Neuron-Targeted Caveolin-1 Improves Molecular Signaling, Plasticity and Behavior Dependent on the Hippocampus in Adult and Aged Mice

Supplemental Information

Stereotactic Injection

Mice were anesthetized with 5% isoflurane mixed with 30% O_2, balanced with N_2 and then mounted onto a stereotaxic frame (Kopf; Berlin, Germany). Anesthesia was maintained with 1.4% isoflurane throughout the procedure. Hair was removed from top of head and a dorsal skin incision was made to expose the skull. Skin was retracted and bilateral burr holes were made using a V35 electric hand piece (Buffalo Dental). Burr holes were approximately 4 mm anterior to posterior (0 to -4 A-P from bregma) from the coronal suture and 4 mm laterally from the sagittal suture modified from a previously described protocol (1). The dura was carefully incised and a stereotaxic needle, attached to a precision micromanipulator (Neurostar – Berlin, Germany), was positioned directly on top of the bregma. Hippocampal-targeted injections were controlled using Injectomate software (Neurostar – Berlin, Germany). Injections were made using a 33 gauge 10 µl Hamilton Gas Tight syringe. At each coordinate, the needle was lowered at a rate of 0.32 mm/sec. After 60 sec, 0.5 µl of adeno-associated virus serotype 9 (AAV9) containing synapsin-red fluorescent protein (*SynRFP*) or synapsin-caveolin-1 (*SynCav1*) was injected over 60 sec (0.5 ml/min injection rate) at 3 locations in each hemisphere with an indwelling time of 1 min (injection point 1: 1.82 posterior and 1.15 right lateral side from bregma, and 1.7 mm depth: injection point 2: 2.30 posterior and 2.25 right lateral side from bregma, and 1.75 mm depth; injection point 3: 2.80 posterior and 2.5 right lateral side from bregma, and 2.00 mm depth). Viral titer = 10^9 genome copies/ml. This injection protocol confirmed protein expression in the entire hippocampus (dorsal to ventral and rostral to caudal) as well as long-term protein expression.

Biochemical Characterization of Membrane/Lipid Rafts

Mice were sacrificed by rapid decapitation (5% isoflurane) and the whole brain was quickly removed and hippocampal tissue (bilateral, CA regions and dentate gyrus combined, 50-100 mg) was dissected and homogenized using a carbonate lysis buffer (500 mM sodium carbonate, pH 11.0) containing protease and phosphatase inhibitors (2). Protein was quantified by Bradford assay and normalized to 0.5 mg/ml. Sucrose was dissolved in MBS (25 mM MES and 150 mM NaCl, pH 6.5) buffer to prepare 80%, 35% and 5% solutions (2). Sucrose gradients were prepared by adding 1 ml of 80% sucrose followed by 1 ml of sonicated sample with brief vortexing followed by 6 ml of 35% sucrose followed by 4 ml of 5% sucrose. Gradients were spun in an ultracentrifuge using a SW-41 rotor at 39k rpm at 4°C for 3 h. Fractions (1 ml) were collected from the top of each tube starting at 4 ml to 12 ml. Samples were run as individual fractions and f4-5 (buoyant fractions; BF) and f10-12 (heavy fractions; HF) combined for WB. Samples were run on 10% or 4-12% bis-tris gels (Life Technologies). After transfer to PDVF membranes and blocking, membranes were incubated overnight with primary antibodies for Cav-1 (Cell Signaling #3238, 1:1000), TrkB (BD Biosciences 610102, 1:1000), or CT-B (Invitrogen C-22841, 1:1000) and conjugated to species-specific secondary antibodies and HRP. Densitometric analysis (arbitrary units) was conducted as previously described (2).

Fear Conditioning Behavior

Fear conditioning and extinction training took place in 4 acrylic chambers (30 cm wide x 20 cm deep x 19 cm high, Med Associates Inc., St. Albans, VT, USA) placed in sound attenuating boxes as previously described (3, 4). Foot shocks were delivered through the floor consisting of 36 stainless steel rods wired to a shock generator. Presentation of unconditioned stimuli (US: scrambled foot shock) and conditioned stimuli (CS: auditory tone) were controlled by computer (Med Associates Inc.), and the animal's movements were continuously monitored by real time

video recordings. Freezing was determined using analysis software (Video Freeze, Med Associates Inc., St. Albans, VT; ANY-MAZE, San Diego Instruments, San Diego, CA).

Training: After an acclimation period (2 min), mice were presented with a tone (CS: 90 dB, 5 kHz) for 30 s that co-terminated with a foot shock (US: 2.0 s, 1.0 mA) in dark chambers. A total of three tone-shock pairings were presented with a varying intertrial interval of 30-90 s. Freezing was measured during each CS to measure fear acquisition level across groups. The chambers were cleaned with 70% ethanol after each session.

Context Fear: Twenty-four hours later, mice were re-exposed to the fear conditioning chamber for 8 min to test recall of contextual fear and freezing was averaged across the entire session.

Cued Fear: Twenty-four hours after context fear, mice were tested for recall of cued fear. To remove contextual cues, the chambers were altered across several dimensions (odor - scent; visual – light chambers and walls were altered via plastic inserts; tactile - new floor covering) to minimize generalization from the conditioning context. The session started with a 3 min acclimation period, during which time no tones were presented (“pre-tone” period), then 10 blocks 5 CSs were presented for 30 s each with an inter-trial interval of 5 s. Freezing was recorded during each CS presentation. For analysis, total freezing was averaged as total freezing during all CS presentations. The chambers were cleaned with soapy water after each experiment.

Open Field Activity

Open field activity allows assessment of basic activity and general behavior/anxiety of the mouse. Locomotion was recorded and analyzed by a computerized video tracking system (Noldus XT 7.1, Leesburg, VA). Animals were habituated to the testing room; spontaneous locomotion was assessed in a white plexiglass open field box (41 × 41 × 34 cm enclosures) for

10 minutes. Recorded parameters were distance moved (cm), velocity (cm/second), and time spent in the center of the arena represented by 50% of the total arena (seconds).

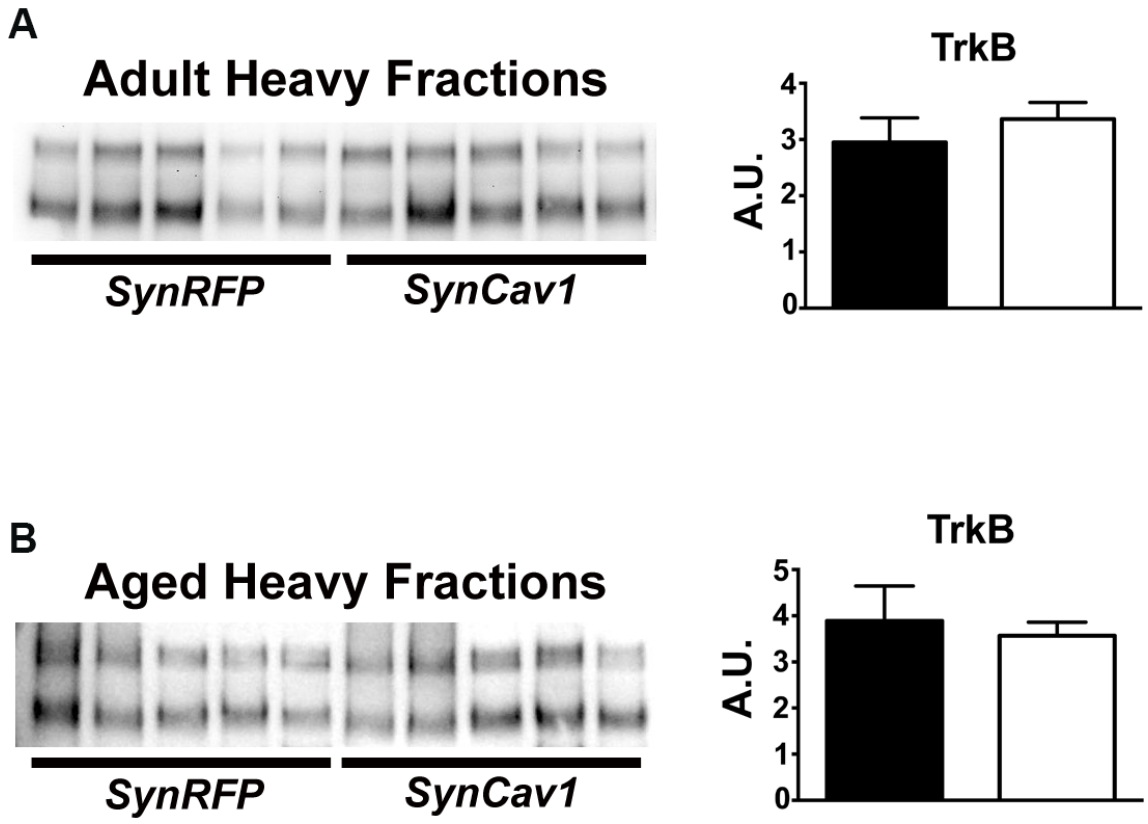

Figure S1. *SynCav1* gene delivery does not produce a difference in TrkB protein expression in heavy fractions from adult and aged hippocampi. Following sucrose density fractionation of hippocampal tissue from adult (**A**) and aged (**B**) mice that received either *SynRFP* or *SynCav1* (as shown in **Figure 1, main manuscript**) were probed for TrkB. Graph represents quantitation of TrkB protein expression in pooled heavy fractions 11 & 12 isolated from 10 adult hippocampi (**A;** *SynRFP* vs *SynCav1*; M ± SEM 2.95 ± 0.43 vs 3.36 ± 0.29; t_8 = 0.79; p = 0.45; n = 5/group) and 10 aged hippocampi (**B;** *SynRFP* vs *SynCav1*; M ± SEM 3.89 ± 0.76 vs 3.57 ± 0.30; t_8 = 0.39; p = 0.70; n = 5/group). All fractions were generated from equal protein loading of 0.5 mg/ml. Data represent arbitrary units (A.U.) mean ± SEM. Significance was assumed when $p \leq 0.05$.

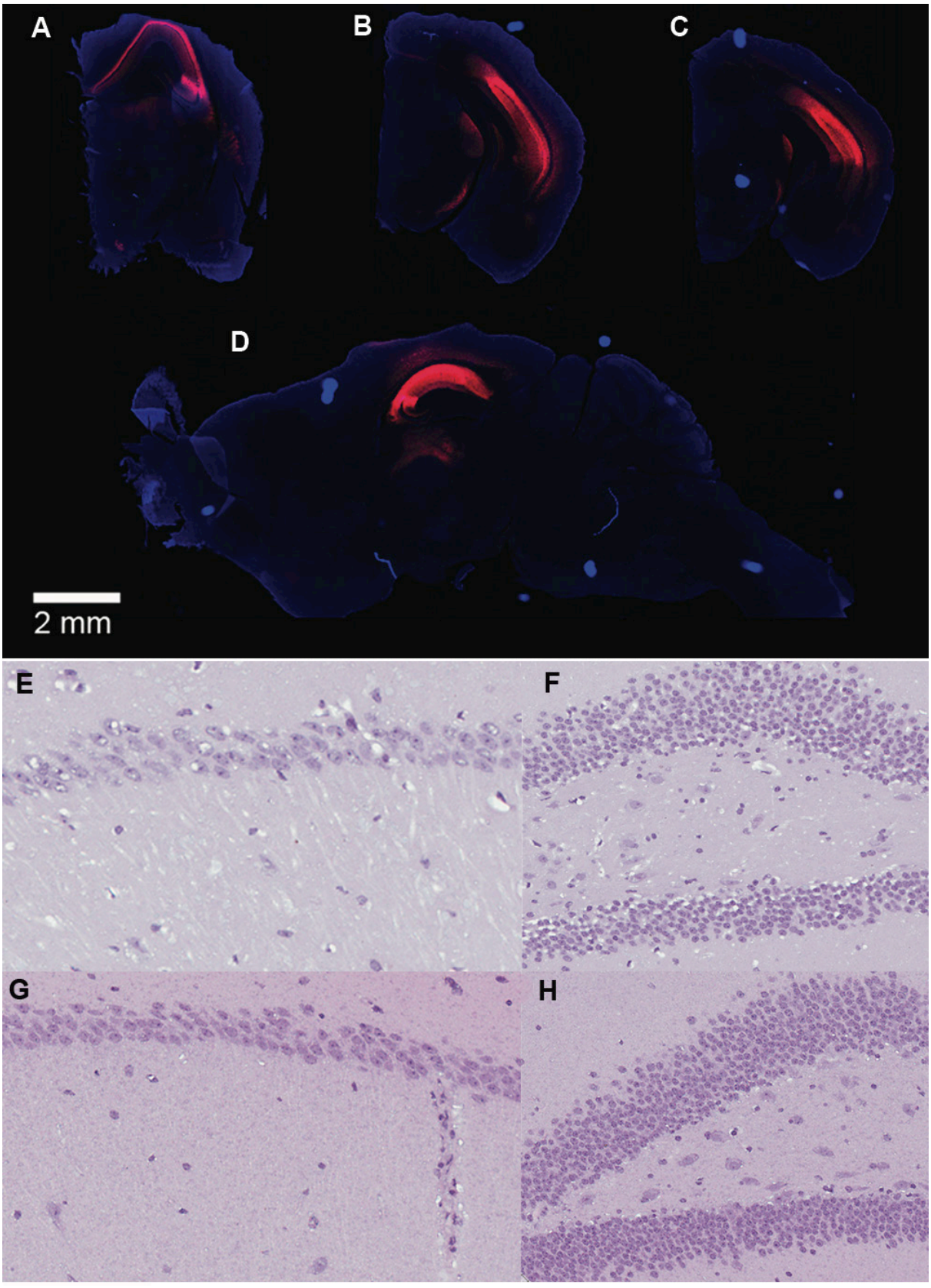

Figure S2. Long-term *SynCav1* overexpression in the hippocampus in aged animals is

not associated with gross anatomical changes. Immunofluorescence microscopy in 20 m aged mice (gene delivery at 10 m) confirmed hippocampal-targeted and long-term protein expression using an AAV9-synapsin driven viral vector (**A-D**); Red, RFP; blue, DAPI (1:5000). Bright field microscopy of hematoxylin and eosin stained hippocampal tissue demonstrates no apparent gross histological differences in either CA1 or DG between *SynRFP* (**E**, CA1; **F,** DG) and *SynCav1* (**G**, CA1; **H**, DG) 10 m post viral vector delivery (**E-H**).

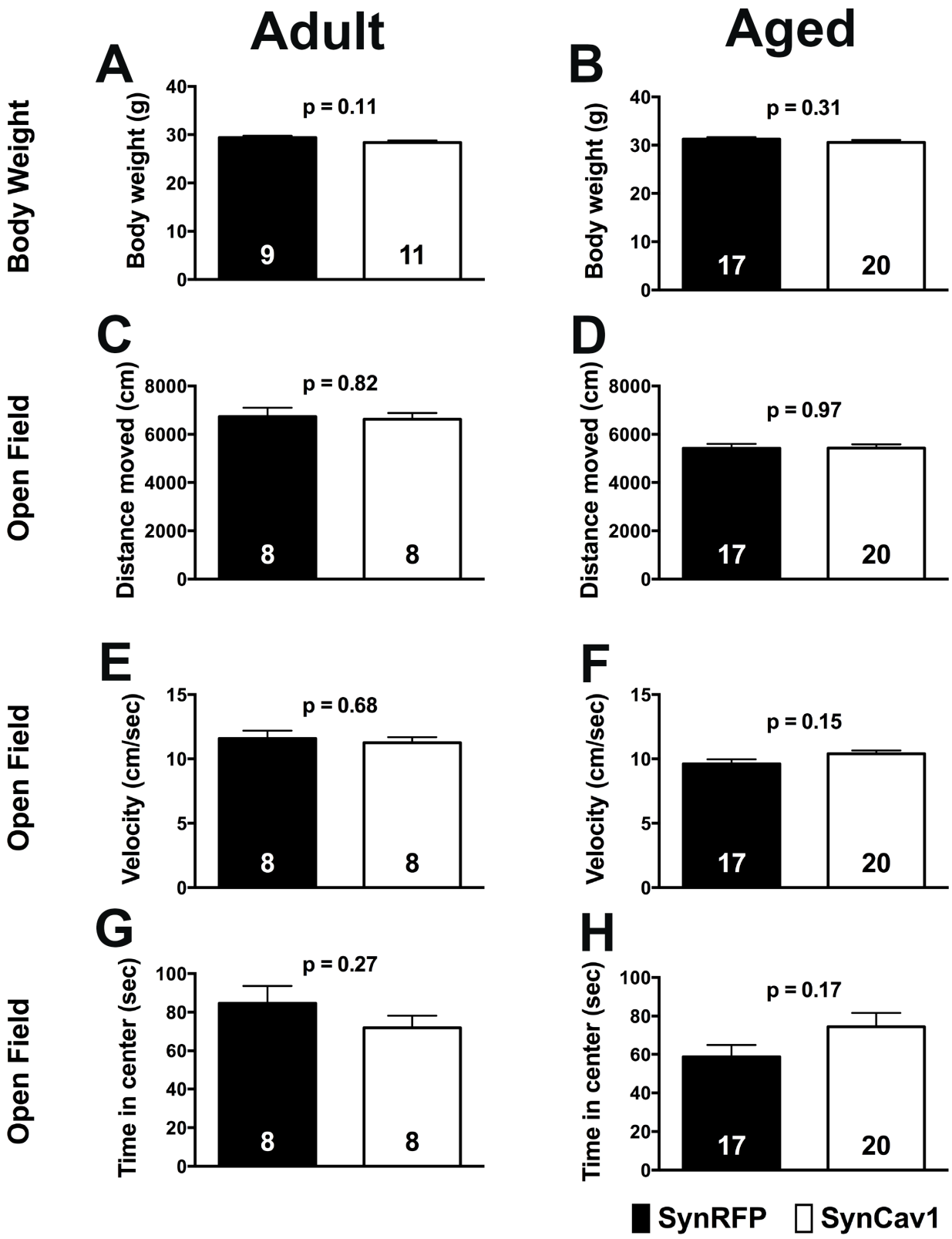

Figure S3. *SynCav1* overexpression does not alter body weight and does not produce gross motor impairment or anxiety-like behavior in adult and aged mice. We performed an unpaired Student's *t*-test or Mann-Whitney test after testing for normality and exclusion of

outliers using ROUT's method (Q = 1%). There was no statistically significant effect from gene on **(A-B)** body weight (**Adult:** *SynRFP* vs *SynCav1*; M ± SEM 29.38 ± 0.41 vs 28.39 ± 0.41; t_{18} = 1.67; p = 0.11; n = 9-11/group; **Aged:** *SynRFP* vs *SynCav1*; Median 31.6 vs 30.65; U = 136; p = 0.31; n = 17-20/group); **(C-H)** and performance in the open field test (**C-D;** distance moved; **Adult:** *SynRFP* vs *SynCav1*; M ± SEM 6731 ± 374.1 vs 6624 ± 258.3; t_{14} = 0.26; p = 0.82; n = 8/group; **Aged:** *SynRFP* vs *SynCav1*; M ± SEM 5420 ± 178.6 vs 5429 ± 154.4; t_{35} = 0.04; p = 0.97; n = 17-20/group; **E-F;** velocity; **Adult:** *SynRFP* vs *SynCav1*; M ± SEM 11.58 ± 0.66 vs 11.26 ± 0.44; t_{14} = 0.42; p = 0.68; n = 8/group; **Aged:** *SynRFP* vs *SynCav1*; Median 9.21 vs 10.12; U = 115; p = 0.15; n = 17-20/group; and **G-H**; time in center; **Adult:** *SynRFP* vs *SynCav1*; M ± SEM 84.66 ± 9.00 vs 71.95 ± 6.26; t_{14} = 1.16; p = 0.27; n = 8/group; **Aged:** *SynRFP* vs *SynCav1*; Median 55.66 vs 66.80; U = 118; p = 0.17; n = 17-20/group). Data represent mean ± SEM. Significance was assumed when $p \leq 0.05$.

Supplemental References

1. Niesman IR, Schilling JM, Shapiro LA, Kellerhals SE, Bonds JA, Kleschevnikov AM, *et al.* (2014): Traumatic brain injury enhances neuroinflammation and lesion volume in caveolin deficient mice. *Journal of neuroinflammation*. 11:39.
2. Head BP, Peart JN, Panneerselvam M, Yokoyama T, Pearn ML, Niesman IR, *et al.* (2010): Loss of caveolin-1 accelerates neurodegeneration and aging. *PLoS One*. 5:e15697.
3. Maren S (2008): Pavlovian fear conditioning as a behavioral assay for hippocampus and amygdala function: cautions and caveats. *The European journal of neuroscience.* 28:1661-1666.
4. Gresack JE, Risbrough VB, Scott CN, Coste S, Stenzel-Poore M, Geyer MA, *et al.* (2010): Isolation rearing-induced deficits in contextual fear learning do not require CRF(2) receptors. *Behavioural brain research.* 209:80-84.