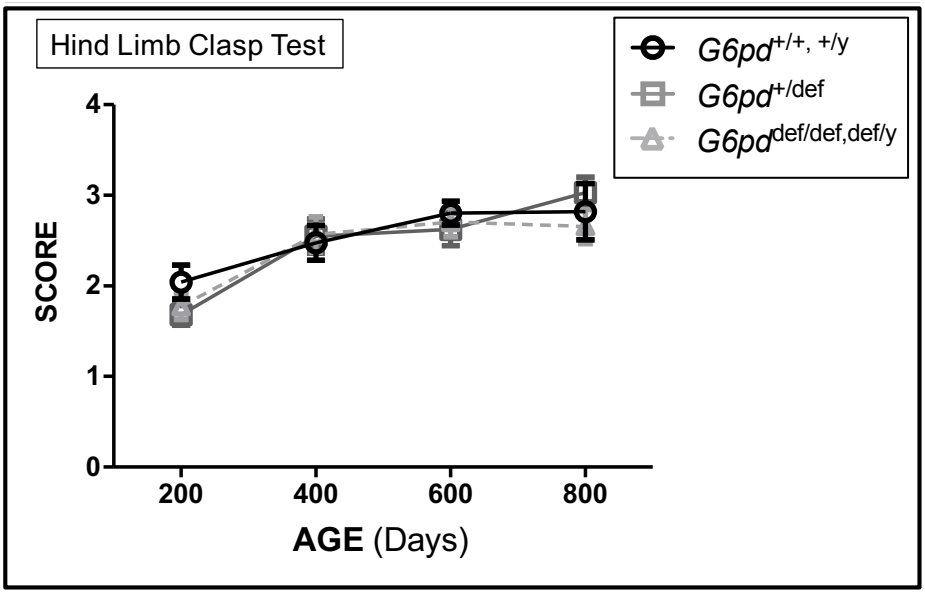

Figure S6: No effect of G6PD-deficiency on the hindlimb clasp test during aging. The hindlimb splay reflex was scored on a scale of 1 - 4, with 4 indicating the worst performance. The final results were grouped according to ages divided into 200-day intervals. No sex differences were apparent, so the data for males and females were combined for analysis. The number of animals per group were as follows: *G6pd* +/+, +/y combined (no alleles mutated) = 33, *G6pd* +/def (one allele mutated) = 52, *G6pd* def/def, def/y combined (all alleles mutated) = 37. No differences were found between these groups, but all of the genotypes progressively declined over age ($p<0.0001$, two-way ANOVA with a Bonferroni post-hoc test).

Figure S7

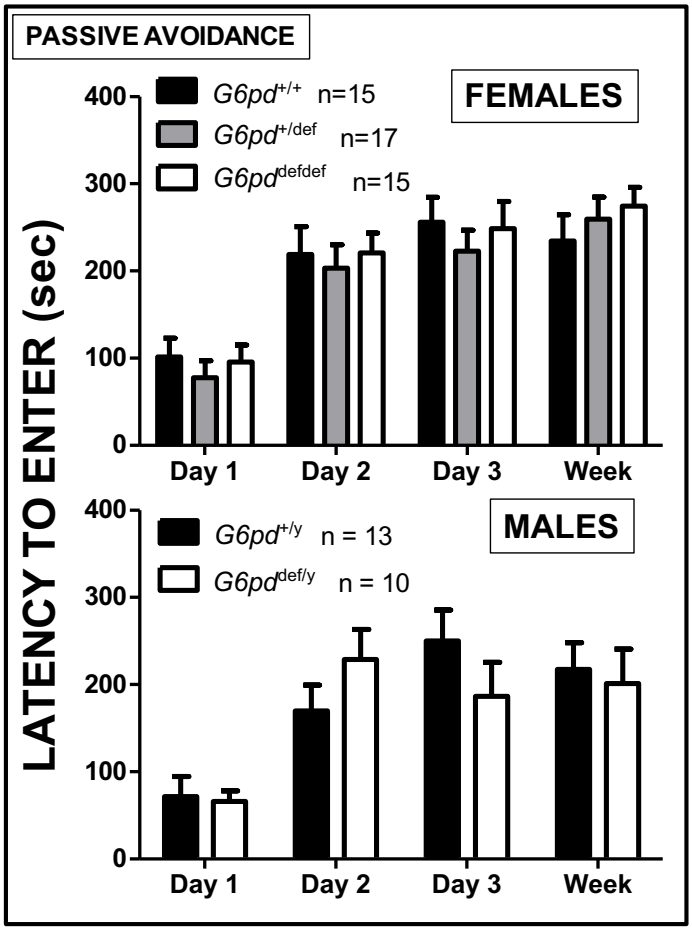

Figure S7: No effect of G6PD deficiency on cognitive function measured via passive avoidance in aging mice. Aging mice (12-18 months) were placed in a two-chamber cage with one dark (safe) chamber and one light (unsafe) chamber. The mice were allowed to explore the light chamber for 20 sec with a closed door to the dark chamber. The door was opened, the time to enter the dark chamber was recorded and the animal received a mild shock when entering the dark area. The following day the subject was placed back into the light chamber and the latency to enter was recorded after the door to the dark chamber was opened. The test was repeated for one more day and again 1 week later. No differences were found for any G6PD genotype. The number of mice for each genotype is shown in the figure. Statistical analyses were performed using two-way ANOVA, followed by a Bonferroni post-hoc test. The minimum significance level used throughout was $p<0.05$.

Figure S8

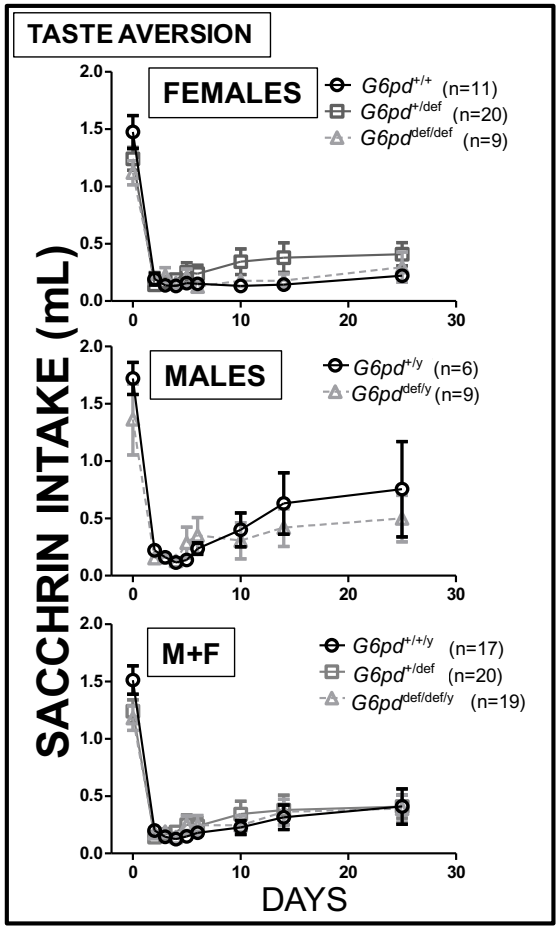

Figure S8: Taste aversion. Aging mice were acclimatized for 7 days to a 7-hr drinking cycle (animals received water for only 7 hr during a 24-hr period). On Day 0, the mice were given a 0.5% saccharin solution for the first 30 min of the drinking cycle and this was paired with a 4% body weight dose of 0.14 M LiCl i.p. to induce a feeling of malaise. At 2, 3, 4, 5, 6, 10, 14 and 25 days later, the saccharin was presented again for the first 30 min of the drinking cycle and the amount of saccharin solution consumed was measured. No sex- or G6PD-dependent differences were observed. The number of mice for each genotype is shown in the figure. Statistical analyses were performed using two-way ANOVA, followed by a Bonferroni post-hoc test. The minimum significance level used throughout was $p<0.05$.

Figure S9

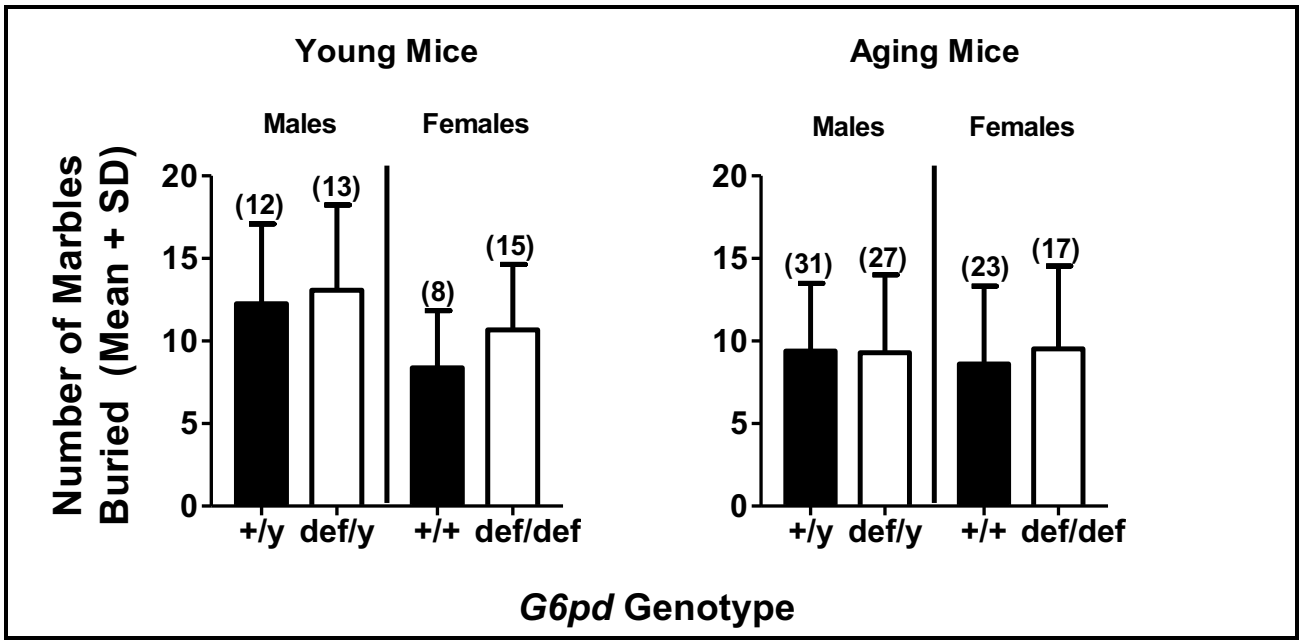

Figure S9. Effect of G6PD deficiency on repetitive behaviour. The marble burying test was conducted to assess repetitive burying behaviour. The number of marbles buried is counted and used as a measure of repetitive behaviour. Marbles were considered buried if 50% or more of their surface area was covered by the bedding. G6PD wild-type (+/y) normal males were compared to mutant G6PD-deficient (def/y) counterparts. G6PD wild-type (+/+) normal females were compared to mutant G6PD-deficient (def/def) counterparts. **Left panel:** young mice (2-5 months). **Right panel:** aging mice (12-18 months). Statistical analyses were performed using Student's t-test. The minimum significance level used throughout was $p<0.05$. No sex- or G6PD-dependent differences were observed.

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