Social Isolation Exacerbates Schizophrenia-like Phenotypes via Oxidative Stress in Cortical Interneurons

Supplemental Information

Supplemental Methods and Materials

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde. Brains were post-fixed with the same solution overnight, and then coronally sectioned by Vibratome with 35-μm thickness. Brain sections were washed with phosphate buffered saline (PBS) three times and blocked with 10% normal goat serum in PBS, followed by overnight incubation at 4°C with gentle shaking in primary antibodies. After three washes in PBS, sections were incubated in the dark in Alexa 555, Alexa 488 or Alexa 350-conjugated goat anti rabbit- or mouse-IgG (1:300; Invitrogen, Carlsbad, CA) for two hours. Sections were mounted in Vectashield. The primary antibodies used were mouse anti-8-hydroxy-2′-deoxyguanosine (8-OH-dG) (1:30; JalCA, Shizuoka, Japan), rabbit anti-4-Hydroxy-2-nonenal (4-HNE) (1:1000; ADI, San Antonio, TX), mouse anti-parvalbumin (PV) (1:5000, Swant, Bellinzona, Switzerland), rabbit anti-PV (1:2000; EMD Chemicals, Philadelphia, PA), rabbit anti-calretinin (1:2000; Chemicon, Billerica, MA), mouse anti-Calbindin (1:1000; Swant), rabbit anti-neurogranin (1:5000; Chemicon), mouse anti-Reelin (1:1000; Millipore, Billerica, MA), rabbit anti-GFP (1:2000, Invitrogen), rabbit anti-β-galactosidase (1:2000; MP Biomedicals, Inc., Santa Ana, CA), mouse anti-glial fibrillary acidic protein (GFAP) (1:1000; Chemicon), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1) (1: 2000; Wako Chemical USA, Richmond, VA), and rat anti-CD68 (1: 500; AbD Serotec, Raleigh, NC). Confocal images were obtained on a Zeiss LSM 510 confocal microscope

at the National Institute of Neurological Disorders and Stroke Light-Imaging Facility. Immunoreactive (IR) signals were quantified by NIH Image J after being converted to gray scale. Relative IR from medial prefrontal cortex (mPFC) or S1 area (covering through layer I-VI) of each animal was normalized by the average value from age-matched group-housed fGluN1 controls.

Non-radioisotopic Double *In Situ* Hybridization

DNA fragments corresponding to mouse PV complementary DNA (cDNA) (GenBank accession number: NM_013645) and PGC-1a cDNA (GenBank accession number: NM_008904.2) were amplified by reverse transcription polymerase chain reaction (RT-PCR) using the following primers: 5' GGGCCTGAAGAAAAAGAACC 3' and 5'AGTACCAAGCAGGCAGGAGA 3' for PV (1), and 5'GACAGTGTGTGTGTGTGTGTCC 3' and 5' TATCAGAGGCCATGCTAGTGAA 3' for PGC-1a (exon 13). To detect mouse PV and PGC-1a mRNAs, the complementary RNA (cRNA) probe for PV was labeled with 2, 4-dinitrophenyl (DNP), and the cRNA probe for PGC-1a was labeled with digoxygenin (DIG). Double non-radioisotopic *in situ* hybridization was performed as previously described (2). Briefly, 18-μm fresh frozen brain sections were post-fixed for 15 min in 4% paraformaldehyde-PBS, permeabilized in 5 μg/ml proteinase K in 1x PBS for 10 min at room temperature, and acetylated in 0.25% acetic anhydride in triethanolamine 10 mM (pH 8.0) for 10 min. DIG-labeled PGC-1a mRNA antisense probe and DNP-labeled PV mRNA antisense probes were then hybridized together at 56°C for 48 hours. After washing with graded SSC buffers, DIG probes were reacted with sheep alkaline phosphatase-conjugated anti-DIG (1:2000; Roche, Mannheim, Germany), and DNP probes were reacted with rabbit anti-DNP (1:400; Invitrogen). DIG signals

were then detected using NBT-BCIP (blue precipitate), and DNP signals were detected with peroxidase reaction using 3-amino-9-ethylcarbazole (AEC, red precipitate; Vector Laboratories) after incubation with a biotinylated anti-rabbit secondary antibody, followed by avidin-biotin complex formation.

Western Blot

The mPFC from fGluN1 control or knockout (KO) mice was dissected out and homogenized on ice in 400 μl extraction buffer (10 mM NaPPi, pH 7.5, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 2 mM dithiothreitol, 500 μM benzamidine, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 25 μg/ml Trypsin inhibitor). The supernatant was obtained by centrifugation at 1,700 *g* for 10 min at 4^{o}C. Protein levels were determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). Hippocampal extracts was denatured in Laemmli buffer (Sigma-Aldrich). Equivalent amounts of protein were loaded for SDS-PAGE. Following electrophoresis, proteins were transferred onto Immobilon-P membranes (Millipore), blocked with 5% milk in TBS with 0.05% Tween-20, and blotted with primary antibody followed by the HRP-labeled secondary antibody. The reaction was developed with ECL plus (Amersham Pharmacia Biotech, Piscataway, NJ). Antibodies included rabbit polyclonal anti-PGC-1α (sc-13067, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-β-actin (Sigma-Aldrich). Western blot results were quantified using KODAK Molecular Imaging Software (Carestream Health Molecular Imaging, New Haven, CT).

Quantitative RT-PCR

Medial PFC from fGluN1 control or KO mice was dissected and total RNA was extracted using Trizol reagent (Invitrogen). Genomic DNA was digested with DNase RQ (Promega, Madison, WI) and removed by RNeasy Cleanup Kit (QIAGEN, Valencia, CA). RNA concentration and quality were examined by NanoDrop (Thermo Fisher Scientific, Waltham, MA). One microgram of RNA was used for reverse transcription with Superscript II (Invitrogen) and oligo-dT in a 20 µl reaction. 180 µl H_2O was added at the end. One µl cDNA was used for real-time PCR with QuantiTect SYBR Green PCR Kit (QIAGEN). Primers used were:

PGC-1a: AGCCGTGACCACTGACAACGAG and GCTGCATGGTTCTGAGTGCTAAG,

Superoxide dismutase 1 (SOD1): CAAGCGGTGAACCAGTTGTG and TGAGGTCCTGCACTGGTAC,

Superoxide dismutase 1 (SOD2): GCCTGCACTGAAGTTCAATG and ATCTGTAAGCGACCTTGCTC,

Glutathione peroxidase 1 (GPX1): GTCTCTCTGAGGCACGATCCG and TTCCGCAGGAAGGTAAACAGC,

Catalase (CAT): ACCCTCTTATACCAGTTGGC and GCATGCACATGGGGCCATCA,

Nox1: CTTCCTCACTGGCTGGGATA and TGACAGCGTTTGCGCAGGCT,

Nox2: CCAACTGGGATAACGAGTTCA and GAGAGTTTCAGCCAAGGCTTC,

Nox4: TTAAACACCTCTGCCTGCTC and CTTCTTGTTCTCCTGCTAGG,

IL-6: ATGGATGCTACCAAACTGGAT and TGAAGGACTCTGGCTTTGTCT, and

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): TGTGTCCGTCGTGGATCTGA and TTGCTGTTGAAGTCGCAGGAG.

Real-time PCR was conducted on a Smartcycler (Cepheid, Sunnyvale, CA).

Behavioral Analysis

The male Ppp1r2-cre/fGluN1 KO mice and their age-matched fGluN1 littermates (homozygous), were maintained under a 12:12-hour dark:light cycle with food and water *ad libitum*. All behavioral tasks, except the Y maze (see below), were performed during the light phase by an investigator blind to genotype/treatment. All animals were moved from the mouse colony room to a holding room adjacent to the behavioral test room in their home cages at least one hr before testing. Behaviors were evaluated as previously described (2).

Elevated Plus Maze

Anxiety-like behaviors were assessed using an elevated plus maze. The maze (Mikes Machine Co., Attleboro, MA) consisted of two open arms (30 x 5 cm) with 3 mm high ledges and two closed arms (30 x 5 cm) with 16 cm-high opaque walls. The floor of the arms and a central square (5 x 5 cm) were made of white plastic and were elevated 42 cm from the room floor. The maze was placed in the center of a room (10 x 10 ft) and was homogenously illuminated (about 100 lux across arms). Each mouse was place in the central square facing the open arm opposite to the investigator. Mouse behavior was automatically video-recorded for 5 min and animal position was determined by automatic video tracking (ANY-maze; Stoelting Co., Wood Dale, IL).

Locomotor Activity in an Open Field

The activity of animals was monitored in a 40 x 40 cm VersaMax chamber (AccuScan Instruments, Columbus, OH) and detected through photobeam breaks for 30 min. Data were analyzed every 3 min.

Y-maze Spontaneous Alternation Test

The Y-maze apparatus, which was made of transparent Plexiglas, had three identical arms (40-cm long x 4.5-cm wide x 12-cm high) placed at 120° with respect to each other. Each mouse was placed at the end of one arm facing the center and allowed to freely explore the apparatus for 8 min. Animal behaviors were recorded through a video camera mounted above the maze and evaluated using 'ANY-maze' video-tracking system (Stoelting Co.). The pattern of entries into each arm was visually scored over eight minutes after the first entry (with a cut-off of 2 min). Alternation behavior was defined as consecutive entries into each of all three arms without repeated entries, as on overlapping triplet sets (i.e., ABC, BCA…). Percent of spontaneous alternation was calculated as the ratio of actual (= total alternations) to possible (= total arm entries minus 2) number of alternations x 100. Total entries were scored as an index of ambulatory activity in the Y-maze, and animals with scores below 12 were excluded. All experiments were conducted during the first 2 to 3 hours of the initial dark phase (6:00 PM to 9:00 PM) to maximize exploratory activity.

Acoustic Startle Reflex

Acoustic startle reflex was carried out using SR-LAB startle chambers (San Diego Instruments, San Diego, CA). Briefly, a Plexiglas cylinder was mounted on a platform in a ventilated sound-attenuated cubicle; a high-frequency loudspeaker mounted on the ceiling produced all acoustic stimuli. Movements within the cylinder were detected and transduced by a piezoelectric accelerometer attached to the Plexiglas base, digitized, and stored by the computer. Background sound levels (70 dB) and calibration of the acoustic stimuli were confirmed with a digital sound level meter. The session began with a 5 min acclimation period follow by 42 trials delivered in blocks of six. Each block contained one no-stimulus trial and 5 trials with auditory

stimuli of different intensities (bursts of 40-ms white noise pulse at 84, 90, 100, 110, and 120 dB) delivered in pseudo-random order with an average inter-trial interval (ITI) of 15 ms (10-20 ms). Beginning at the stimulus onset, 65 readings of 1 ms duration were recorded to obtain the peak startle amplitude. The average startle response was calculated for each trial.

Prepulse Inhibition

Testing was conducted in SR-LAB startle chambers. Each session consisted of 42 trials, following a 5 min acclimation period. Six different trial types were presented; the no-stimulus trials, trials with the acoustic startle stimulus (40 ms; 120 dB) alone, and trials in which a prepulse stimulus (20 ms white noise at 72, 74, 78, and 84 dB) had an onset 100 ms before the onset of the startle stimulus. The different trial types were presented in blocks of six, in randomized order within each block, and with an average ITI of 15 ms (range: 10-20 ms). Each session was initiated with a 5 minute acclimation period of background noise (70 dB) followed by 7 successive 120 dB habituation tones (40 ms long, ITI = 15 ms). These trials were not included in the analysis. Measures were taken of the startle amplitude for each trial, defined as the peak response during a 65 ms sampling window that began with the onset of the startle stimulus. An overall analysis was performed for each subject's data for levels of prepulse inhibition at each prepulse sound level (calculated as 100 - ((response amplitude for prepulse stimulus and startle stimulus together/response amplitude for startle stimulus alone) X 100)).

Two-Bottle Saccharine Preference Test

During six days of experiment, animals were single-housed in their home cage and had access to two drinking tubes. Day 1-2, mice were provided water vs. water; Day 3-4, water vs. saccharine 0.03%; and Day 5-6, water vs. saccharine 0.06%. The position of the tubes was

switched every 24 hr to prevent any possible side bias. Solution preference ratios were calculated as: preference (%) = saccharine intake/(saccharine intake + water intake) X 100.

Nest Building Test

Each animal was transferred to a clean mouse cage containing fresh bedding and a single pressed cotton square nestlet (2.7 g; Ancare, Bellmore, NY) approximately one hour before the dark phase. Cages remained on their original racks in the colony room throughout the experiment. Any unused nestlet material was weighed the next morning by collecting un-shredded cotton pieces more than approximately 0.1 g.

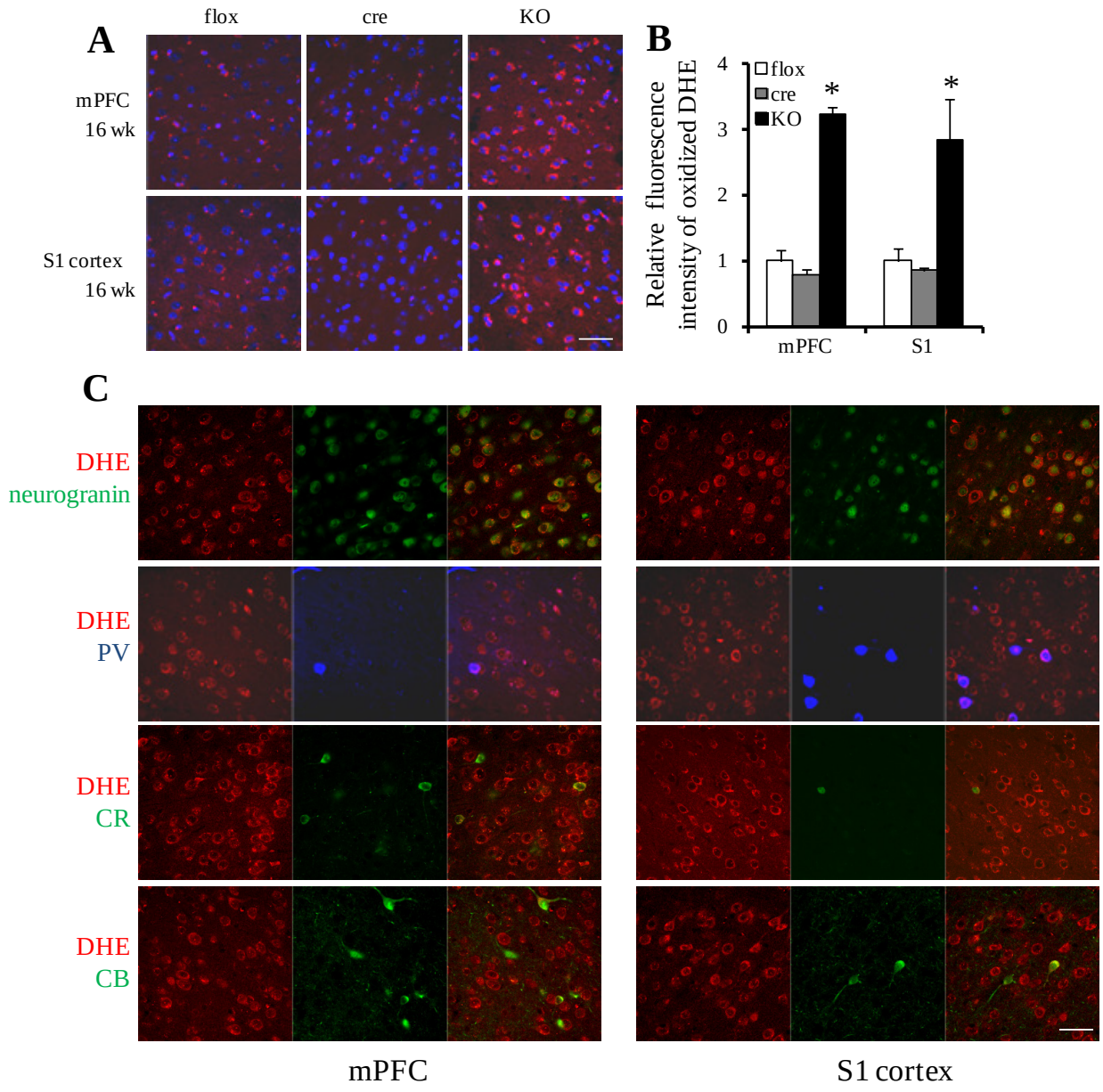

Figure S1. (A) Group-housed Ppp1r2-cre mice (cre), homozygously floxed-GluN1 (flox) or knockout (KO) animals at the age of 16 weeks were injected with dihydroethidium (DHE) (4~5 animals per group). Reactive oxygen species (ROS) levels in medial prefrontal cortex (mPFC) and S1 cortex were visualized by oxidized DHE (in red) in sections counterstained with DAPI (blue). **(B)** Relative ROS levels in both mPFC and S1 cortex were much higher in 16-week-old KO animals, compared to age-matched flox and cre controls. One-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test, $*p < .05$. **(C)** Characterization of cells with elevated ROS in cortex. Coronal sections from post-weaning social isolated Ppp1r2-cre/fGluN1 KO animals that received DHE injection at the age of 8 weeks were stained with neurogranin (NG, excitatory neuron marker), parvalbumin (PV), calretinin (CR) or calbindin (CB) antibody. Red fluorescence indicated oxidized DHE by ROS. Neurogranin-, calretinin- and calbindin-positive neurons are indicated by green fluorescence (Alexa 488) and parvalbumin positive neurons are indicated by blue fluorescence (Alexa 350). Oxidized DHE positive cells could be excitatory neurons, CR+, CB+ (indicated by yellow) or PV+ (indicated by purple). Scale bar in (**A)** and (**C)**: 50 µm.

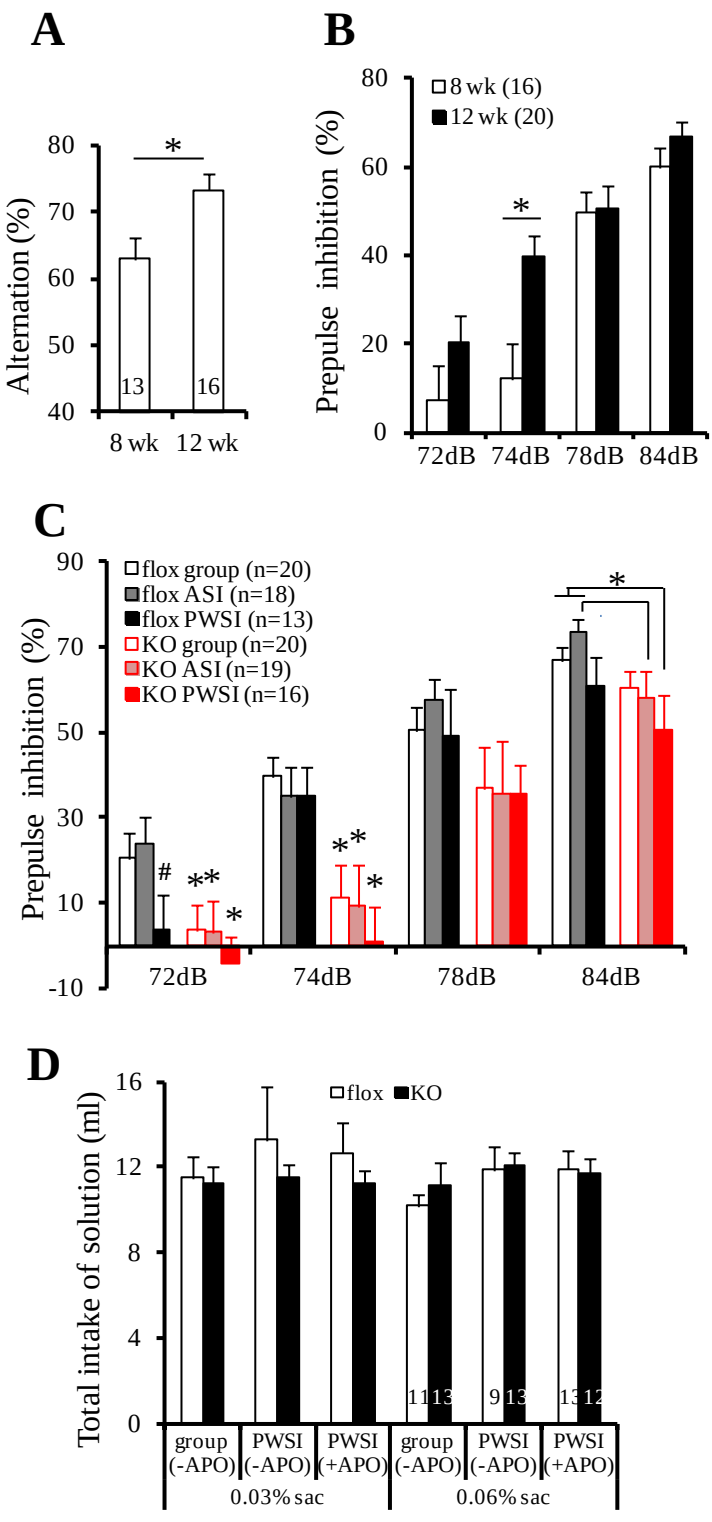

Figure S2. (**A**) The animals at the age of 8 weeks were premature to run spontaneous Y-maze alternation. Group-housed flox control animals showed lower alternation in Y-maze conducted at 8 weeks-old compared with 12 weeks-old. Two-tailed unpaired Student *t*-test, **p* = .015. (**B**) fGluN1 control mice at 8 weeks old did not show robust prepulse inhibition (PPI) compared to those at 12 weeks old. *Post hoc* Fisher least significant difference (LSD) test,**p* = .0034. (**C**) Ppp1r2-cre/fGluN1 knockout (KO) mice showed PPI impairment at the age of 12 weeks regardless of housing condition. Repeated measures analysis of variance showed significant genotype effect ($F(4,97) = 5.73$, $p = 0.00035$), no housing-condition effect ($F(8,194) = 1.23$, $p = 0.28$). *Post hoc* Fisher's LSD test **p* < .05 compared with group-housed or ASI flox controls. ASI: adolescent social isolation (single-housed from the age of 7 weeks); PWSI: post-weaning social isolation. Flox control animals showed PPI impairment at 72 dB after PWSI but not after ASI. *Post hoc* Fisher's LSD test #*p* < .05 compared to group-housed and ASI flox controls. (**D**) The total intake volumes, *i.e.*, the sum of saccharine intake and water intake, in two-bottle saccharine preference test were similar regardless of genotype, housing condition and drug treatment. Number of animals used is indicated in parentheses or inside plot bars. APO, apocynin.

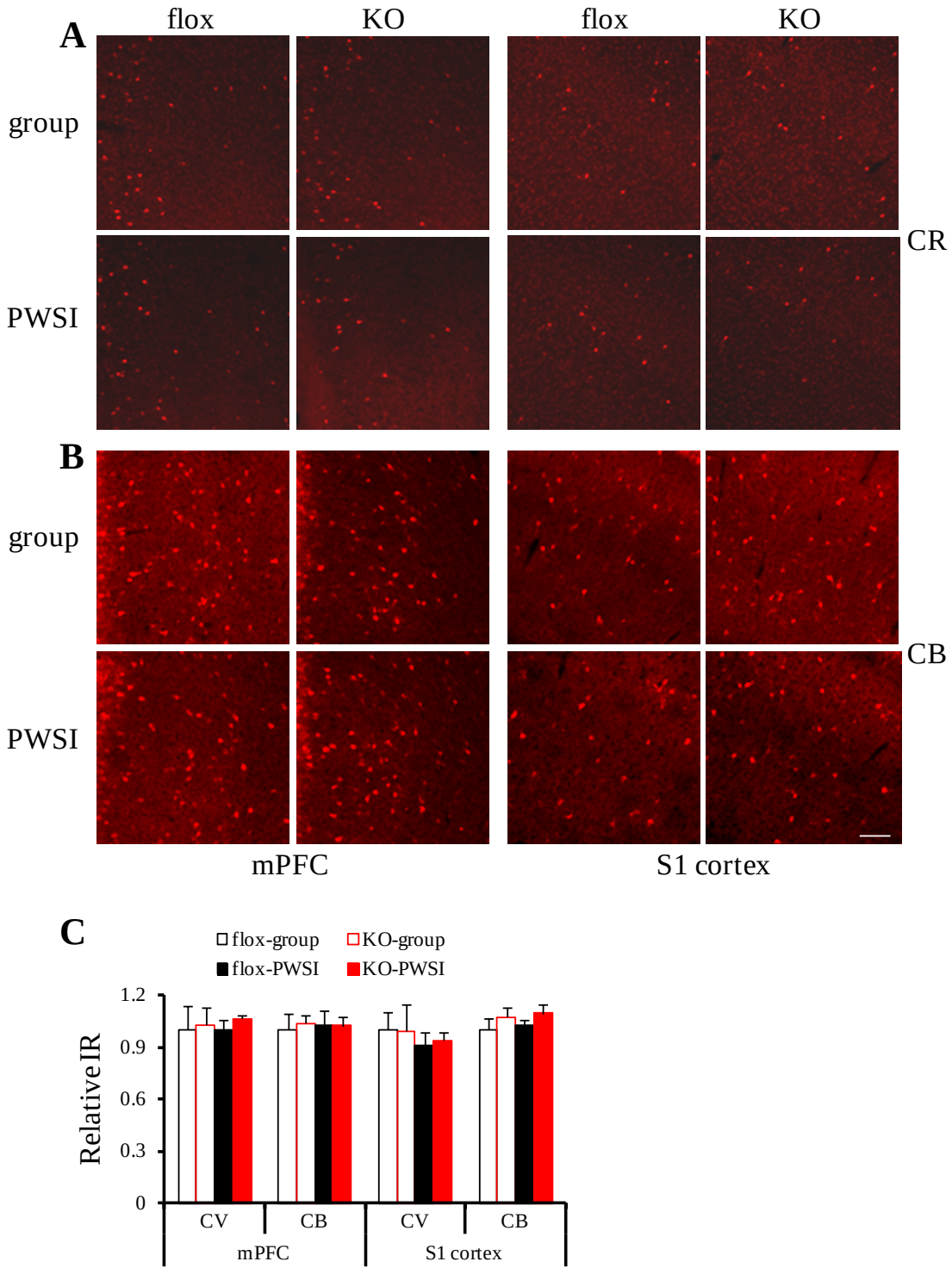

Figure S3. No differences in relative calretinin (CR) or calbindin (CB)-immunoreactivity (IR) between Ppp1r2-cre/fGluN1 knockout (KO) and flox control mice regardless of housing conditions in medial prefrontal cortex (mPFC) or S1 cortex. **(A** & **B)** Brain sections from group-housed or post-weaning social isolation (PWSI) animals (4-6 animals for each group, 8 weeks-old) were immunostained with CR or CB antibody and visualized with Alexa 555. (**C**) Relative fluorescence intensity from mPFC or S1 area was normalized by the average value from age-matched group-housed fGluN1 controls. Scale bar: 100 µm.

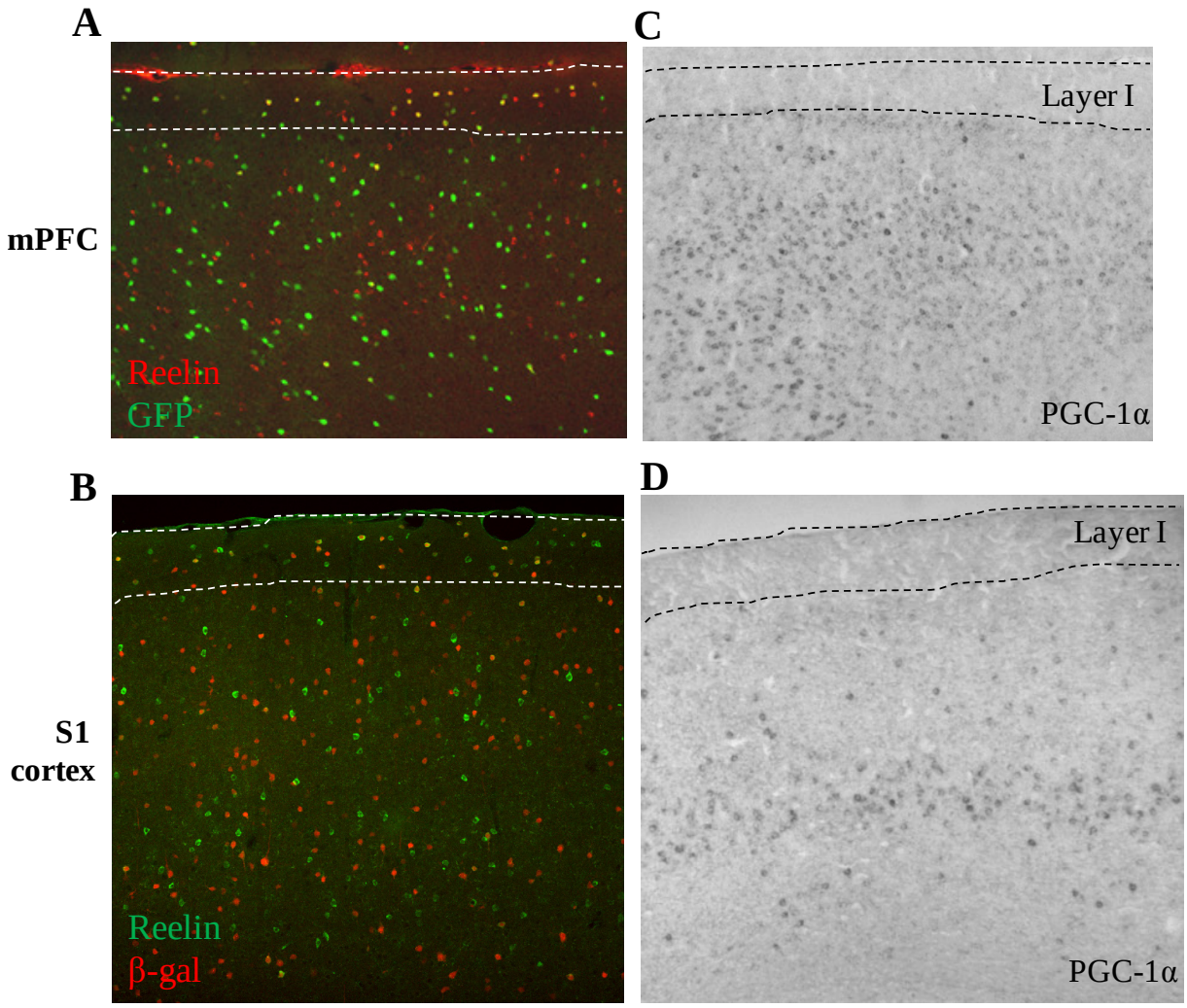

Figure S4. Cre targeted Reelin-positive neurons are predominantly localized in layer I of medial prefrontal cortex (mPFC) and S1 cortex, whereas PGC-1a was not expressed in layer I. (**A** & **B**) Double staining with Reelin and green fluorescent protein (GFP) antibodies using mPFC sections from reporter line Ppp1r2-cre/floxed-YFP (**A**, Reelin in red and GFP in green), or with Reelin and β-gal antibodies using S1 cortex sections from reporter line Ppp1r2-cre/floxed-LacZ (**B**, Reelin in green and β-gal in red) revealed their colocalization appeared only in Layer I. (**C** & **D**) PGC-1a is absent in layer I of mPFC (**C**) or S1 cortex (**D**) as shown by *in situ* hybridization using PGC-1a probe.

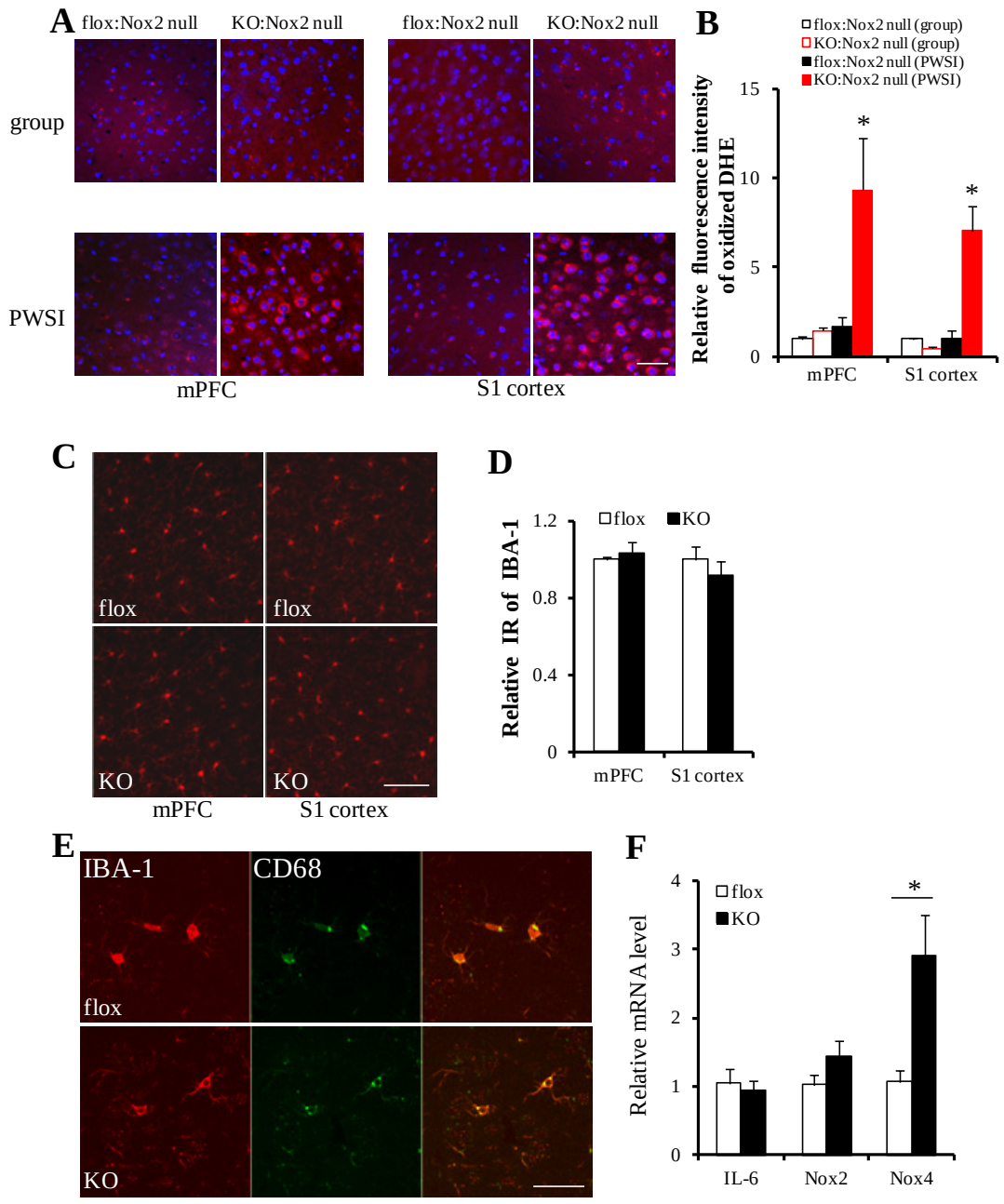

Figure S5. (**A-B**) Depletion of Nox2 in mouse brain did not alleviate social-isolation induced exacerbation of oxidative stress in the medial prefrontal cortex (mPFC) and S1 cortex (**A**). Fluorescence intensity of oxidized DHE was quantified for each group and compared to the average value of group-housed flox controls (**B**), **p* < .05, two-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test. Animals used in A & B were all at the age of 8 weeks. flox: Nox2 null, flox controls with Nox2 ablation, KO: Nox2 null, Ppp1r2-cre/fGluN1 KO with Nox2 ablation. (**C-E**) Oxidative stress was not due to microglial activation. Morphology visualized by microglial marker anti-Iba1 (**C**) or its immunoreactivity intensity (**D**) was not significantly altered in the cortex of knockout (KO) mice. CD68 was minimally stained in KO mice, suggesting microglial activation was not observed in KO animals using activation marker CD68 (**E**). (**F**) Quantitative real-time polymerase chain reaction showed higher Nox4 mRNA levels in the mPFC of KO mice, otherwise close to the detection limit level

in the fGluN1 controls; however there was no detectable change in IL-6 and Nox2 expression. Unpaired student's *t*-test, **p* < .05. Note that Nox1 mRNA was below detection level in these tissues. Animals used in C-F were all at the age of 16 weeks, group-housed. Scale bar: 50 μm (A), 100 μm (C), 40 μm (E). PWSI, post-weaning social isolation.

Supplemental References

1. Tanahira C, Higo S, Watanabe K, Tomioka R, Ebihara S, Kaneko T, Tamamaki N (2009) Parvalbumin neurons in the forebrain as revealed by parvalbumin-Cre transgenic mice. *Neurosci Res* 63:213-223.
2. Belforte JE, Zsiros V, Sklar ER, Jiang Z, Yu G, Li Y, *et al.* (2010): Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat Neurosci* 13:76-83.