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Supplementary Materials for

Ligand-independent activity of the ghrelin receptor modulates AMPA receptor trafficking and supports memory formation

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The PDF file includes:

Fig. S1. Blockade of the ligand-independent activity of GHS-R1a decreases the synapse density in mature primary cultured hippocampal neurons.
Fig. S2. Blockade of the ligand-independent activity of GHS-R1a increases the cell surface diffusion of GluA2-AMPARs.
Fig. S3. Administration of an inverse agonist of GHS-R1a does not impair overall movement.
Legends for movies S1 and S2

Other Supplementary Material for this manuscript includes the following:

(available at stke.sciencemag.org/cgi/content/full/14/670/eabb1953/DC1)

Movie S1 (.mp4 format). Application of the GHS-R1a agonist MK-0677 induces the translocation of PLCδPH-GFP from the plasma membrane into the cytosol.
Movie S2 (.mp4 format). Application of the GHS-R1a inverse agonist SP-A increases the plasma membrane levels of PLCδPH-GFP.

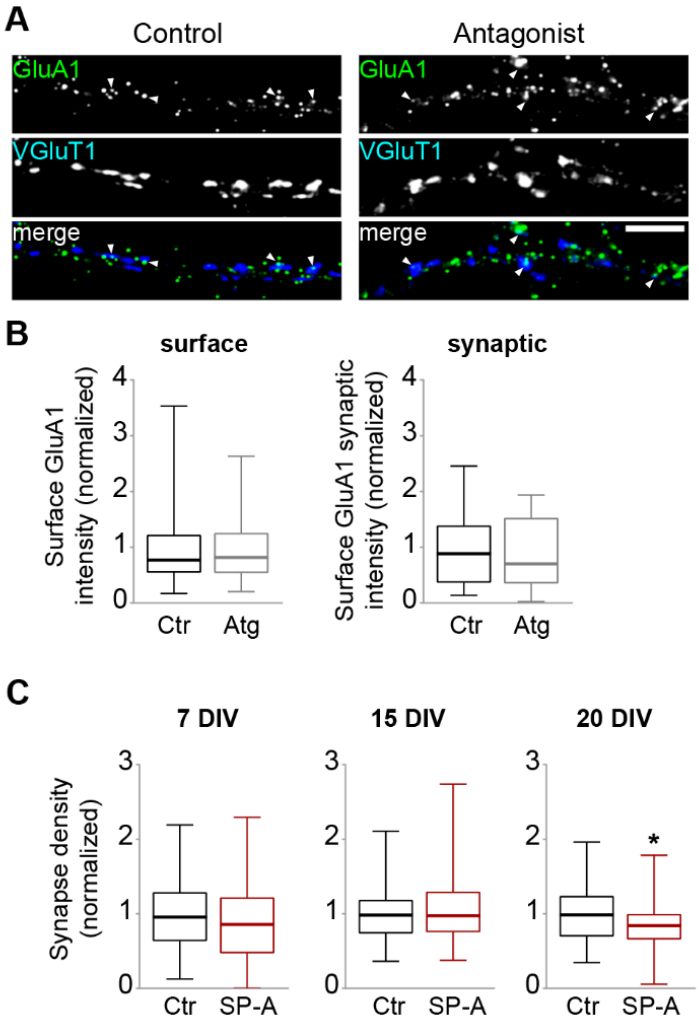

Fig. S1. Blockade of the ligand-independent activity of GHS-R1a decreases the synapse density in mature primary cultured hippocampal neurons. (A) Hippocampal neurons at 15 days in vitro (15 DIV) were incubated with GHS-R1a antagonist JMV2959 (100 μM for 20 hours) and immunostained for surface GluA1 (green) and VGluT1 (blue) under non-permeabilizing conditions. Scale bar, 5 μm. Arrowheads indicate VGluT1-colocalized GluA1-AMPARs. **(B)** Quantification of data described in (A): total fluorescence intensity of GluA1 cell-surface puncta (surface) and total fluorescence intensity of GluA1 synaptic clusters (VGluT1-colocalized), normalized to density of VGluT1 clusters. Ctr, control; Atg, antagonist. Results are shown as the median relative to control cells from 2 independent experiments (each condition, n = 22 neurons), compared by the Mann-Whitney test: U = 239 and 222, respectively; P = 0.9537 and 0.6503, respectively. **(C)** 7 DIV, 15 DIV and 20 DIV hippocampal neurons were incubated with GHS-R1a inverse agonist SP-A (1 μM for 20 hours) and analyzed for synapse density inferred from PSD95- and VGluT1-positive puncta. Results are shown as the median relative to control cells from 4 or 5 independent experiments (each condition, n = 60 neurons at 7 and 15 DIV, 75 neurons at 20 DIV), and compared by Mann-Whitney test: U = 1592, 1778 and 2221, respectively; and P = 0.2772, 0.9105 and 0.0260(*), respectively. Data are related to those in Fig. 2.

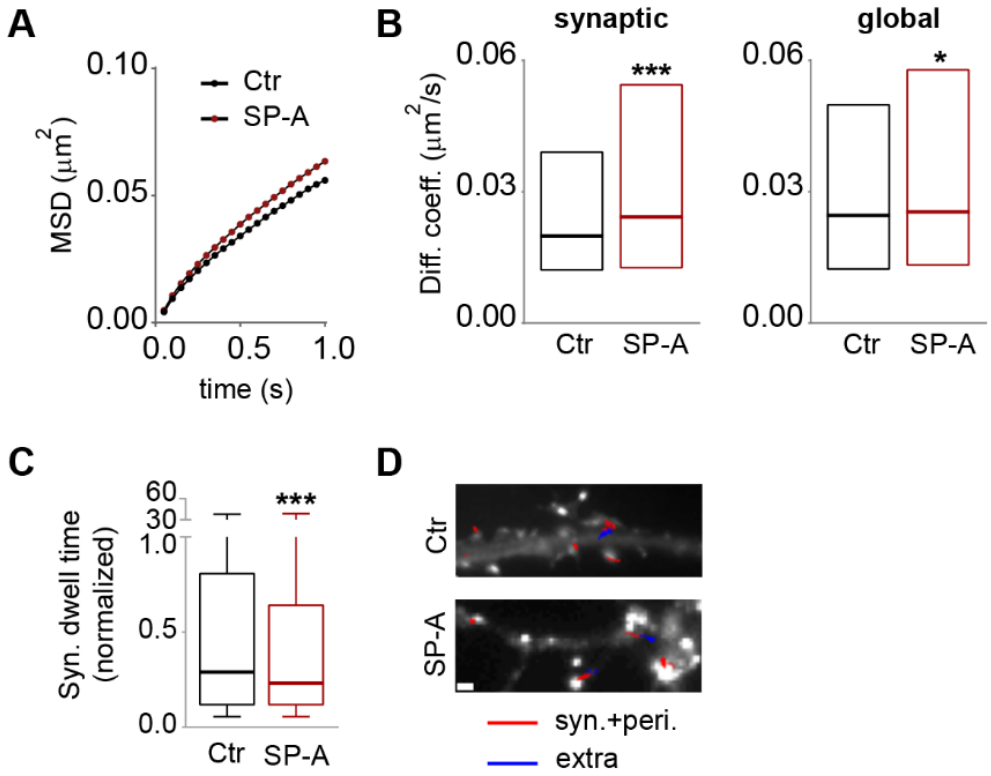

Fig. S2. Blockade of the ligand-independent activity of GHS-R1a increases the cell surface diffusion of GluA2-AMPARs. (A to D) Hippocampal neurons transfected with Homer1C-GFP at 11 DIV were either untreated (Ctr) or incubated with SP-A (1 μM) for 1 hour at 15 DIV prior to assessing GluA2 surface diffusion using quantum dots-labelled antibody for GluA2 (QD-GluA2). Shown are (A) the GluA2 mean square displacement (MSD) versus time; (B) the surface diffusion coefficient of synaptic (left) and global (right) single QD-GluA2; (C) the median synaptic dwell time of GluA2; and (D) representative, reconstructed GluA2 trajectories in the synaptic and extrasynaptic compartments (red and blue, respectively), each in control and SP-A–treated cells. Scale bar (D), 1 μm. A minimum of 33 cells and 4356-2288 trajectories were analyzed in 3 independent experiments. Data were compared by Mann-Whitney tests: U = 8807 and 927240, respectively in (B), and U = 17648853 in (C); **P* < 0.05 (*P* = 0.0122) and ****P* < 0.0001. Data are related to those in Fig. 5.

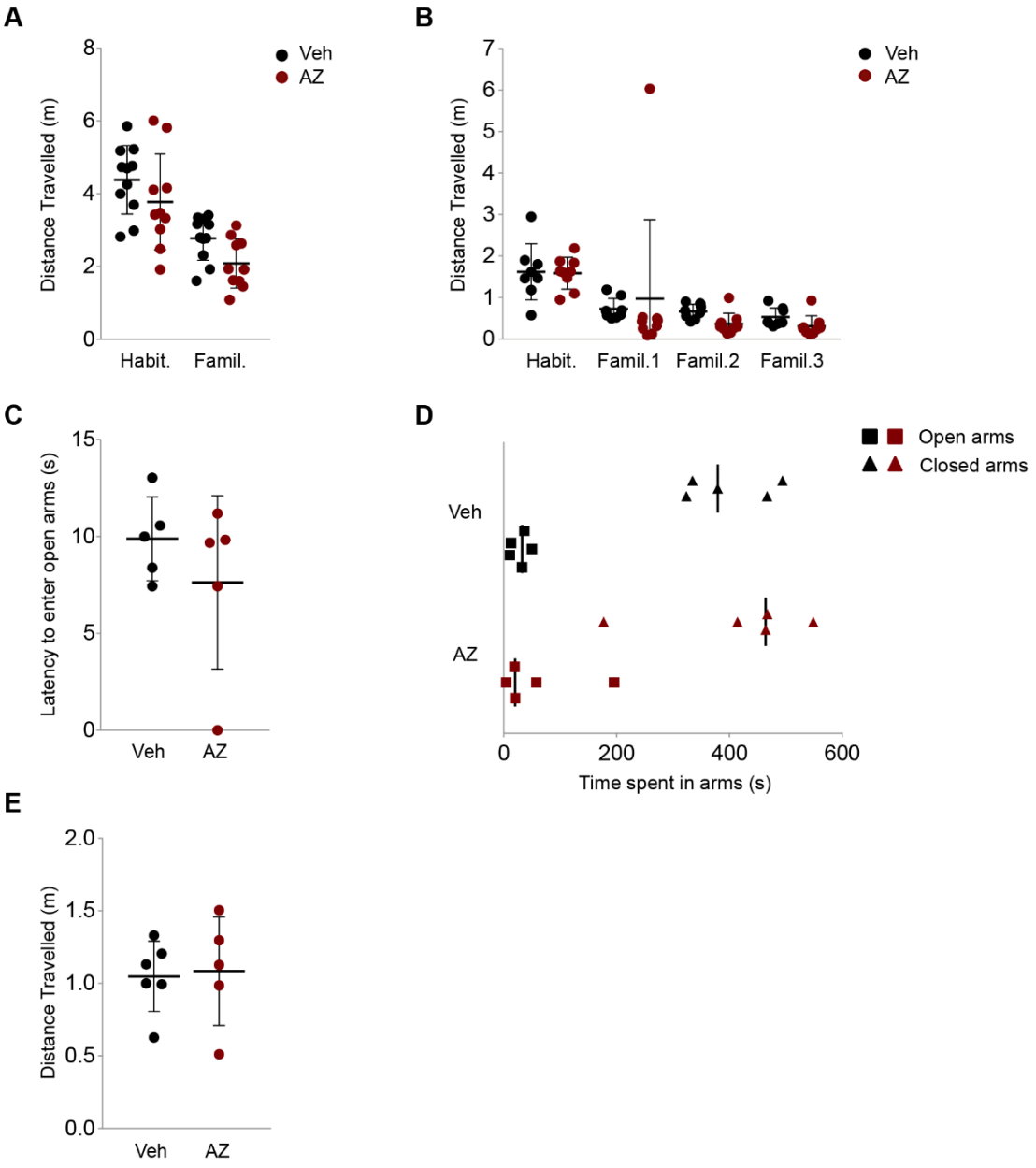

Fig. S3. Administration of an inverse agonist of GHS-R1a does not impair overall movement. (A and B) Male C57/BL6 mice of 8-15 weeks of age received intraperitoneal (i.p.) injections of 100 mg/kg AZ or Vehicle and performed novel object recognition and object displacement recognition tests. (**A**) Distance travelled (mean ± SD) by each animal in the novel object recognition "habituation" and "familiarization" sessions. Comparisons between groups (Veh, n = 11; AZ n = 10) were evaluated by two-way ANOVA and Bonferroni correction for multiple comparisons: variation by session [F(1,19) = 46.6 and ***P < 0.0001] interaction [F(1,19) = 0.03185 and P = 0.8602], and treatment [F(1,19) = 4.093 and P = 0.0574], and multiple comparisons test between groups for habituation (P = 0.2782) and familiarization (P = 0.1853). (**B**) Distance travelled (mean ± SD) by each animal in the object displacement recognition "habituation" and "familiarization" sessions. Comparisons between groups (Veh, n = 8; AZ n = 9) were evaluated by two-way ANOVA and Bonferroni correction for multiple comparisons: variations by session [F(3,45) = 7.903 and P = 0.0002] interaction [F(3,45) = 0.3985 and P = 0.7547] and treatment [F(1,15) = 0.1948 and P = 0.6652], and multiple comparisons test between groups for habituation (P > 0.9999) familiarization 1 (P > 0.9999) familiarization 2 (P > 0.9999) familiarization 3 (P > 0.9999). **(C to E)** Animals of 8-15 weeks

underwent the elevated plus maze test. Behavior was evaluated by (C) latency to enter open arms (s, mean ± SD), (D) time spent on each arm (median), and (E) total movement (cm, mean ± SD). Comparisons between groups (Veh, $n = 5$; AZ, $n = 5$) for latency to enter open arms (C) and median time spent on each arm (E) was assessed using unpaired *t*-tests: $t = 1.016$, df = 8, and $P = 0.3396$; $t = 0.2654$, df = 8 and $P = 0.7974$, respectively. Comparison between groups total movement (D) was evaluated using the Mann-Whitney test: closed arms: U = 10 and $P = 0.6905$; open arms: U = 11 and $P = 0.8413$. Data are related to those in Fig. 7.

Movie S1. Application of the GHS-R1a agonist MK-0677 induces the translocation of PLCδPH-GFP from the plasma membrane into the cytosol. Spinning disk confocal live cell imaging of DIV15 WT rat hippocampal neuron co-expressing mCherry (shown in Fig. 1) and PLCδPH-GFP. The cell was imaged every 30 s, for a total period of 60 min. MK-0677 (1 µM) was added at 6 min. Frame rate: 15 fps. Scale bar: 10 µm. Movie is supplied online in mp4 format and play through VLC player.

Movie S2. Application of the GHS-R1a inverse agonist SP-A increases the plasma membrane levels of PLCδPH-GFP. Spinning disk confocal live cell imaging of DIV15 WT rat hippocampal neuron co-expressing mCherry (shown in Fig. 1) and PLCδPH-GFP. The cell was imaged every 30 s, for a total period of 60 min. SP-A (1 µM) was added at 6 min. Frame rate: 15 fps. Scale bar: 10 µm. Movie is supplied online in mp4 format and play through VLC player.