Supporting Online Material for

Bidirectional Control of Social Hierarchy by Synaptic Efficacy in Medial Prefrontal Cortex

Fei Wang Jun Zhu, Hong Zhu, Qi Zhang, Zhanmin Lin, Hailan Hu*

*To whom correspondence should be addressed. E-mail: hailan@ion.ac.cn

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Corrected 11 October 2011: The author’s SOM follows; an older version was posted in error.
Materials and Methods

Animals. C57/BL6 male mice, at 10 - 14 weeks of age and of similar body weights, were used for most of the behavior tests, unless otherwise noted. Mice were group-housed, 4 in a cage, for 2 weeks before behavioral experiments. Animal husbandry and all experimental procedures were performed in strict compliance with the animal use and care guidelines of the Institute of Neuroscience, Chinese Academy of Sciences.

Tube test. The tube test assay was adapted from Lindzey et al. (1). It employed a transparent Plexiglas tube with 30-cm length and 3-cm inside diameter, a size just sufficient to permit one adult mouse to pass through without reversing the direction. For training, each mouse was released at alternating ends of the tube and ran through the tube, sometimes with the help of a plastic stick pushing at its back. Each animal was given eight training trials on each of two successive days. Following this, animals were tested daily with three further training trials before the test trials. During the test trial, two mice were released simultaneously into the opposite ends and care was taken to ensure that they met in the middle of the tube. The mouse that first retreated from the tube within 2 min was designated the “loser” of that trial. In rare cases when no mice retreated within 2 min, the tests were repeated. Between each trial, tube was cleaned with 75% ethanol. From trial to trial, the mice were released at either end alternatively. Within each cage of four males, paired encounters were staged, using a round robin design, such that each mouse would encounter every other mouse of the group only once, so six possible pairs were tested in all. Each mouse was ranked by their winning times that could vary between 0 - 3.

In the tube test with reward, the tube was connected at each end to a goal box that was 5 cm × 5 cm × 5 cm. Mice were first deprived of food (80% of normal weight), then trained with the food reward in the goal box in the opposite end. During test trials, the goal box didn’t contain the food reward.

For comparison with other five dominance assays (except for VBS and barber test), tube tests were performed first and ranks were stable (identical in at least 4 continuous daily trials) before proceeding to other tests, which were then scored “blindly” without knowing the tube test ranking. We would like to emphasize the importance of having “stable” tube test rank, which we typically get in 30% cages after 10 days of testing, before moving on to other measures or manipulations.

Locomotion test. Mice were placed in a 30 cm × 30 cm open field for a 15-min period (2). Data were acquired and analyzed by the Noldus software.

Visible burrow system. The VBS apparatus and experimental protocol were adapted from Arakawa et al. (3). In brief, the burrow system was comprised of an open field area (61 cm ×61 cm) connected to a series of transparent tunnels and three opaque chambers. Food and water were available in the open field area, which was illuminated on a 12:12 hr light/dark cycle. Specifically, our VBS included narrow ramps that lead to food and water, accessible to only one animal at a time. In this experiment, cages of four male mice were housed in a VBS for 7 days. Weight change of each animal was calculated as the weight before subtracted from weight after VBS.
**Barber test.** From more than 80 cages of male C57/BL6 mice, we found 7 cages, in which there was one mouse with intact whisker (barber) and the rest 3 mice with whiskers trimmed or plucked (recipient). These mice usually aged > 14 weeks. In 3 of these 7 cages, there were additional hair losses on other body parts in recipient mice.

**Agonistic behavior test.** Mice housed together for a while do not exhibit extensive aggressive behavior towards each other. However right after switched to a new cage, especially a dirty cage previously inhabited by other mice, they tend to engage a lot more in agonistic activities presumably due to the need of claiming territory in the new environment. Taking advantage of this fact, we videotaped the mice with stable tube test rank right after the cage switch for 20 min. The number of offensive behaviors (lateral attacks, boxing, mounting and chasing) subtracting the number of defensive behaviors (flight, freezing and underside exposure during fight) was used to calculate the dominance score for each mouse. 16 out of 23 cages tested showed obvious dominant-subordinate agonistic pattern.

**Urine-marking test.** For a cage of four male mice, six possible pairs of urine marking patterns were tested (2 pairs/day, total 3 days) using the round robin design. Each pair of mice was placed in opposite sides of a wire mesh partition in a two-chamber cage (26 cm × 21 cm × 26 cm)(4). Sheets of filter paper were spread below each cage to collect urine deposited by the animals. Mice were maintained in the urine-marking cages for 2 hrs and then returned to their home cages. Urine marks on the filter paper were visualized using the UV light source. The number and size of the marks and their distance from the partition were scored blindly to the tube test result. 8 cages of animals were tested, yielding 48 pairs of results, among which 42 revealed obvious dominant-subordinate urine pattern.

**Ultrasonic vocalization assay.** Mice were individually tested for ultrasonic vocalization in one side of a partition in a two-chamber cage (5). After habituation for 10 minutes, a female was introduced in the opposite side and ultrasounds produced by the mice were monitored (UltraVox, Noldus) for 5 minutes. The Mini-3 ultrasound detector was set at 60 kHz, with the audio filter at level 7. The chamber was cleaned with 0.5% bleach between two tests. In rare occasions when the female mouse urinated, test would be re-done.

**Virus construction and packaging.** For viral constructs, GFP-Ras, GFP-Rap, GFP-GluR4 and RFP-R4Ct were cloned in psinrep5 vector as previously described (6, 7). Recombinant *Sindbis* viruses were generated with standard techniques (8, 9). In brief, the plasmids were linearized and RNA was transcribed *in vitro*. The RNA was then simultaneously transfected into BHK-21 cells by electroporation with helper RNA. The culture medium containing the virus was collected 48hrs later and filtered through 0.2 µm filter unit (Sartorius) before ultracentrifugation. We found this filtering step is important for the health of infected neurons. Virus was dissolved in PBS and titer was tested *in vitro* to be above 10^{11}/µl.

**Stereotactic injection and histology.** Mice were anesthetized with ketamine (100 mg/kg, i.p.)/xylazine (8 mg/kg, i.p.). Microelectrodes were directed at the AC/PL region of mPFC. The stereotaxic coordinates according to the mouse brain atlas of Paxinos & Franklin (2004) were: AP, +2.43 mm; ML, ± 0.28 mm; DV, -1.81 mm, angled 14° toward the midline in the coronal plane. 1 ~ 1.5 µl virus was injected in each side. The
injection sites were examined at the end of the behavior experiments and only data from mice with correct injections were analyzed. Brain slices infected with Ras, Rap and R4Ct virus were examined directly under fluorescent microscope. Slices infected with GluR4 virus were stained with antibody against GFP before microscopic examination. To calculate the infection rate, standard landmarks were used to identify sub-regions according to the brain atlas. For each animal, counting was done on 3 consecutive 30-µm-thick sections from Bregma +2.22 to +1.98 mm (120 µm apart) using ImageJ. Numbers of total neurons were derived from counting the NeuN signal of corresponding subregions in parallel coronal sections of a standard un-infected animal. Infection rates were obtained by dividing the number of labeled cells by the number of total neurons. Average infection rate per section was calculated for each animal and used for correlation analysis.

**Electrophysiology.** Freshly isolated brain slices containing mPFC were cut with a Vibratome in a chamber filled with chilled (2-5°C) cutting solution containing the following (in mM): 212 sucrose, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 7 MgCl₂ and 10 glucose. Whole-cell recording was made from layer V pyramidal neurons of mostly AC and PL subregions from Bregma: +1.7 to + 2.2 mm coronal slices (300 um thick) using a patch-clamp amplifier under infrared differential interference contrast optics. Microelectrodes were made from borosilicate glass capillaries and had a resistance of 4-7 MΩ. Whole-cell recording was made at 29 ± 1°C. The internal solution contained the following (in mM): 115 CeMeSO₃, 20 CsCl, 10 HEPES, 2.5 MgCl₂, 4 Na₂ATP, 0.4 Na-GTP, 10Na- phosphocreatine and 0.6 EGTA. Data acquisition and analysis were performed by using a digitizer and pClamp 10 software.

For mEPSC recordings, neurons were held at -60 mV in voltage-clamp mode in presence of picrotoxin (100 µM), AP5 (100 µM) and TTX (1 µM). Data were filtered at 1 kHz and acquired at 5 kHz. Cumulative distributions were generated using 30 consecutive mEPSCs from each cell. Brain slices were made and recorded from Rank-1 and Rank-4 animals on the same day, to avoid potential differences induced by prolonged absence of a group member. The order was randomized.

For paired recordings of evoked EPSC from viral infected brain slices, a bipolar tungsten stimulation electrode was placed in the layer II/III mPFC, and pulses of 40 µs duration were applied at 1 Hz. Layer V pyramidal neurons were held at -60 mV to record AMPAR-EPSCs and at +40 mV to record NMDAR-EPSCs, in the presence of picrotoxin (100 µM) and 2-chloroadenosine (2 µM). Pairs of neighboring infected and uninfected neurons were recorded either simultaneously, or sequentially with one recorded immediately after another using the same stimulus intensity. Data were filtered at 2 kHz and acquired at 5 kHz. AMPAR EPSC amplitudes were measured by subtracting the peak at -60 mV by the baseline. NMDAR-EPSC amplitudes were measured by subtracting amplitude at 60ms after stimulation onset by the baseline. 60 EPSCs were averaged for each cell.

**c-Fos Immunohistochemistry staining and counting.** Mice were habituated for the tube for three days, during which they were trained consecutively to cross the tube in alternative directions for six times. On the test day, after habituated in the experimental environment for 3 hrs, two cagemate mice were released simultaneously into the opposite ends and encountered in the middle of the tube. Winner and loser were then determined. To enhance the c-Fos expression signals, the tube test was repeated for five
times between the same pair of winner and loser mice. Two other mice from the same cage were used as control and allowed to cross the tube five times without encountering another mouse. After tube test, mice were videotaped for 2 hrs to ensure that there were no fights within the group.

Two hours after the last tube test, mice were deeply anesthetized with 10% chloral hydrate and perfused transcardially with 0.1M phosphate-buffer saline (PBS, pH=7.4) followed by 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight in the same fixative and cryoprotected in 30% sucrose in PBS. Coronal brain sections (30 µm) were serially cut using a freezing microtome divided for 6 interleaved sets. Immunohistochemistry for c-Fos was conducted as previous described (10). Briefly, staining for c-Fos was conducted using rabbit polyclonal c-Fos antibody (1:5000; Calbiochem) and with biotinylated goat anti-rabbit secondary antibody (Vector laboratories) with 3, 3’-diaminobenzidine (DAB) as chromogens.

Number of c-Fos-positive cells was counted “blindly” with respect to assignment of mice group. The entire extent of medial prefrontal cortex from Bregma: +2.58 to +1.54 mm was included for calculation. AC, PL and IL subregions were divided according to the brain atlas of Paxinos & Franklin (2004). c-Fos-stained nuclei were identified by dark brown particles. The number was counted using 6 consecutive brain sections (150 µm apart) from an interleaved set using Image-Pro Plus and average number per section was calculated.

**CaMKII, Parvalbumin and Gad67 immunostaining and counting.** 1 µl of *Sindbis*-RFP-R4Ct virus was injected into mPFC unilaterally. After 24 hrs of expression, mice were perfused and brains were taken. Immunofluorescence staining for CaMKII and parvalbumin were conducted as previously described (11). Briefly, sections were incubated in rabbit anti-CaMKII primary antibody (1:500, Epitomics) or rabbit anti-parvalbumin antibody (1:1000, Calbiochem). The next day, sections were washed and placed in biotinylated goat anti-rabbit secondary antibody (1:400, Vector laboratories) overnight. After washing, sections were incubated in SA-HRP (1:500, PerkinElmer) for 90 min, then washed again and incubated in Fluorophore tyramide amplification reagent (1:100, PerkinElmer) for 25 min. Finally, sections were washed, mounted and coverslipped. For Gad67 counting, injection was done in Gad67-GFP knock-in mice (kindly provided by Dr. Yuchio Yanagawa, Guma University Graduate School of Medicine, Japan) (12) and fluorescence was directly visualized without staining.

Sections were assessed for the number of infected cells, CaMKII or Gad67-positive cells and double-labeled cells. The numbers were counted in three sections (each containing 100 to 150 infected cells) from each animal using Image-Pro Plus.
Supplementary Notes

Supplementary Note 1

Our choice of using GluR4 as the tool for synaptic perturbation was based on the following reasoning:

Multiple lines of work from hippocampus suggest that among the four AMPAR subunits, GluR4 is the one that can potentiate synaptic transmission at basal conditions when overexpressed. GluR2/3 mostly replace the existing AMPARs in the synapses, so overexpression of GluR2/3 doesn't increase synaptic transmission (13). GluR1 and GluR4 both undergo activity-dependent insertion in the synapses. But they differ in that GluR4 can be driven into synapse by spontaneous activity (14, 15), whereas GluR1 insertion requires LTP-like stimulus (13), or, co-expression of constitutively-active CaMKII (16). When overexpressed, GluR1 is restricted from synapses and does not change basal transmission until upon LTP or experience-dependent stimulus (17, 18). In contrast, overexpression of GluR4 can cause synaptic potentiation under basal conditions. In cortex, this rule also holds true: GluR4 overexpression changes transmission at all types of cortical synapses examined, whereas GluR1 alters transmission only at a subpopulation of synapses and only after experience-dependent activity (19). Therefore we chose GluR4 over GluR1 for synaptic manipulation.

We would like to emphasize that the molecules used in this study are tools to probe circuit function. We do not infer endogenous function of these molecules in the dominance behavior. Endogenously, GluR1 is more abundant than GluR4 in mPFC, and could be more relevant for the synaptic plasticity associated with dominance behaviors.

Supplementary Note 2

On the depression effect of R4Ct construct. Although GluR4 is expressed in mPFC (20) and its expression in layer V neurons can be further supported by in situ result from the Allen Brain Institute (http://mouse.brain-map.org/data/compare/ivt.html?ispopup=true&include=71358628), its level is less abundant than that of GluR1. From our data, the synaptic depression caused by the R4Ct virus is larger than expected from interfering with GluR4 alone (Fig. 4D). We think this is most likely due to the high homology in the membrane proximal region (MPR) of R1 and R4 as shown below.

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\begin{align*}
R1Ct & 13 \quad LIGGLGLAMLVALIEFCYKSREKRGKGFCLIPQ+SINEAIR & 55 \\
& L+GGLGLAMLVALIEFCYKSR+E+KRMK & ++EAIR \\
R4Ct & 1 \quad LVGGLGLAMLVALIEFCYKSRAEKRMK---------LTFSEAIR & 36
\end{align*}
\]

This region is important for GluR1 synaptic trafficking (21, 22). Therefore, we believe R4Ct inhibits the synaptic delivery of both R1 and R4.

References:

S2. V. Krishnan et al., Cell 131, 391 (2007).
Fig. S1. Additional features of the dominance tube test. (A) Summary graph showing the stability of rank, which was measured repeatedly over 7 days. Stability percentage is defined as the percentage of animals retaining the same rank position as in the previous day. (B) Weight of rank-1 plotted against weight of rank-4 mouse. (C) Locomotion of rank-1 mice plotted against that of rank-4 mice in open field test. (D) Center duration percentage of rank-1 mice plotted against that of rank-4 mice in open field test. n = number of animals. Error bars = s.e.m.
Fig. S2. Additional correlations among different dominance measures. (A-C) Contingency table showing the correlation between the results of tube test and those of VBS assay, agonistic behavior test and ultrasound test, respectively. Number of animals in each category is displayed. (D-F) Contingency table for correlations among barber assay, agonistic behavior and urine marking assays. (G) Summary table of the correlation significance, as measured by the fisher’s exact test. NA: no data available for the comparison.
Fig. S3. A schematic model showing the relationship among different dominance behaviors. Dominance is a common dependable factor underlying the six behaviors examined, in addition to various factors that contribute to each individual behavior. To verify that a manipulation alters dominance but not these other factors, more than one dominance assay should be used.
Fig. S4. Property of Sindbis Virus. (A - D) Sindbis virus preferentially infects pyramidal neurons. RFP-R4Ct virus was injected into the mPFC of wild-type (A, C) or GAD67-GFP knock-in (B) mice. Note that the virus is mostly excluded from the Gad- and parvalbumin-positive neurons. (D) Quantification of infection in (A) and (B). (E - F) Sindbis virus does not affect basic electrophysiological properties of infected neurons as revealed by effects on the input resistance (E) and leak currents (F) of whole-cell patched mPFC neurons. GFP, Ras and Rap virus infected neurons are recorded 12 - 24 hr post infection. GluR4 and R4Ct virus infected neurons are recorded 36 - 48 hr post infection. Two-tailed student t-test *: p < 0.05; ***: p < 0.001; error bars = s.e.m.
Fig. S5. Summary of the effects of each viral construct on the mPFC AMPAR-mediated EPSCs obtained from the pair recording configuration. For each pair, the average amplitude of AMPA-R EPSCs of the infected neuron is plotted against those of its uninfected neighbor. Error bars = s.e.m.
Fig. S6. Time-dependent effect of Ras virus on AMPAR- and NMDAR-EPSCs in mPFC layer V pyramidal neurons.
Fig. S7. Viral targeting in mPFC subregions of mice used for behavior tests. (A) Anterior-posterior positions where majority of the virus expression locates. Left is example of brain slices taken from an animal injected with R4Ct virus. Subregion boundaries are indicated with dotted lines. Right are corresponding schematics adapted from the atlas of Paxinos & Franklin 2004. (B) - (C) Summary on viral infection from 5 Rank-1 animals injected with R4Ct virus. (B) Average number of infected cells per slice in each cortical subregions from Bregma +2.22 to Bregma +1.98mm. (C) The induced rank changes (averaged over time point 12 to 72hr), plotted against the average infection rates in the three subregions –PL, AC and M2 from Bregma +2.22 to Bregma +1.98mm.
Fig. S8. Summary of effects on all mice injected with different viruses. Each line indicates the tube-test dynamics of one injected animal. Warm colors indicate increase while cool colors indicate decrease in rank. Rank numbers at the left indicate the starting rank position of each injected animal.
Fig. S9. Effects of injecting R4Ct-expressing Sindbis virus in M1 motor cortex on tube-test dynamics. (A) Example of RFP-R4Ct virus injection sites. (B) Each line indicates the tube-test dynamics of one injected animal. Grey color indicates no change of rank in all 4 animals injected. (C) Effects of RFP-R4Ct virus on the AMPAR and NMDAR-mediated EPSCs of M1 cortical neurons. ***: p < 0.001; error bars = s.e.m.
Fig. S10. Manipulations of synaptic efficacy in mPFC bi-directionally modulate ultrasound production towards a female mouse. (A) Natural log transformed duration of ultrasonic vocalization (sec) toward a female before and after viral injection. Each line represents data from one animal. (B, C) Comparisons of the number (B) and duration (sec) (C) of 50-70 kHz ultrasounds towards a female mouse before and after GluR4 and R4ct virus injection. Control mice were un-injected cagemates of the injected mice. Since GluR4- and R4Ct- expressing virus were injected into low and high-rank mice respectively, control mice were correspondingly divided into the low (Rank-3 and 4) and high (Rank-1 and 2) groups. The p value was obtained by two-tailed student t-test on ln-transformed data. *: p < 0.05; **: p < 0.01; error bars = s.e.m. (D) Ranks in ultrasound test before and after injection with GluR4 and R4ct virus.
References


Supporting References


