Maternal lupus and congenital cortical impairment

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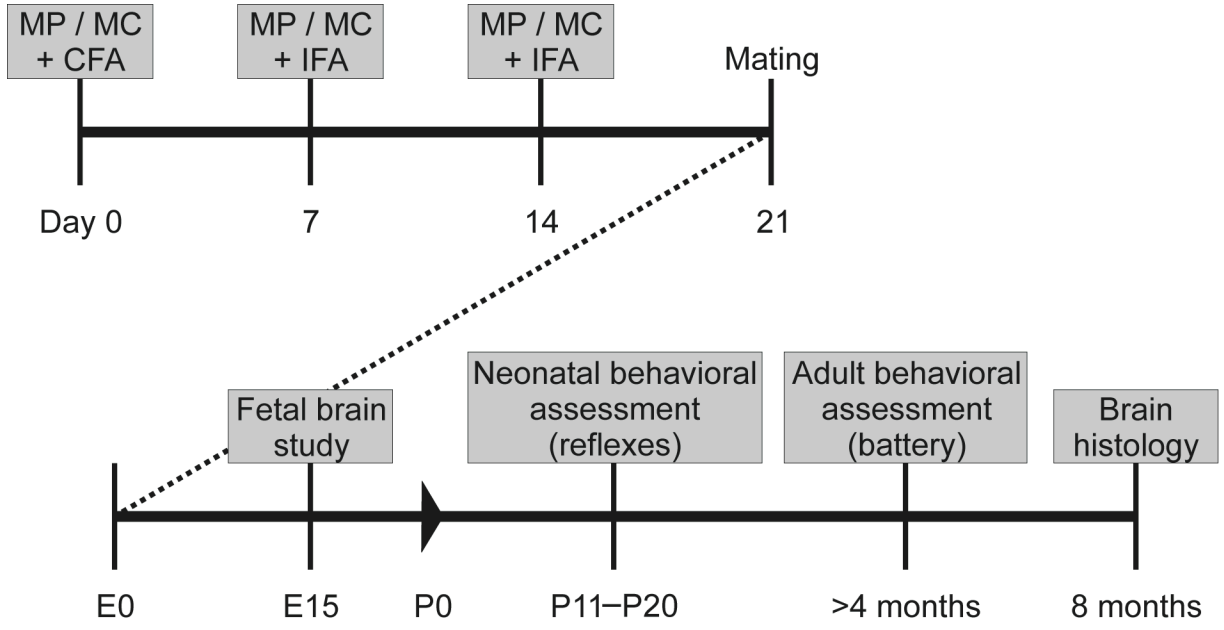
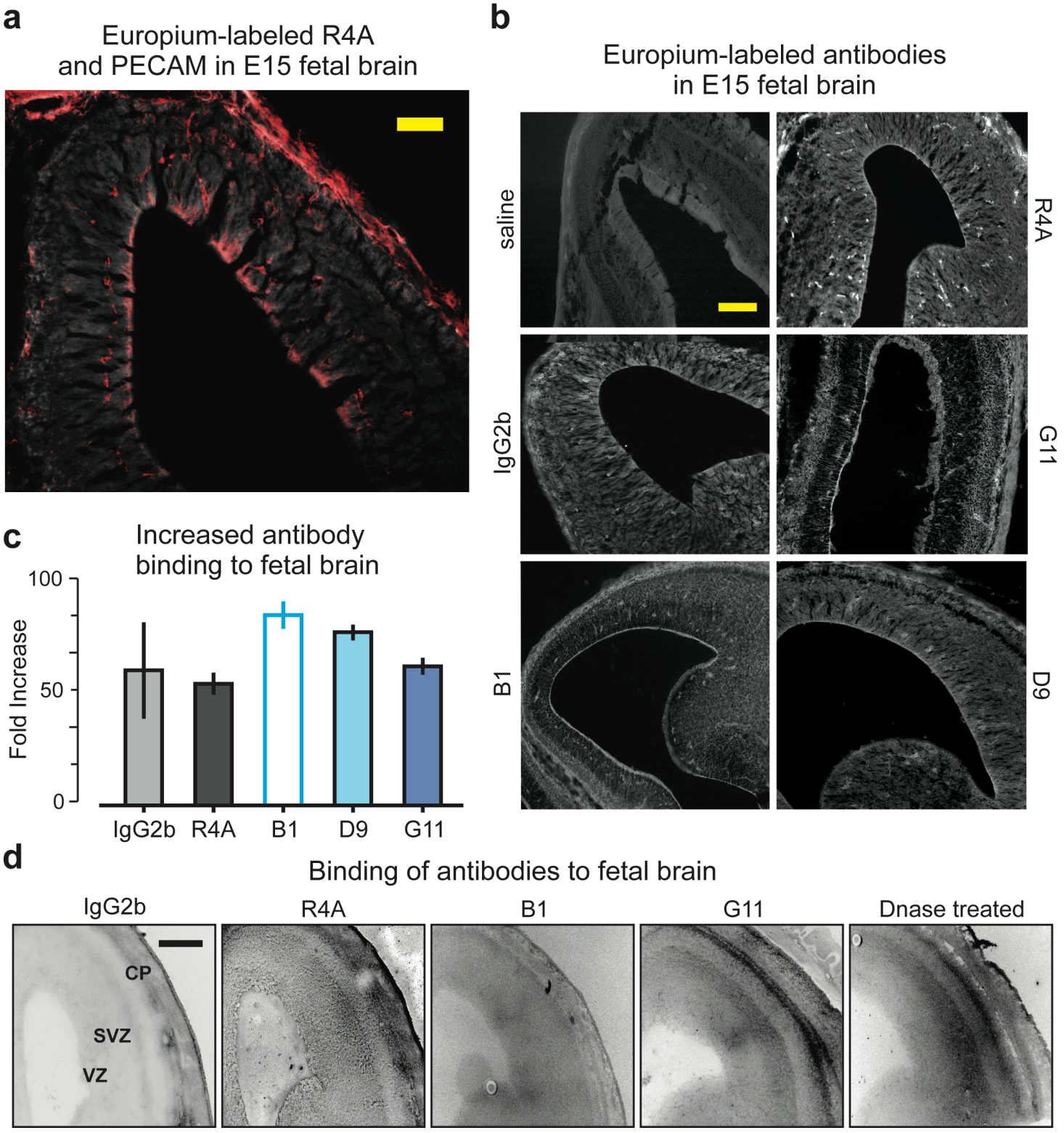
SUPPLEMENTARY INFORMATION

Supplementary Figure 1: Experimental design of a mouse model for maternal lupus.
BALB/c adult female mice were immunized with octamerized DWEYSVWLSN (MP, 100 μg) in complete Freunds adjuvant (CFA) or polylysine core (MC, 100 μg) in CFA. Two subsequent boosts in incomplete Freunds adjuvant (IFA) were administered 7 days apart. MP and MC mice were then mated and, at embryonic day 15 (E15), the fetal brains were harvested and processed. Pup cohorts born to MP and MC mice were assessed for developmental reflexes during postnatal days 11–20 (P11–P20). A battery of behavioral tasks was performed on these mice as adults (age > 4months). Histological analysis was performed on the brains of adult mice subsequent to behavioral testing.

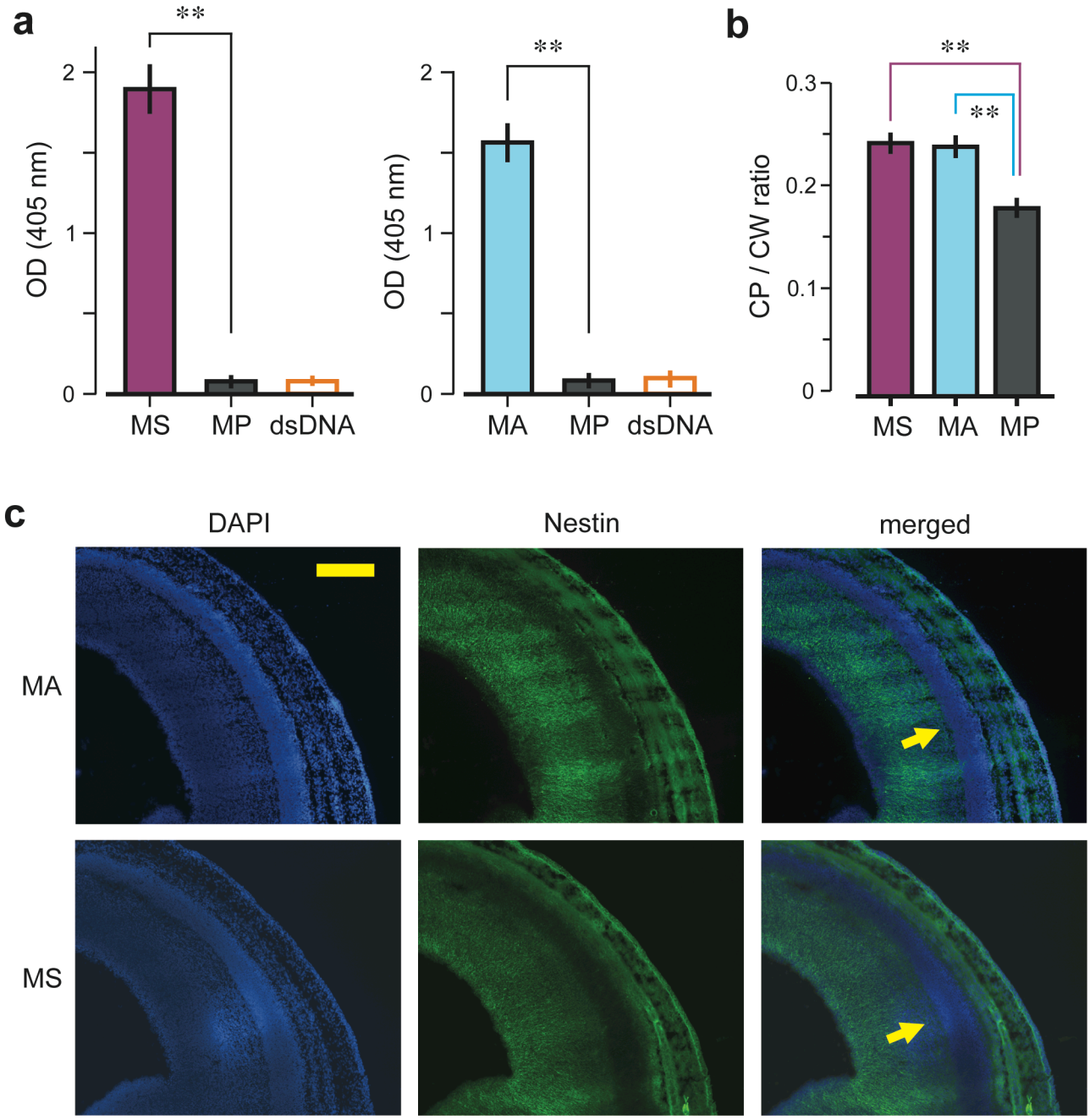


Supplementary Figure 2: Maternal transfer of lupus autoantibody to fetal brain.
METHODS: The mouse monoclonal DNA-specific, NMDAR-specific AAb, R4A, was conjugated to europium (Eu). Labeling of R4A to Eu was accomplished with DELFIA Eu-N1 DTA Chelate (PerkinElmer), a Eu^{3+}-chelate of N1-[p-(3,5-dichlorotriazinyl) benzyl] diethylenetriamine - N^1,N^2,N^3 - tetraacetic acid reagent. The chelate was dissolved in 10 mM sodium acetate (pH 4.8) and aliquoted. The reagent was kept on ice before use and unused aliquots were frozen and stored at –20 °C. The labeling reactions were performed using 1 mg of protein (at 2–5 mg ml^{-1}) and a 20-fold molar excess of a chelate according to the manufacturer's instructions. Excess chelate was removed by a PD-10 desalting column (GE Healthcare) followed by an extensive dialysis. Purity was confirmed by precipitation of a small aliquot of IgG by an excess of Protein G (GE Healthcare). The concentration of IgG was established by ELISA, with IgG2b as a standard. The Eu concentration on labeled protein was determined to be 7–8 per IgG molecule using Eu^{3+} standard in DELFIA Enhancement Solution. The fluorescence was measured on 1420 VICTOR^3 Multilabel Counter (PerkinElmer). Eu-labeled R4A (100 μg per dam) was administered intravenously to pregnant dams on E15 and, 24 h later, the embryos were harvested for cortical histology. (**a**) Visualization of Eu-labeled R4A in coronal fetal brain sections (white label) shows that it is transferred from the maternal circulation to the fetal circulation. Eu-R4A is detected in fetal brain. The tissue is also stained with Ab to CD-31 (red label) to confirm that perfusion at the time of sacrifice removed blood from the circulation and that Eu-R4A was present in the tissue. Scale bar = 500 μm **(b)** All monoclonal Abs are transferred from the mother to the fetal brain. The mouse Abs R4A and IgG2b and the human Abs G11, D9, and B1 are Eu-labeled as above and administered intravenously to pregnant dams. Tissue is assessed for Eu-Ab. Saline is

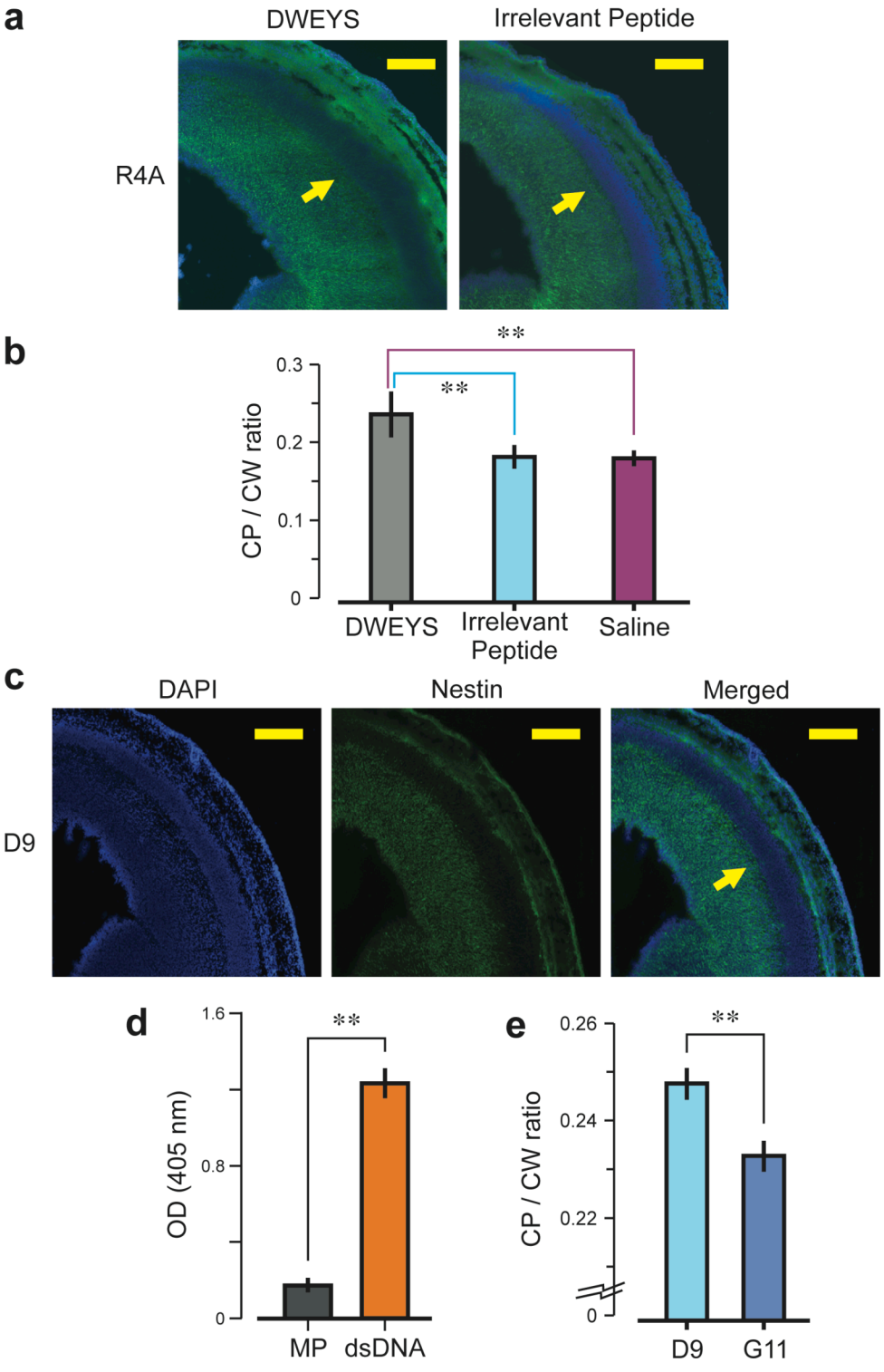
used as a control. Scale bar = 250 μm. **(c)** Eu counts per mg of brain is determined for 3–5 pups as well as the dam for each Ab. **(d)** Binding of mouse and human monoclonal DNA-specific, NMDAR-specific AAbs to fetal brain is assessed for the control isotype Ab, IgG2b (25 μg ml^{-1}), the mouse DNA-specific, NMDAR-specific AAb, R4A (25 μg ml^{-1}), the control human isotype Ab, B1 (25 μg ml^{-1}) and the human DNA-specific, NMDAR-specific AAb, G11 (25 μg ml^{-1}). Both R4A and G11 show strong binding in the fetal cortex, as evidenced by the dark staining in CP. The pattern of binding of R4A and G11 reflect the distribution of NMDAR, rather than the transport of serum AAb to brain (as in supplementary Fig. 2a). To confirm that G11 binding in the cortex was due to NMDAR reactivity, and not to cross reactivity to DNA, the coronal cortical sections were treated with DNase I (20μg ml^{-1}) for 20 min at 37°C before antibody labeling. G11 still shows robust binding in DNase I-treated cortical sections.



Supplementary Figure 3: Fetal brain from mice immunized with octamerized WSDYEVWLSN (MS) and AAAAAVWLSN (MA).
(a) BALB/c female mice were immunized with MP, MA or MS as described in Methods. Post-immune sera were assayed (by ELISA) for reactivity to DWEYSVWLSN (MP), dsDNA, and the respective immunizing peptide. The left graph shows that serum diluted 1:1000 from MS-immunized mice displays reactivity to WSDYEVWLSN. The right graph shows that serum from MA-immunized mice reacts to AAAAAVWLSN (1:1000 diluted). **(b)** MS and MA fetal brains exhibited normal CP / CW ratios, comparable to MC brains, and significantly higher than MP brains (* $P < 0.005$, Mann-Whitney U tests, MS vs. MP, MA vs. MP). Values represent means ± s.e.m. For each assay, 3 fetal brains per dam were analyzed. **(c)** Representative MA and MS brains were stained with DAPI and nestin for the measurement of the CP (indicated by the arrows in the merged panels). Scale bar = 250 μm.



Supplementary Figure 4: (a) Peptide-mediated protection from R4A disruption. The DNA-specific, NMDAR-specific monoclonal AAb, R4A was administered intravenously (200 µg) to E12 pregnant mice. Subsequently, at 0h, 24h, and 48h, DWEYSVWLSN peptide (D isoform), NEMQSSRLRE peptide (which is not bound by R4A) or saline was administered by intraperitoneal injection (200 µg). The D peptide containing DWEYS was able to counter the deleterious effect of R4A. The panels show representative coronal sections of fetal brains, harvested at E15, stained with DAPI and nestin. **(b)** The peptide containing the DWEYS sequence prevented thinning of the CP (mean ± s.d., ** $P < 0.001$, Mann-Whitney U tests). **(c)** The human monoclonal DNA-specific AAb, termed D9, causes no fetal brain pathology. Pregnant BALB/c mice were injected on day E12 with D9 (200 µg). On E15, mice were sacrificed and fetal brains were processed. The representative coronal sections of D9-exposed fetal brains demonstrate absence of neocortical abnormality. **(d)** D9 displays no cross-reactivity with DWEYS peptide (MP) but has strong dsDNA binding. **(e)** D9-treated fetal brains were normal with respect to CP/CW ratio when compared to G11-treated brains (* $P < 0.005$, Mann-Whitney U tests). Values represent means ± s.e.m. For each assay, 3-5 fetal brains per dam were analyzed.



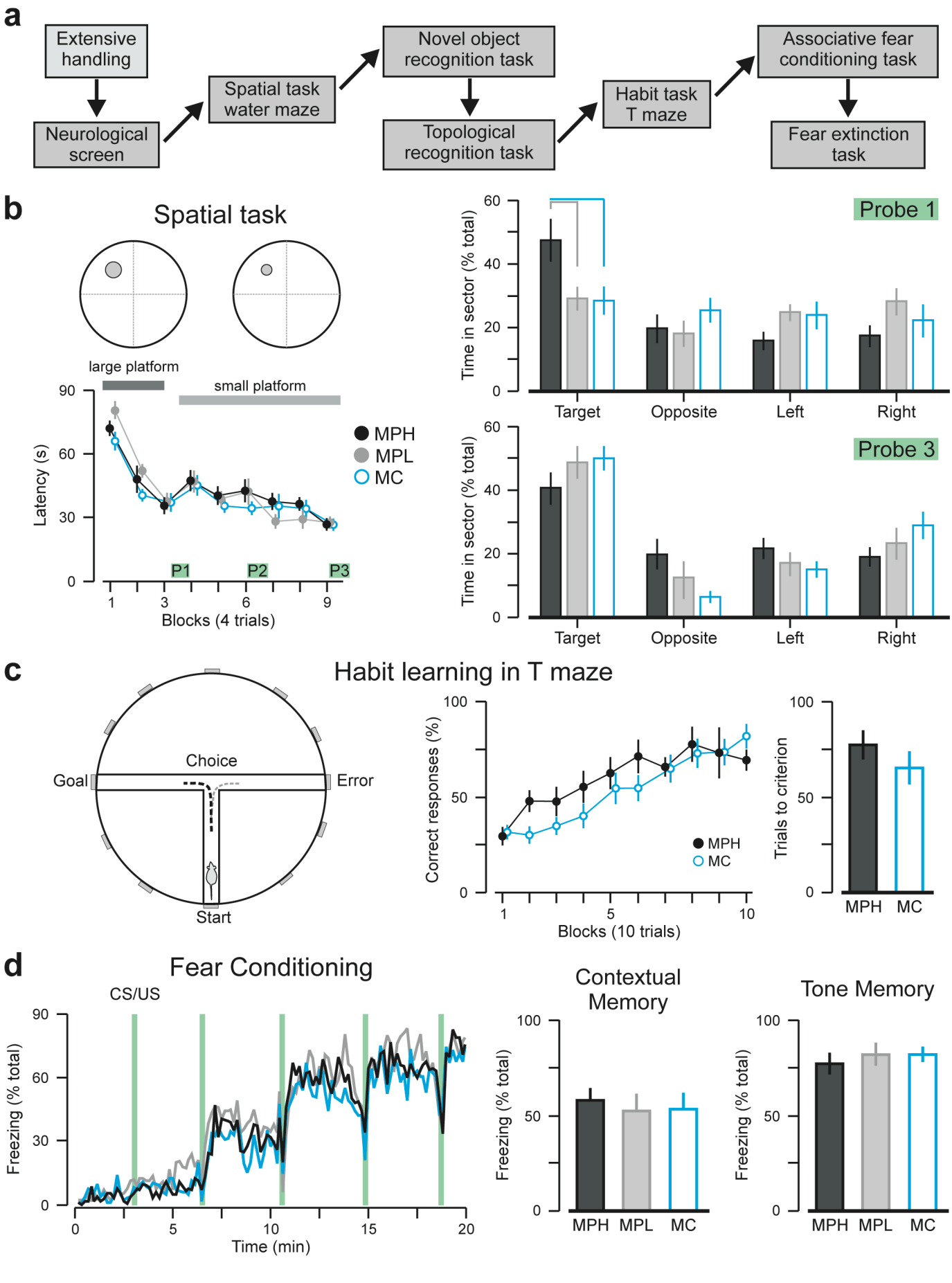
Supplementary Figure 5: Behavioral assessment of adult offspring.

(a) Experimental design for the behavioral battery. Adult male offspring from MPH (*n* = 10), MPL (*n* = 8) and MC (*n* = 10) groups underwent a series of standardized tasks, which were selected on the basis of the brain regions that are known to play a crucial role for successful performance on each task (detailed in the legend for each task). Mice were housed in cohorts of 4 animals and were kept on a reverse light cycle. Initially, all animals were extensively handled for 2 weeks. Each mouse was placed in the experimenter's hand for 5 min sessions, 2–3 times per day. We found that considerable handling was required for BALB/c mice to become habituated to the experimenter and the general testing conditions. The battery proper started with a neurological screen, covering 48 variables, which was similar to the first phase of the so-called SHIRPA protocol (further described in supplementary Table 1). Following the screen, each cohort of mice was tested in the spatial task (Morris water maze), the novel object recognition task, the topological task and the habit task (in the T maze). The order of the tasks was different for each cohort to counter-balance undesirable order effects. The inter-task interval was 2–5 days. The battery ended with the fear conditioning task followed by the fear extinction task.

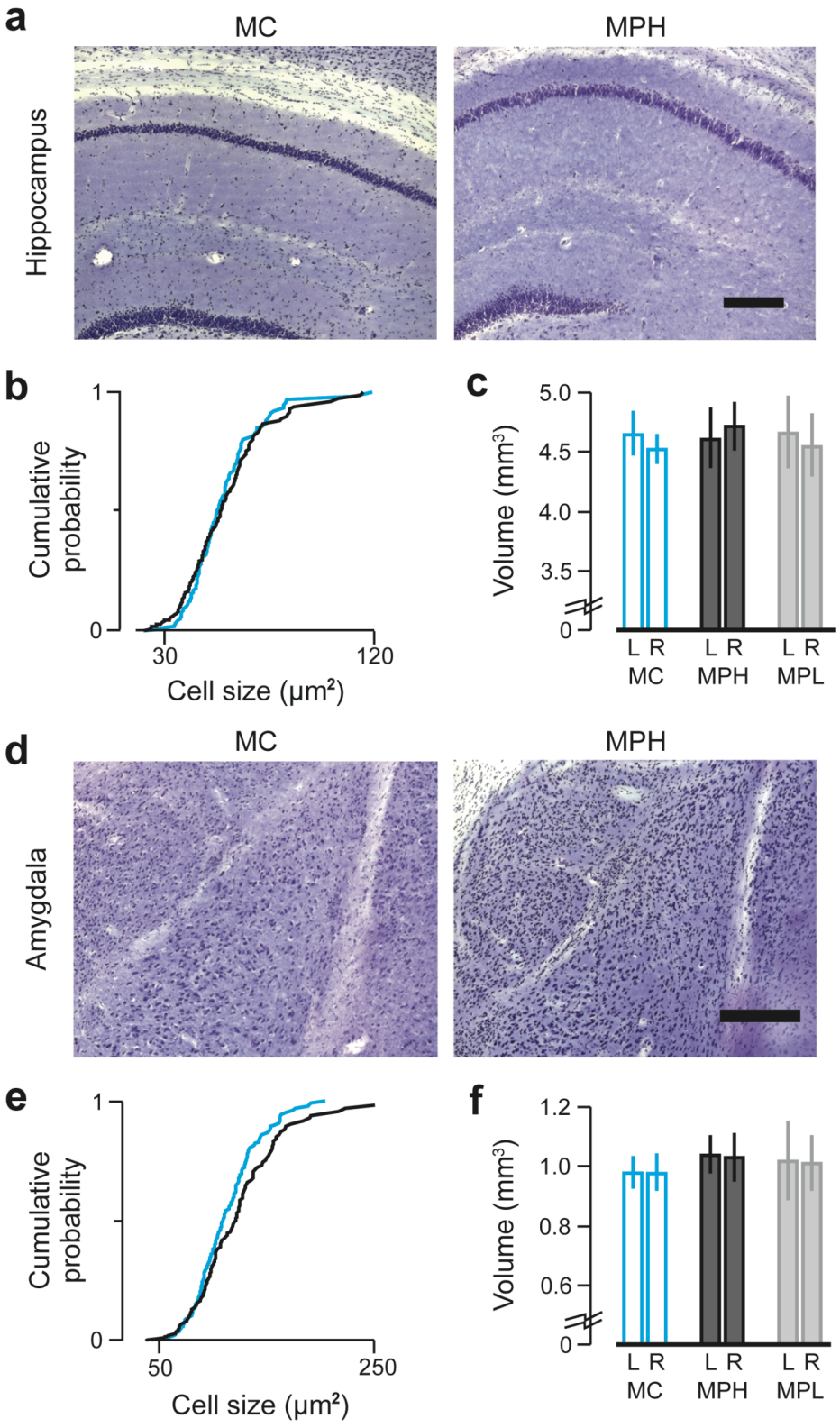
(b) Spatial learning and spatial memory were normal in MPH mice. The Morris water maze task is exquisitely dependent on the integrity of the hippocampus. The animals are required to navigate within a pool as they search a hidden platform (slightly below the water), which they can only find with the use of distally located cues. Probe tests, in which the platform is removed from the pool, are used to measure how well he animals have memorized the location of the hidden platform. This task is well known to depend on hippocampal NMDARs. We used an apparatus consisting of a circular pool (diameter, 160 cm), filled with water, which was placed in a darkened room. A few objects that were distant from the pool, and were focally illuminated from below, served as distal spatial cues. A top-mounted camera attached to the AnyMaze software allowed monitoring and analysis of the animals' performance with lab-written routines. For the task, mice were trained to find a platform that was always in the same location within the pool. As shown in the left diagram, the task was divided into 2 phases: in the first phase, mice were trained to find a hidden platform of "large" diameter (24 cm) for 12 trials (4 trials per day, inter-trial interval of ~30 min). Each trial started from 1 of 4 possible release points (N, S, E, and W), which were chosen randomly, and lasted until the mouse reached and climbed the platform. If the animal was not able to reach the platform in 90 s, the trial was stopped and the animal was gently guided to the platform before being rescued from the pool and thoroughly dried. After trial 12, a first probe test (P1) was performed: each mouse swam for 60 s with the platform removed from the pool. For analysis, the maze was divided into 4 sectors; "target" (in which the platform was located during training), "opposite" (in relation to the target), "left" (L) and "right" (R). The fraction of time the mouse spent of each sector was calculated with routines within AnyMaze. The second phase of the task consisted of trials 13-36, in which the mice found a platform of "small" diameter (16 cm). A second probe test (P2) occurred after trial 24, and a third probe test (P3) occurred at the end of training. We found that the 3 groups of mice displayed similar spatial learning curves, as they decreased their latency to find the hidden platform across trials. Analysis of the probe tests (at right) revealed that the 3 groups displayed comparable spatial memory by the end of training. MPH mice actually showed a higher spatial bias in probe 1 (*, $P < 0.05$, *t* test, MPH vs. MC) and probe 2 ($P < 0.05$, *t* test, MPH vs. MC, data not shown), as they spent longer time in the target sector of the pool.

(c) Habit learning in a T maze task was normal in MPH mice. This task assessed the integrity of the striatal system. As depicted in the leftmost diagram, the apparatus for this test was a circular maze (85 cm diameter), with the floor and perimeter wall (30 cm high) made of clear plastic, joined with waterproof sealant. The maze was filled with water (25°C) to a depth sufficient to wet the underside of the belly of mice (~2cm). The wall contained 12 equally spaced cylindrical holes (4 cm diameter, 5 cm long), corresponding to a 12-hr clock. The lower edge of the holes was 3 cm above the maze floor. For the task, a T-maze (clear plastic arms, 3 cm wide, 15 cm high) was fitted snugly inside a circular maze, so that the arms ended in the holes at 3-hr, 6-hr, and 9-hr (in this case, the 6-hr arm was the start arm). This configuration of holes was different for each mouse. The hole in the start arm and one of the others were sealed with black plugs, while the third hole led to an exit black pipe. Thus, from the center of the maze, the exit appeared similar to the decoy. A black curtain surrounded the periphery of the maze and a soft incandescent light illuminated from above. The task consisted in training the mice to move from the start arm and turn, either left or right; into the goal arm (the diagram shows the goal on the left side). Each mouse was trained to always make the same turn to solve the task. Mice were randomly assigned for left or right turning. For each trial, the mouse was given 120 s to find the exit. If the animal does not succeed in that period, it was gently guided to the exit. The habit task was completed when a mouse found the exit in 9/10 consecutive trials, or 100 trials have elapsed. As observed in the right panels, we found that the different groups of mice displayed similar habit learning curves.

(d) Associative fear conditioning and fear memory were normal in MPH mice. This task consisted of pairing a tone (the conditioned stimulus, CS) with an electric shock (the unconditioned stimulus, US) delivered on the foot pads. Mice learned to associate the tone-CS with the shock-US and thereafter froze in anticipation of the shock after hearing the tone, thus displaying auditory conditioning. Moreover, mice also learned to associate the context in which they received the noxious stimulus (i.e., the context became a CS) and thereafter froze when placed in the environment in which they were shocked, thus displaying context conditioning. Studies using this paradigm have determined that the amygdalar circuit and the auditory inputs to the amygdala are required for the execution of this task of emotional memory. The fear conditioning procedure occurred as follows: on the day before conditioning (day 1), mice were habituated to the training and testing chambers for 10 min. Habituation was counterbalanced between groups to control for possible order effects. On the day of conditioning (day 2), mice were given 3 min to acclimate to the conditioning chamber. This was followed by the presentation of 5 pairings of a 20-s tone-CS (5kHz, 80dB) that co-terminated with a foot shock-US (1s, 0.6mA). The CS-US pairings are indicated with vertical bars in the leftmost graph in (**c**). The inter-trial interval was 90-120 s. We found that the 3 groups of mice displayed similar fear acquisition curves (graph at left), as they increased their freezing across CS-US pairings. After conditioning, mice were returned to their home cages. On the day of testing (day 3), the fear responses conditioned to the tone-CS and the conditioning apparatus (context-CS) were tested separately. Testing for contextual memory took place in the conditioning chamber. The mice were placed in the conditioning chamber and were allowed to explore for 5 min (to give them time to recognize the context), after which the duration of freezing was measured for 5 min. We found that the 3 groups of mice displayed similar contextual fear memory (bar graph in middle panel). Responses conditioned to the tone-CS were measured in the testing chamber. After a brief acclimation period to the testing chamber, the mice received 2 test tones (20 s, 5kHz, 80dB; interval, 100 s). We found that the 3 groups of mice displayed similar tone fear memory (bar graph in panel at right).


a
Extensive handling
Neurological screen
Spatial task water maze
Novel object recognition task
Topological recognition task
Habit task T maze
Associative fear conditioning task
Fear extinction task
b
Spatial task
large platform
small platform
Latency (s)
Blocks (4 trials)
P1
P2
P3
MPH
MPL
MC
Probe 1
Probe 3
Time in sector (% total)
Target
Opposite
Left
Right
c
Habit learning in T maze
Choice
Goal
Error
Start
Correct responses (%)
Blocks (10 trials)
Trials to criterion
d
Fear Conditioning
CS/US
Freezing (% total)
Time (min)
Contextual Memory
Tone Memory

Supplementary Figure 6: Morphological analysis in the adult hippocampus and amygdala.
(a) Coronal sections (cresyl violet) of the mid-hippocampal region (dorsal CA1 region, and the superior blade of the dentate gyrus) prepared from MPH and MC mice. **(b)** The cumulative probability distributions for neuronal size were comparable for MC and MPH in the hippocampus. **(c)** Regional volumes (in mm^3, mean ± s.d.) of hippocampus were also comparable for MC, MPH and MPL mice. **(d)** Coronal sections (cresyl violet) of the dorso-lateral amygdala in MC and MPH mice show similar organization and normal neurons. MPL histology from these regions was also normal (data not shown). **(e)** The cumulative probability distributions for cell size were equivalent for MC and MPH in the amygdala. (f) Regional volumes (in mm^3, mean ± s.d.) of amygdala were also comparable for MC, MPH and MPL mice.



Supplementary Table 1: Neurological screen shows unaltered behavioral variables in MPH mice. The table lists the medians (25% / 75% inter-quartile) for the variables in the order they were measured. We assessed the integrity of sensory and motor systems with an observational screen of 48 variables (modified from Irwin, S., Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse, *Psychopharmacologia* 13: 222-57, 1968) which was similar to the first phase of the SHIRPA protocol (Rogers, D.C. *et al.*, Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm Genome*, 8:711-3, 1997). The variables were divided into 5 categories which measured muscle and lower motoneuron function, spinocerebellar function, sensory function, neuropsychiatric function, and autonomic function. For each mouse, the screen was performed in ~15 min by a trained experimenter. Basal activity and body position was assessed in a cylindrical glass flask (height 15 cm, diameter 11 cm) followed by transfer to an arena (55 x 33 cm^2) and manipulations using tail suspension for measuring visual acuity, grip strength, body tone, and reflexes. Subsequently, each mouse was restrained in a supine position to record autonomic responses, skin color, limb tone, and abdominal tone. Salivation and provoked biting were also recorded. Measuring the righting reflex and negative geotaxis completed the screen. Throughout the procedure, instances of fear, irritability, aggressiveness towards the experimenter, vocalizations and abnormal behavior (such as seizures) were recorded.

Variable	Scale range	MC (*n* = 10)	MPH (*n* = 10)	MPL (*n* = 8)
Coat	[0-4]	0 (0 / 1.5)	0 (0 / 0)	0 (0 / 0)
Hair Length	[0-3]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Hair Morphology	[0-2]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Body Position	[0-8]	4 (4 / 5)	4 (4 / 5)	5 (3.5 / 5)
Spontaneous Activity	[0-8]	3.5 (3 / 5)	3 (2.5 / 4)	5 (4.5 / 5)
Respiratory Rate	[0-3]	2 (2 / 2)	2 (2 / 2)	2 (2 / 2)
Tremor	[0-2]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Defecation, arena	#	4.5 (3 / 5)	5 (2 / 7)	5 (3 / 7.5)
Defecation, whole screen	#	10 (7 / 13)	8 (6 / 9)	6.5 (3.5 / 10.5)
Urination	[0-1]	0.7 (0 / 1)	1 (0 / 1)	0.5 (0 / 1)
Transfer Arousal	[0-6]	3.5 (2 / 4.5)	3 (2 / 4)	4 (3.25 / 4.5)
Latency	sec	7 (4 / 11)	10 (6 / 11.5)	3.5 (1.5 / 7)
Locomotion	#	3 (0 / 5)	3 (1 / 4)	4 (3.5 / 4.5)
Piloerection	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Palpebral Closure	[0-2]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Startle Response	[0-3]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Gait	[0-3]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Pelvic Elevation	[0-3]	2 (2 / 2)	2 (2 / 2)	0 (0 / 2)
Tail Elevation	[0-2]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Touch Escape	[0-3]	1.25 (1 / 2)	1.5 (1 / 2)	1 (1 / 1.25)
Positional-Passivity	[0-4]	0.5 (0 / 2)	0 (0 / 0.5)	0 (0 / 1)
Trunk Curl	[0-1]	0 (0 / 0)	1 (0 / 1)	0 (0 / 0)
Limb Grasping	[0-1]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Visual Placing	[0-4]	2.5 (2 / 3)	3 (3 / 3)	2 (2 / 2.5)
Grip Strength	[0-4]	1.75 (1.5 / 3)	3 (2 / 3)	2 (2 / 2.5)
Body Tone	[0-2]	1.5 (1 / 2)	1 (1 / 1)	1 (1 / 1)
Pinna Reflex (left)	[0-2]	1 (1 / 1.5)	1 (1 / 1.25)	1 (0 / 1.25)
Pinna Reflex (right)	[0-2]	1 (1 / 1.5)	1 (1 / 2)	1 (0 / 1.75)
Corneal Reflex (left)	[0-3]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Corneal Reflex (right)	[0-3]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Toe Pinch (left)	[0-4]	2 (2 / 3)	1.5 (1 / 3)	3 (2.5 / 3)
Toe Pinch (right)	[0-4]	2 (2 / 3)	1.5 (1 / 3)	3 (2 / 3)
Body Length	mm	97.5 (95 / 102)	96 (95 / 98)	94 (93 / 96.5)
Tail Length	mm	94 (91 / 94)	87 (85 / 87)	84.5 (84 / 86)
Lacrimation	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Whisker Morphology	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Provoked Biting	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0.5)
Salivation	[0-2]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Heart Rate	[0-2]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Abdominal Tone	[0-2]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Skin Color	[0-2]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Limb Tone (left)	[0-4]	1.75 (1 / 2)	2 (1 / 2)	1 (1 / 2)
Limb Tone (right)	[0-4]	1 (1 / 2)	2 (1 / 2)	1 (1 / 2)
Wire Maneuver	[0-4]	0 (0 / 0.5)	0 (0 / 1)	0 (0 / 0.5)
Righting Reflex	[0-3]	1 (0 / 1)	0 (0 / 0)	0 (0 / 0)
Contact Righting	[0-1]	1 (1 / 1)	1 (1 / 1)	1 (1 / 1)
Negative Geotaxis	[0-4]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Fear	[0-1]	0.25 (0 / 1)	0.5 (0 / 1)	0 (0 / 0.5)
Irritability	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0.5)
Aggressiveness to Experimenter	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Vocalization	[0-1]	0 (0 / 1)	1 (0 / 1)	1 (0 / 1)
Abnormal Behavior	[0-1]	0 (0 / 0)	0 (0 / 0)	0 (0 / 0)
Body Weight	grams	30.4 (28.9 / 31.2)	27.85 (25.9 / 28.4)	26.38 (25.5 / 27.35)