SUPPLEMENTARY INFORMATION

Animals and experimental design

Male Fischer x Brown Norway F1 hybrid rats were obtained at 2- and 17 months-of-age (moa) and acclimated to a reverse light cycle (off 9:00, on 21:00) over 4 wk prior to initiation of irradiation/sham irradiation. Animals were housed in pairs (same age and treatment) in wire-topped cages with ad libitum access to food and water (bottles). Rats were progressively habituated to handling and transport so that by the end of the fourth week animals remained calm when held by the scruff and inverted, abdomen-up and head down, in preparation for anesthesia by intraperitoneal injection. Procedures were performed in low light and during the dark, waking phase of the rats' cycle; carts with dark cloth covers were used to transport rats between the housing facility and the irradiator or testing laboratory. Rats received 40 Gy total dose whole brain irradiation in eight fractions of 5 Gy (fWBI) each over a 4 wk period; details of irradiation procedures have been reported previously (1). Treatment of 24 rats per group included: 3 moa sham irradiated, 3 moa fWBI, 18 moa sham irradiated, and 18 moa fWBI. Rats were weighed twice per week during irradiation, once weekly for 5 weeks following irradiation and then approximately every 2-3 weeks for the remainder of the study (1). Longitudinal testing for cognitive function was assessed using spontaneous novel object recognition (NOR) and novel object location recognition (NOL) tasks. Each rat was tested on each task at each post-treatment testing time, 3, 6 and 12 mo after irradiation. Given accumulating evidence that the details of behavioral testing protocols greatly impact outcomes and the fact that several aspects of the current study differed from other studies of the effects of WBI on novel object- and novel location memory, detailed procedures are provided below.

Behavioral testing facility

Behavioral testing occurred in a laboratory dedicated to that purpose. The room was equipped with a portable HEPA air filter (Whirlpool 450) and a CD player with a white noise CD, and was lit with red and green LED lighting (< 1 lux). Behavioral testing was performed in an inner 6 ft x 9 ft room created using office cubicle panels lined on all interior surfaces with Sonex™ Classic Colortec acoustical foam, excluding a 2 ft wide entry door. The open field arena, a polypropylene box 30" square with walls 18" tall (Tamco® Industries #9302, with 6" added height using foam-core board) sat on the bottom shelf of a 4'x6'wire rack surrounded by white curtains. A low light video camera and infrared lights (940 nm, beyond the human or rat visible spectrum) were suspended from a wire shelf set 6 ft above the arena floor, with green LED lights directed upwards and reflected off a green cloth covering the top wire shelf to yield arena lighting of ~1 lux (simulating deep twilight). A fan on the floor of the inner room exchanged air with the laboratory, providing white noise which was supplemented by a Marpec Soundscreen 980A Sound Conditioner. For the NOL, vertical 3" black stripes were hung against the back curtain wall to serve as visual cues for place reference. Pairs of rats in each cage were distinguished by marking the tail of one rat with a black Sharpie marker and a blacklight reactive marker (MAR-CO Invisible blue UV pen) to facilitate accurate identification under either daylight or low light conditions. Daily testing was divided into morning (10:00 – 13:00) and afternoon (14:00 – 17:00) sessions. Rats for a session (typically 4 cages; one pair of rats from each treatment status) were transported in their home cages and remained in darkness on curtained shelves within the laboratory until all rats were tested, after which they were returned to the animal facility. For individual testing, a single cage was placed on the bench top and illuminated briefly by blacklight for animal identification; the appropriate rat was transferred to

the arena for the sample phase. For the delay period, rats were returned to their home cage and placed on an isolated holding shelf in complete darkness, followed by return to the arena for the novelty test phase. Cage-mates were always tested in sequence. After each phase for each rat, the front curtains were clipped open and the floor and walls of the arena were wiped clean with diluted dish soap solution (3 drops Dawn/ L water) followed by 70% ethanol and air-dried two minutes aided by a portable fan attached to the rack above the arena.

Arena, objects and test design

The 30” square arena was virtually divided into four 15” square quadrants; object placement was centered in these quadrants (3” diameter paper circles were taped on the exterior bottom of the semi-translucent arena to visually guide object placement from above). For all NOR trials, two identical objects were positioned in the two distant quadrants and rats were placed into the arena facing the middle of the front wall for the sample phase. After a delay period (1, 6, or 30 min), rats were returned to the arena for a test trial where one object was replaced with an identical third copy, while the other object was replaced by a new item. For NOL trials, using a total of four identical objects, two sample phase objects were located in the two distant quadrants and rats were entered facing either the left or right front corner, for test trials in which moved object appeared in the front right or left quadrant, respectively.

Objects were typically 12-25 cm tall by 8-12 cm in diameter, made of ceramic, metal, plastic or resin, and represented a wide range of colors, textures, shapes and complexity (specifically avoiding detectable odor). General categories include small resin statues, candle holders and plastic cups/jars. To assure that differences in the “attractiveness” of objects did not influence the rats’ behavior in the NOR and NOL, we tested specifically for differences among objects in the

time the rats spent exploring the objects during the sample phase of the NOR6 and NOR30. Despite the variation in object materials, sizes, complexity, etc, the average amount of exploration of each object in the sample phase varied only within a narrow range (coefficient of variation among objects ~20%). The average exploration of two objects was significantly greater than that of most of the other objects; those more “attractive” objects were paired only with each other, never with a less attractive object.

Sufficient replicates of objects permitted single use in each testing session. After use, objects were immersed in Coverage® Plus NPD (Steris) solution, thoroughly rinsed with tap water and air dried with the aid of a bench top fan. The presentation of objects was balanced across rat treatment status and object placement (novel- left or right). Objects were unique among tests within time point after treatment (3, 6, or 12 mo) and re-exposure to an object was limited to >3 mo since previous exposure.

To simulate some conditions used by our collaborators, for the NOR 1 minute delay, the objects were the same as those used in previous studies by M.E. Robbins and colleagues (2-5). These included a plastic jar (object X), 3” by 4” base by 7.5” tall, presented on its side, and a cylindrical metal can (object Y), 6” tall by 4” in diameter, presented standing. There were two copies of each object, which were cleaned by wiping with 70% ethanol and used alternately during testing. The arena was also wiped clean with 70% ethanol and dried with paper towels, as described by our collaborators. We were unable to clean the arena and position the test objects for Noldus tracking in 1 min; thus, our results reflect an average inter-trial delay of 1 min 40 sec. All other aspects of the testing environment used our standard conditions.

Testing, tracking and analysis

At 3, 6, and 12 mo after irradiation, a single test was performed each week in the following order: NOL, NOR 6, NOR 30. In the week before testing started, rats were transported to the lab, handled and placed on the holding shelves to become accustomed to the laboratory environment. On the first week of NOL testing, rats were habituated to the empty arena (with place cues on the back wall - see above) in 5 min sessions on Monday and Tuesday. NOL tests were run for the remainder of the week with 4 min sample phase, 6 min delay, and 2 min test phase. In the second week, rats again were habituated for 5 min to the empty arena (place cues removed) and NOR 6 tests were run for the remainder of the week with 6 min sample phase, 6 min delay, and 3 min test phase. On the final week rats were habituated to the empty arena and NOR 30 testing was performed with 8 min sample phase, 30 min delay, and 3 min test phase. For this longer delay, the second rat in each cage was sampled 5 min into the delay period for the first rat from the cage.

At 12 mo after irradiation, NOR 1 trials were added for rats treated at 3 moa; NOR 1 testing was completed prior to testing in the NOL, NOR 6 and NOR 30 and incorporated elements of the methodology used by our collaborators M.E. Robbins and colleagues in previous studies (2-5). Rats were habituated to the empty arena in 5 min trials over 3 consecutive days and then tested on the fourth day with 3 min sample phase, 1 min 40 sec delay (see above), and 3 min test phase.

All sessions were video recorded for automated analysis using Noldus Ethovision XT 7.0 with simultaneous recording and 3-point live tracking. Proper object positioning was insured by aligning items to outlines drawn using the software and viewed onscreen prior to each rat session. Exploration of objects was defined as time the animals' nose-point was on or within 2cm of an object. Nose-point coordinates within this zone, but when rats were grooming, were deleted from analysis. Discrimination ratios for novel preference (DR) in the test phase were calculated

as (time exploring novel – time exploring familiar) / (time exploring novel + time exploring familiar). Noldus software analysis allows calculations for DR for unlimited intervals within the test phase, which was useful for confirming meaningful selection of test duration (with the possibility that age or treatment might affect outcome). We determined that results for our testing closely followed early studies by Dix and Aggleton (6) and thus report here test phase results for the first 1 min of the NOL and for the first 2 min in the NOR 6 and NOR 30. Note, however, that we report test results in the NOR 1 for the full 3 min test phase, consistent with previous studies in the Robbins laboratory (2-5). Total distance moved (center-point path length) during the sample and test phases also was analyzed using the automated Noldus system. Although tests were balanced across sides of the arena, we also performed DR calculations for the sample phase to discriminate for preference toward the object that would be replaced with a novel object (NOR) or moved (NOL) in the subsequent test phase, in order to reveal any potential bias. In the total of 36 group trials for this entire study (2 treatments x 2 ages x 3 time-points = 12 groups for each of 3 tests), 34 of the groups had sample DR's that did not differ from chance (DR=0), indicating no bias. Both sham and fWBI rats treated at 18 moa had significantly negative DR's in the sample phase of the NOL at 3 mo after treatment, despite very strong preference for the novel/moved object in the test phase. We attribute this bias to 1) potential anxiety in the older rats (this was the very first test trial performed) and 2) the unavoidable location task design; the stable/familiar object was always closer to the rat entry point during the sample phase, in order to permit equidistant object locations in the test phase. Finally, to assess possible effects of fWBI and/or time after treatment on the extent of habituation of exploratory behavior, which might influence discrimination scores, we calculated the Index of Global Habituation (IGH), equal to the difference in the amount of time exploring objects during the sample phase and the amount of

time spent exploring objects in the test phase (Supplemental Figure 2), as described previously (7).

SUPPLEMENTAL REFERENCES

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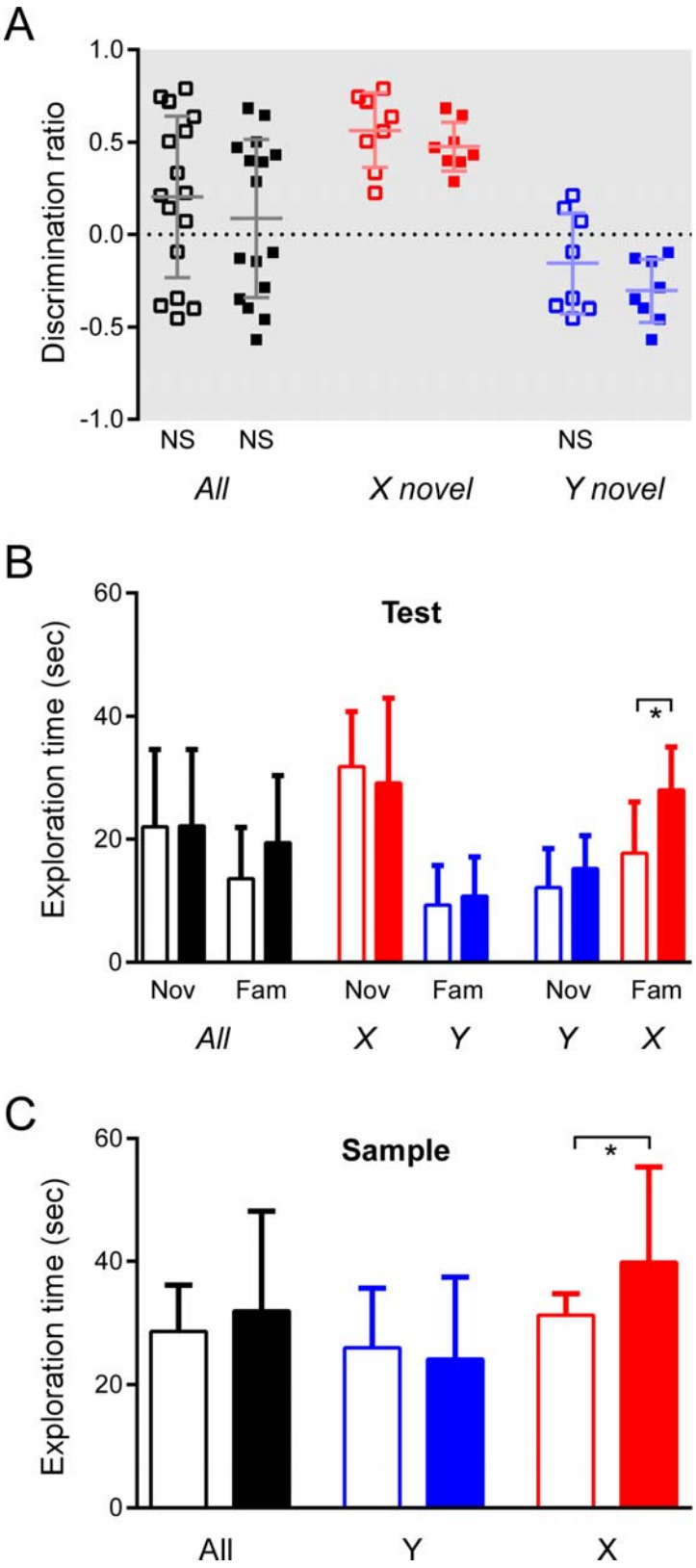
Supplemental Figure 1. NOR 1 Recognition Memory and Exploration. A. Discrimination ratios for novel preference are plotted for individual rats treated at 3 months-of-age and tested in the NOR 1 at 12 mo after fWBI (closed symbols) or sham irradiation (open symbols). The mean ratio also is indicated (with sd). DR values are plotted for all trials (black) and then separately for trials in which object X was the novel object (jar, in red) and for trials in which object Y was the novel object (can, in blue). Groups for which the mean discrimination ratio was not significantly different from chance (NS) are indicated. B. Mean (+ sd) exploration times during the test period of the NOR 1 are shown for all trials and then for the trials separated based on which object was novel in the test phase. The open bar in each pair represents sham irradiated control rats and the filled bar fWBI rats. In the test phase, irradiated rats spent significantly more time than sham control rats exploring object X when it was the familiar object, indicating a perseverative response specific to fWBI rats and to object X. C. The greater exploration of object X by fWBI rats was not limited to the test phase; in the sample phase irradiated but not sham control rats spent significantly more time exploring object X than object Y. $*p < 0.05$ for indicated comparison.

Supplemental Figure 2. Index of Global Habituation. The mean (+ sem) normalized values for the Index of Global Habituation (IGH) are plotted for sham irradiated control (open and light

gray bars) and fWBI rats (black and dark gray bars) treated at 3- or 18 months-of-age and tested in the NOR 30 (A), NOR 6 (B) and NOL (C). Values for each test are presented as percentage of the maximum possible IGH value (duration of the Sample Phase minus duration of the analyzed portion of the Test phase). ANOVA revealed an effect of time after treatment in rats treated as young adults but not in rats treated in middle age. [#]To simplify the presentation of the IGH values for the other groups, the normalized IGH in the NOR 6 for sham rats treated at 18 months-of-age, -0.59 + 2.13, is not shown [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.005$ for indicated comparison.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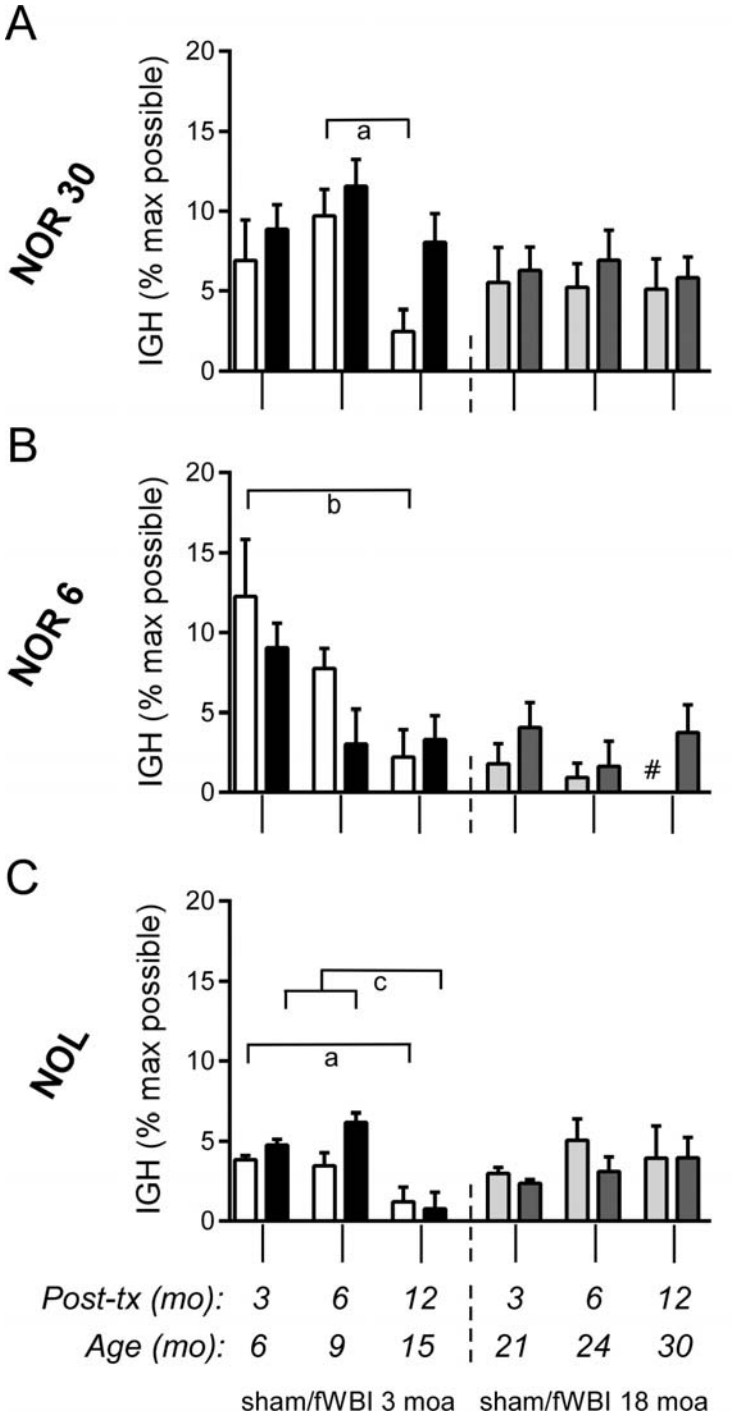
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