Western blot analysis

Total proteins were extracted with RIPA buffer (150mM NaCl, 25mM Tris-HCl pH7.6, 50mM EDTA pH8.0, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS) and their concentration was determined by Bradford's colorimetric assay. Equal amounts of total protein/lane (30 µg/lane) were subjected to SDS-polyacrylamide (SDS-PAGE) electrophoresis under reducing conditions. Gels were electroblotted onto nitrocellulose membrane using a Trans-blot Turbo Transfer system (Cat.1704150, Bio-Rad, USA) for 3 min at 2.5 mA and 25V, and a Ponceau (Cat. R-03021-D50, Advansta, USA) staining was performed to verify the protein transfer efficiency and loading amount. Membranes were blocked with Everyblot blocking solution (Cat. 12010020, Bio-Rad, USA) for 5 min at room temperature and immunoreacted overnight at 4°C with the following primary antibodies (SantaCruz Biotechnology, USA): anti-FAAH (27-Y) (1:1000, Cat. sc-100739), anti-MAGL (C-11) (1:2000, Cat. sc-398942), anti-DAGLα (E6) (1:1000, Cat. sc-398942), anti-CB2 (3C7) (1:2000, Cat. sc-293188), anti-CB1 (2F9) (1:1000, Cat. sc-293419). After washings with TBS-T (TBS supplemented with 0.05% Tween-20), membranes were incubated with the horseradish peroxidase-conjugated anti-mouse secondary antibody for 60 min (1:10000, Cat. 7076, Cell Signaling, USA). Antibodies were diluted in 3% BSA-TBST. Membrane was washed with TBS-T per 5 min, followed by washing in TBS, and processed for chemiluminescence detection (Clarity Western ECL substrate, Cat. 1705060, Bio-Rad, USA). Chemiluminescence signals were detected in a ChemiDoc XRS+ system (Cat.1708265, Bio-Rad, USA) and analyzed by ImageLab Software. Protein levels were normalized to Ponceau staining (1, 2) and expressed as fold changes relative to the correspondent WT group.

References

1. Gilda JE, Gomes AV. Stain-Free total protein staining is a superior loading control to beta-actin for Western blots. Analytical biochemistry. 2013;440(2):186-8.

2. Sander H, Wallace S, Plouse R, Tiwari S, Gomes AV. Ponceau S waste: Ponceau S staining for total protein normalization. Analytical biochemistry. 2019;575:44-53.

Supplementary Figure 1. Timeline of the experiments.

Supplementary Figure 2. Diagrams of rat brain sections showing representative microinjection sites (filled circles) in the hippocampus and amygdala. Only data from animals showing bilateral needle tracks terminating in the hippocampus or amygdala and no damage to the target tissues were included in the final analyses.

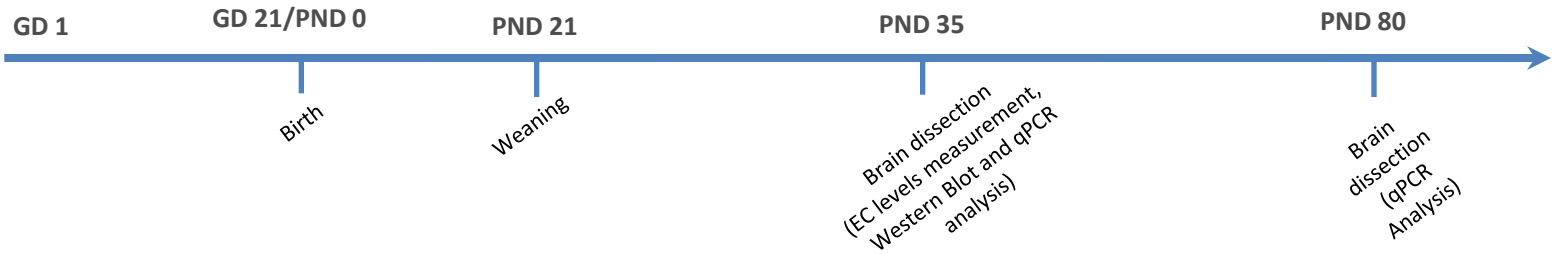
Supplementary Figure 3. Western Blot analysis of the main components of the ECS in the hippocampus (A-E) and amygdala (F-J) of juvenile *Fmr1-$^{\Delta}$exon 8* rats and WT controls. Western blots showing DAGLα (**A**), MAGL (**B**), FAAH (**C**), CB1 (**D**) and CB2 (**E**) protein expression (top panels) and their relative densitometric analysis (bottom panels) in the hippocampus of WT and *Fmr1-$^{\Delta}$exon 8* rats at PND 35. Western blots showing DAGLα (**F**), MAGL (**G**), FAAH (**H**), CB1 (**I**) and CB2 (**J**) protein expression (top panels) and their relative densitometric analysis (bottom panels) in the amygdala of WT and *Fmr1-$^{\Delta}$exon 8* rats at PND 35. The levels of each protein were normalized to Ponceau staining (WT = 4; *Fmr1-$^{\Delta}$exon 8* = 4 animals per group). Data represent mean ± SEM.

Supplementary Figure 4. qPCR analysis of the main components of the ECS in the hippocampus and amygdala of adult *Fmr1-$^{\Delta}$exon 8* rats and WT controls. Fold induction of the enzymes NAPE-PLD (**A, J**), DAGLα (**B, K**), DAGLβ (**C, L**), MAGL (**D, M**), FAAH (**E, N**), and CB1 (**F, O**), CB2 (**G, P**), TRPV1 (**H, Q**), GPR55 (**I, R**) receptor expression in the hippocampus (**A-I**) and amygdala (**J-R**) of *Fmr1-$^{\Delta}$exon 8* rats and WT animals, evaluated at PND

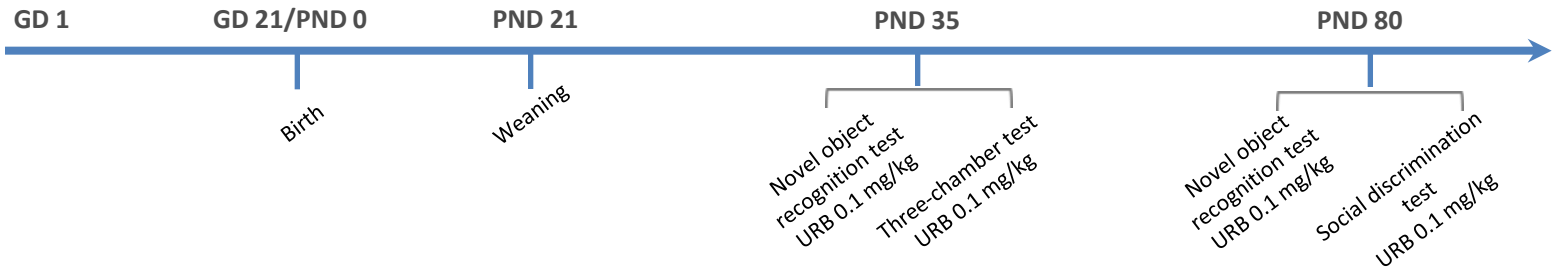
80 (WT = 3-4; *Fmr1-$^{\Delta}$exon 8* = 3-4 animals per group). Data represent mean ± SEM, *p<0.05 and **p<0.01 vs WT group (Student's t-test).

Supplementary Table 1. Forward and reverse primer sequences, used in qPCR experiments, for amplification cycles of enzymes and receptors of the ECS.

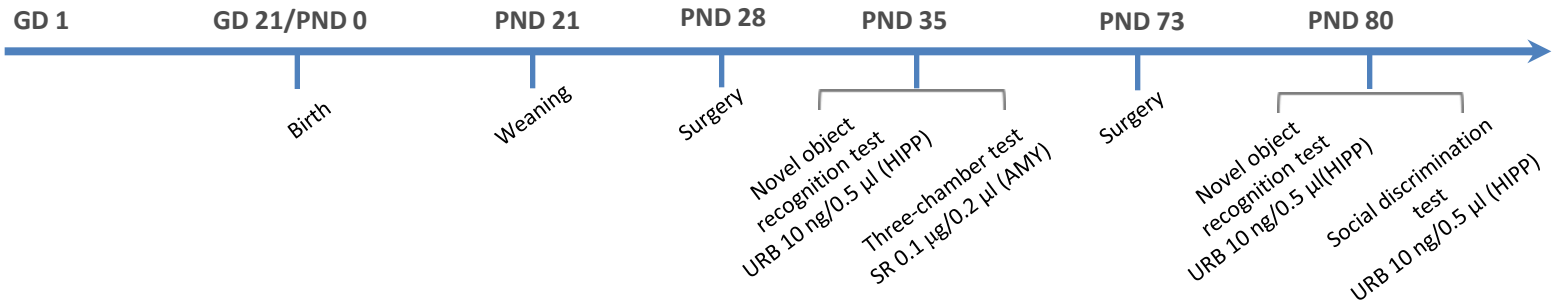
A) Biochemical experiments



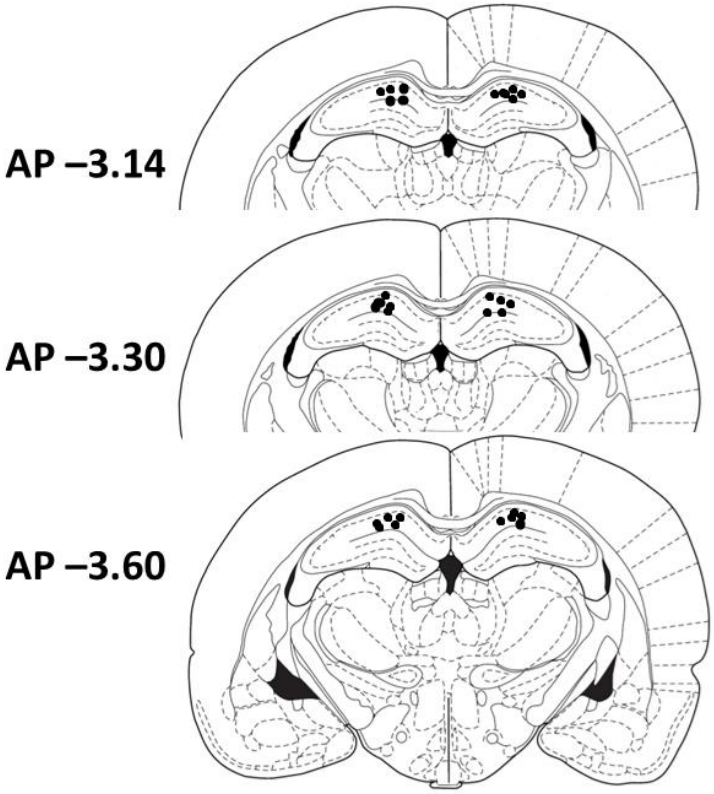
B) Systemic administration of URB597



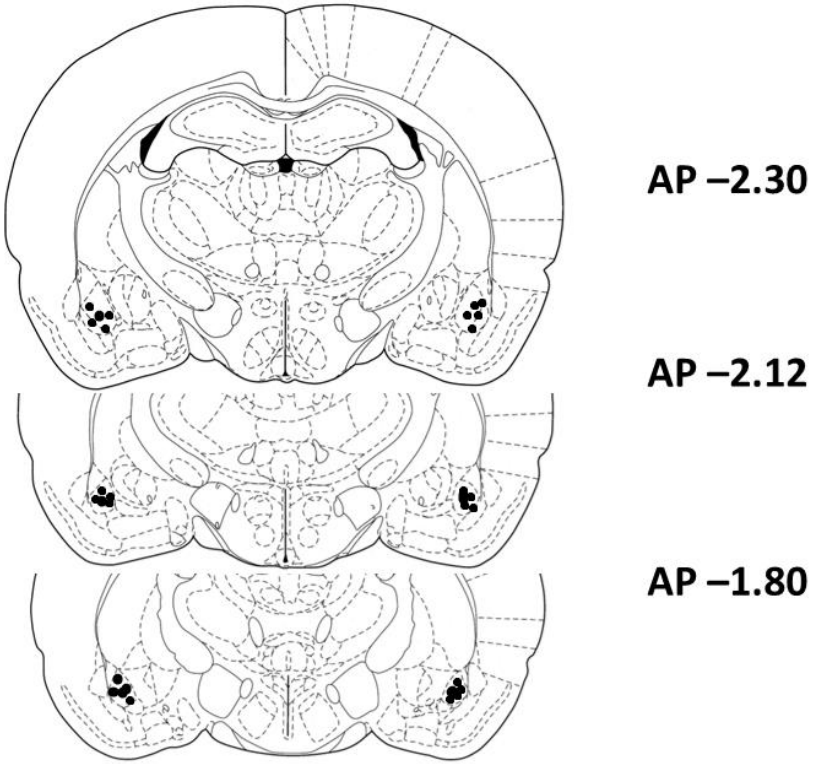
C) Intracranial infusion of URB597 or SR141716A



Supplementary Figure 1



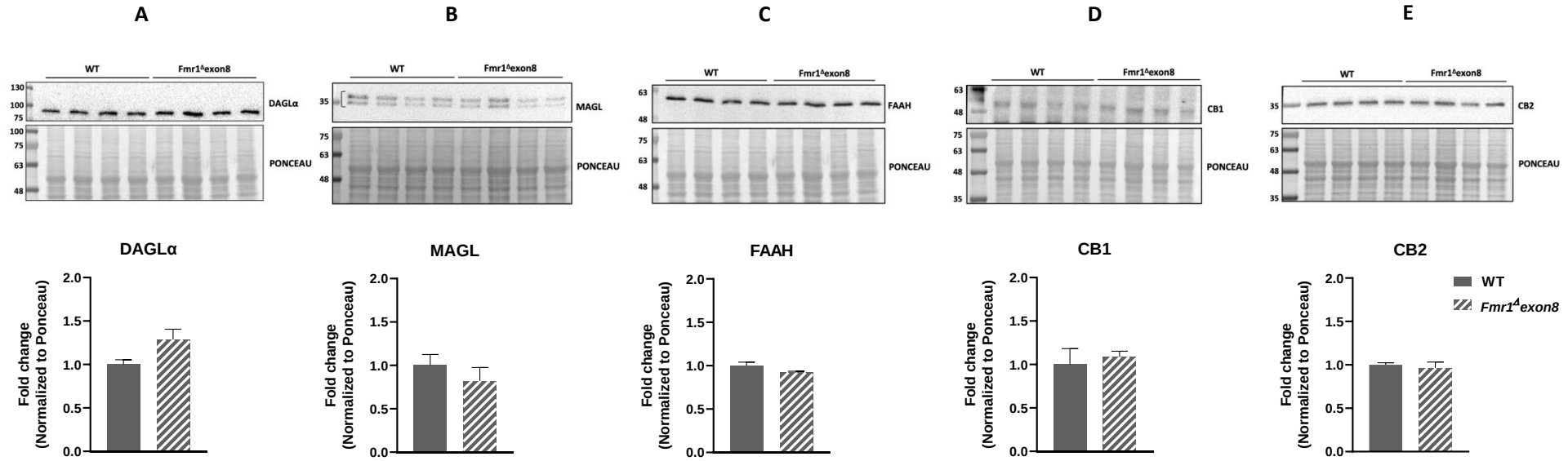
(a) Hippocampus



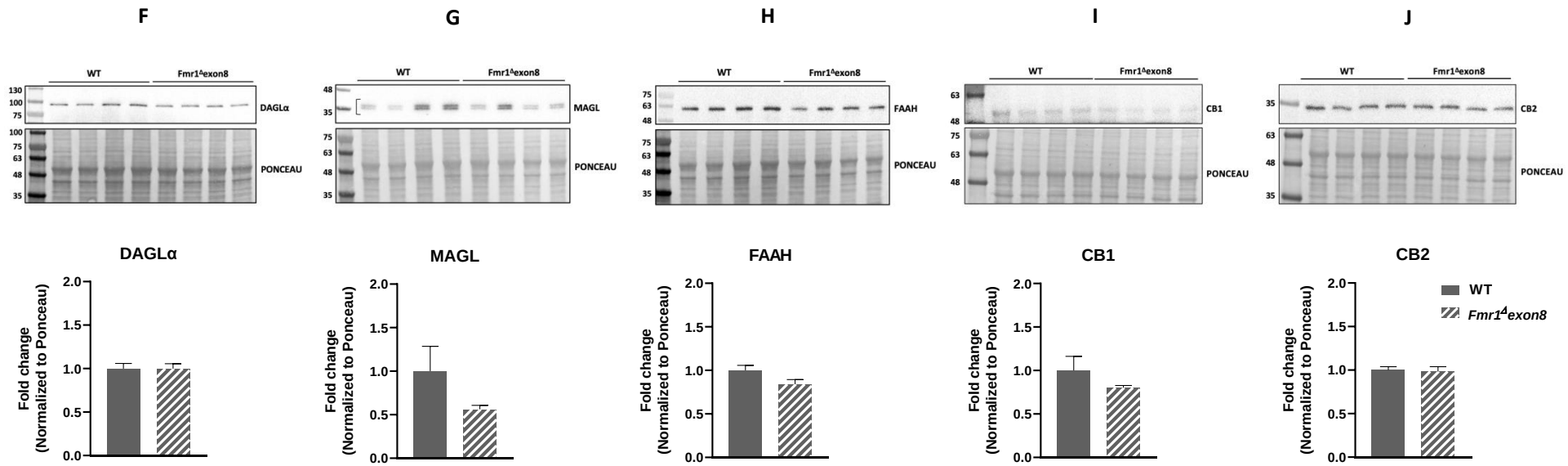
(b) Amygdala

Supplementary Figure 2

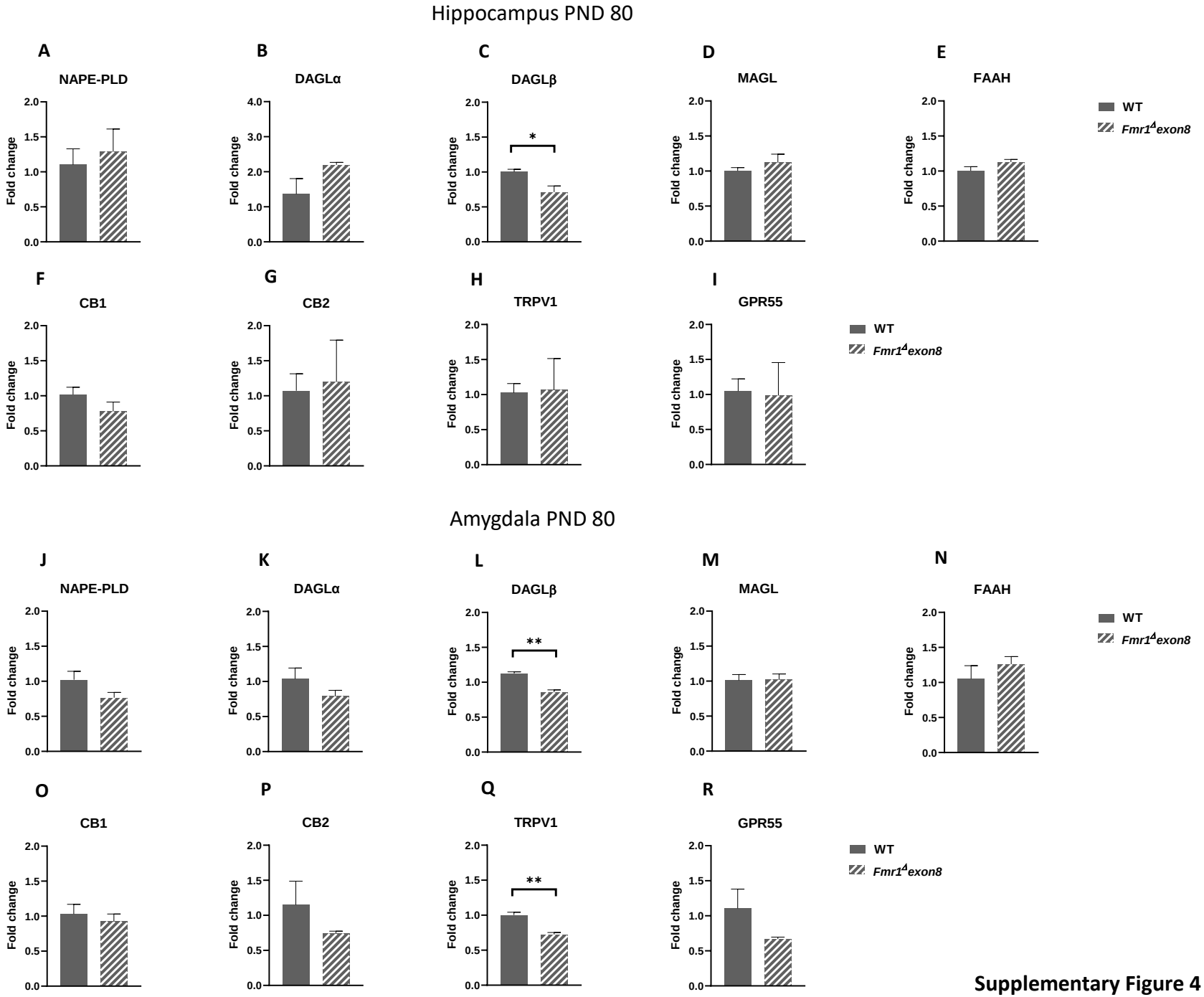
Hippocampus PND 35



Amygdala PND 35



Supplementary Figure 3



Supplementary Figure 4