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**Supplementary Information for**

# A role of anterior cingulate cortex in the emergence of worker-parasite relationship

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**This PDF file includes:**



**Other supplementary materials for this manuscript include the following:**

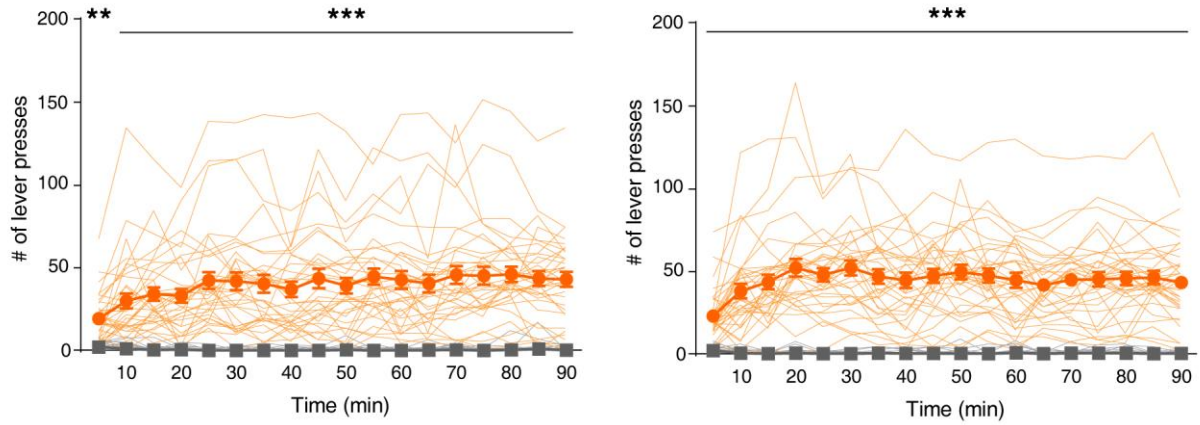


**Fig. S1. Within-session stability of worker-parasite relationship.** Time courses of lever-press behavior during the second (left) and third (right) worker-parasite sessions ($n$ = 34 sessions). Orange, workers; gray, parasites. Thin, soft-colored lines, individual animal data; Large circles/squares and error bars, group means and SEMs. $^{**}P < 0.01$, $^{***}P < 0.001$ (workers versus parasites, post-hoc Sidak's test following two-way repeated measures ANOVA).

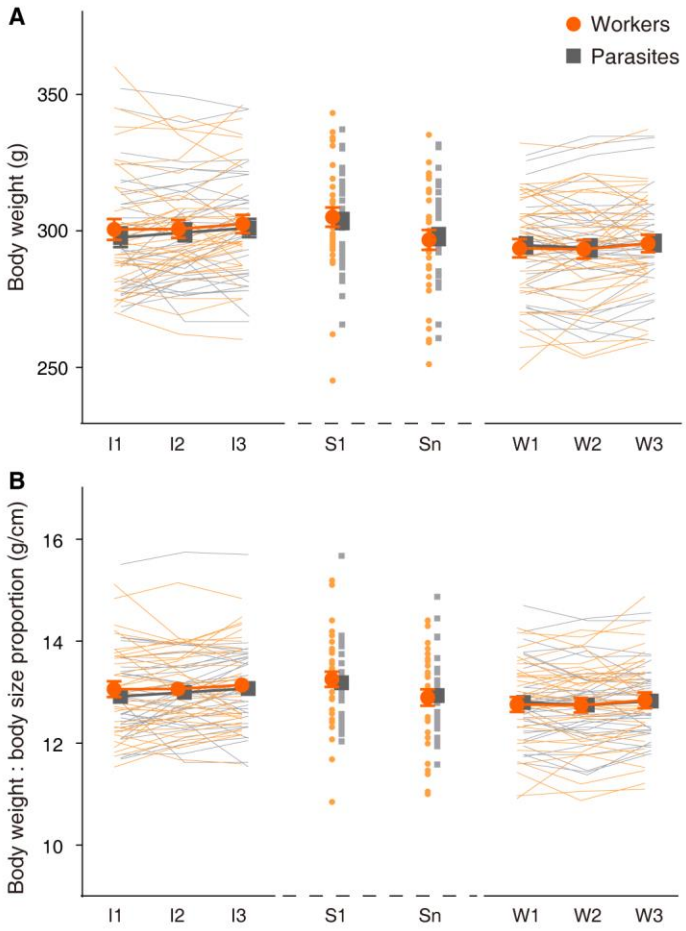


**Fig. S2. Body weights of workers and parasites. (A)** The workers' and parasites' body weights were comparable all through different phases of the experiment ($n$ = 34 rats/groups; two-way mixed ANOVA, test session, $F(7, 462) = 10.10$, $P = 8.9\times10^{-12}$; animal group, $F(1, 66) = 0.014$, $P = 0.906$; test session×animal group interaction, $F(7, 462) = 0.372$, $P = 0.123$). Thin lines and small circles/squares are individual animal data. The individual parasite bodyweight reflects the average of two parasites from the same group. Large circles/squares and error bars are group means and SEMs, respectively. Orange, workers; gray, parasites. (**B**) The body weight: body size (the length between the animal's nose and the root of tail) proportion did not differ significantly between workers and parasites (test session, $F(7, 462) = 10.02$, $P = 1.13\times10^{-11}$; animal group, $F(1, 66) = 0.362$, $P = 0.840$; test session×animal group interaction, $F(7, 462) = 0.362$, $P = 0.924$).

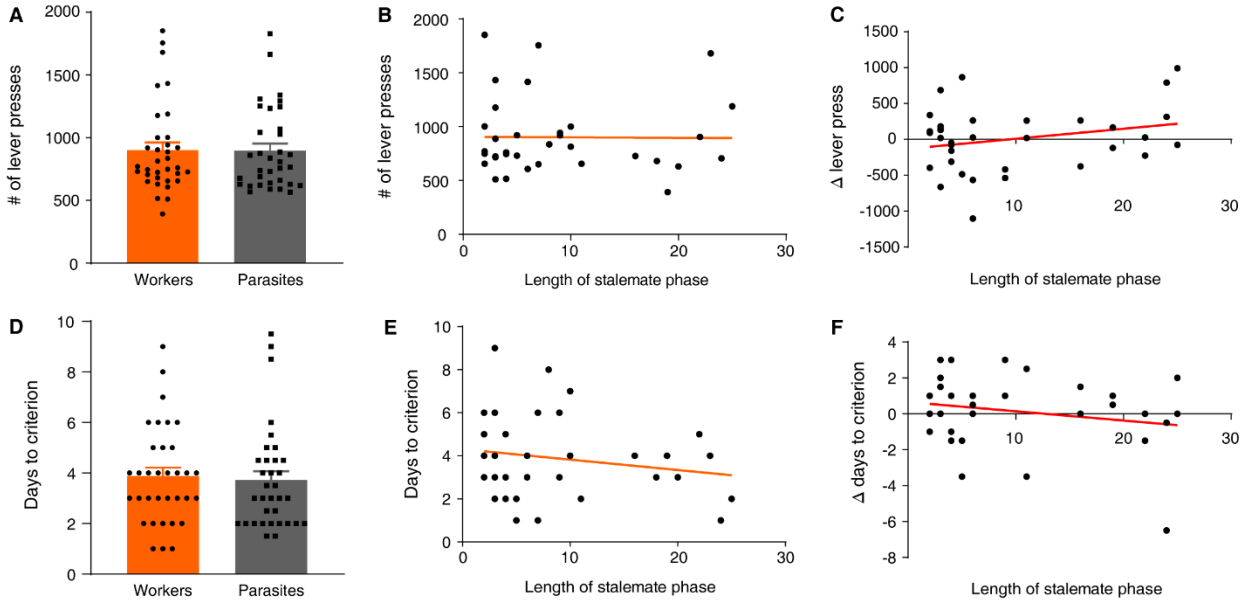


**Fig. S3. Equivalent lever-press behavior of workers and parasites during the individual test.** (**A**) Small circles/squares represent individual animal's lever-press frequency during the final individual-test session. The parasites' lever-press frequency represents the average of two parasites from the same group. Bar graphs and error bars are group means and SEMs (workers versus parasites, paired *t*-test, $t(33) = 0.044$, $P = 0.965$). (**B**) The relationship between worker's lever-press frequency during the final individual-test session (ordinate) and the length of stalemate phase (abscissa). No significant correlation was found ($r = -0.010$; $P = 0.956$). (**C**) The relationship between the difference between worker's and parasite's lever-press frequencies (Δlever press) during the final individual-test session (ordinate) and the length of stalemate phase (abscissa). The parasites' lever-press frequency was averaged from two parasites from the same group before being subtracted from their cohort worker's lever-press frequency. No significant correlation was found ($r = 0.254$, $P = 0.147$). (**D**) Small circles/squares represent individual animal's training days to reach the performance criterion in the individual test (100 lever presses/session). The parasites' fractional (e.g., 1.5, 2.5…) data distribution reflects the means of two parasites from the same group. Bar graphs and error bars are group means and SEMs (workers versus parasites, paired *t*-test, $t(33) = 0.480$, $P = 0.634$). (**E**) The relationship between worker's training days to reach the performance criterion (ordinate) and the length of stalemate phase (abscissa). No significant correlation was found ($r = -0.084$; $P = 0.298$). (**F**) The relationship between the difference between worker's and parasite's training days to reach the performance criterion (Δdays to criterion; ordinate) and the length of stalemate phase (abscissa). The parasites' training days were averaged (two parasites from the same group) before being subtracted from their cohort worker's training days. No significant correlation was found ($r = -0.213$, $P = 0.226$).

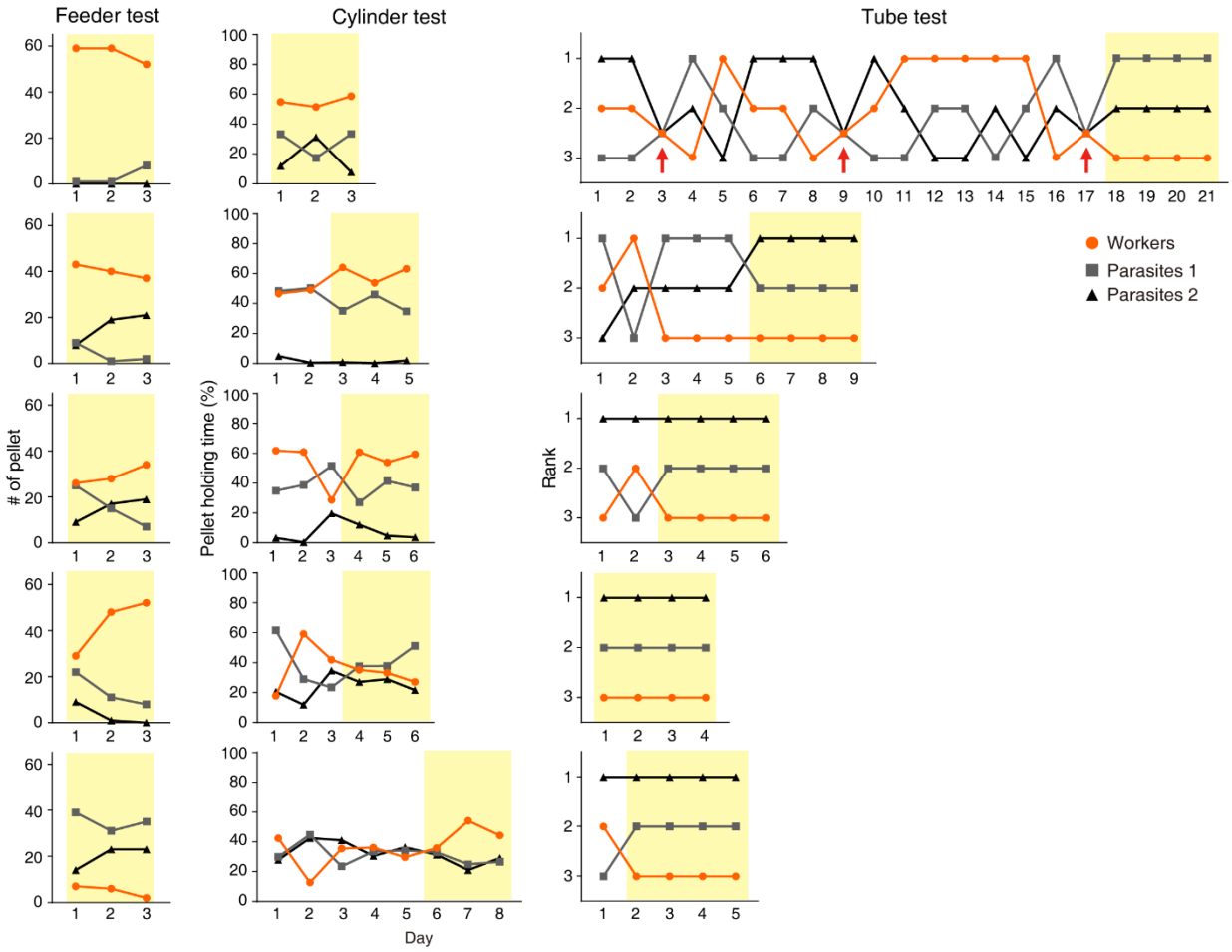


**Fig. S4. Dynamics of social ranks in three social dominance tests.** Shown are daily social ranks of individual groups across feeder, cylinder, and tube tests. Each test continued until the same animal was at the top rank for three consecutive days (feeder and cylinder tests) or all three rats maintained the same ranks for four consecutive days (tube test; yellow shading). Red arrows denote three-way ties in the tube test.

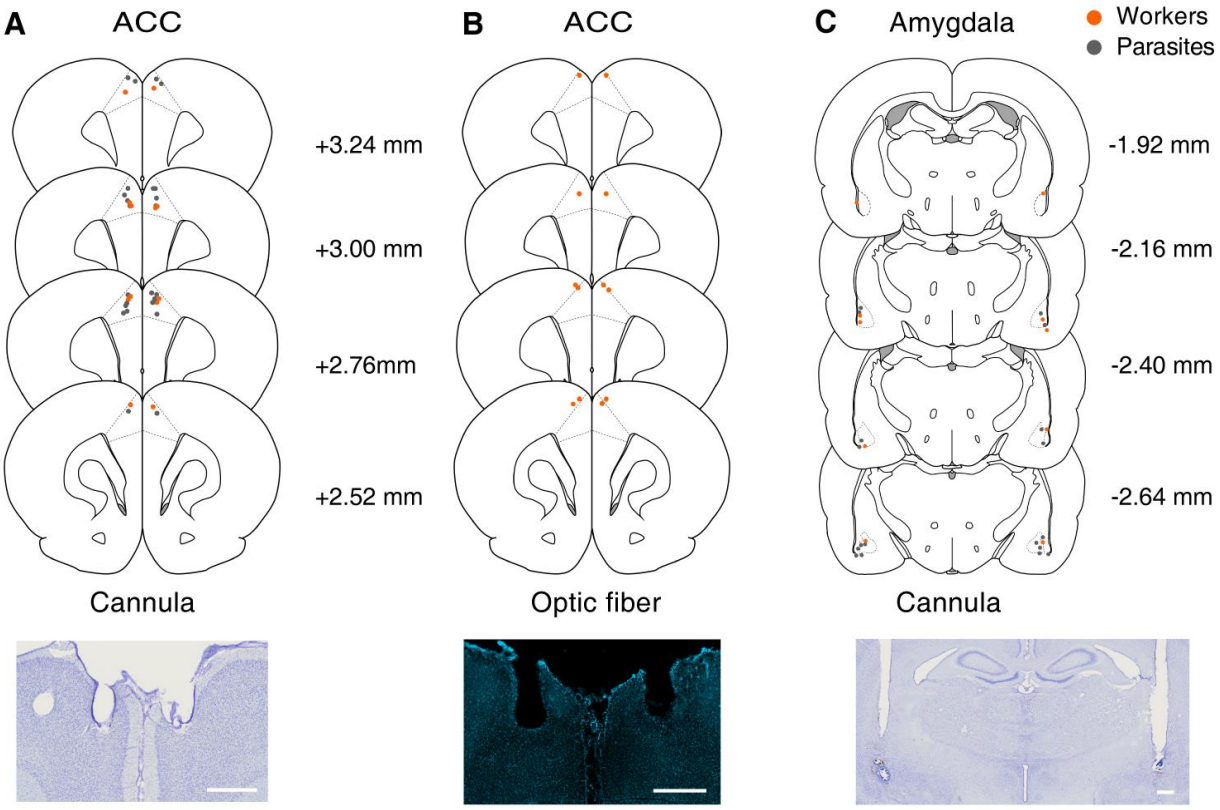


**Fig. S5. Histological verification of cannulae and optical probes.** Locations of cannula and optical probe tips were determined with histological examinations. Shown are coronal section views of the rat brain (numbers indicate distance from bregma in mm; +, anterior; -, posterior to bregma). (**A**) *Top*, circles indicate locations of cannula tips in the ACC in the muscimol-infused worker (orange) and parasite (gray) rats. *Bottom*, a sample coronal section showing tips of cannula tracks in the ACC. **(B)** *Top*, circles indicate locations of optical probe tips in the ACC in optically-stimulated worker rats. *Bottom*, a sample coronal section. (**C**) *Top*, circles indicate locations of cannula tips in the amygdala in the muscimol-infused worker (orange) and parasite (gray) rats. *Bottom*, a sample coronal section. Scale bar, 500 μm.

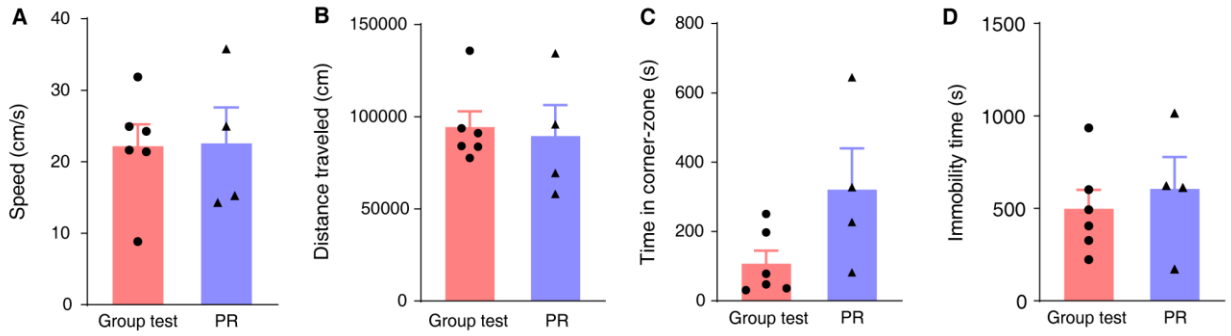


**Fig. S6. Comparisons of various behaviors displayed by the workers during group and individual tests.** Putative behavioral indices of the animal's effort (mean speed and total distance traveled), anxiety (total time spent in corner zones (10 cm radius circle from each corner) and immobility (instantaneous speed < 1.75 cm/s)) were compared between the group test (n = 6 workers) and PR individual test (n = 4 workers). No significant difference was found in any of these measures (*t*-test, speed, $t(8) = 0.077$, $P = 0.940$; distance, traveled, $t(8) = 0.285$, $P = 0.783$; time in corner-zone, $t(8) = 2.019$, $P = 0.078$; immobility duration, $t(8) = 0.578$, $P = 0.579$).

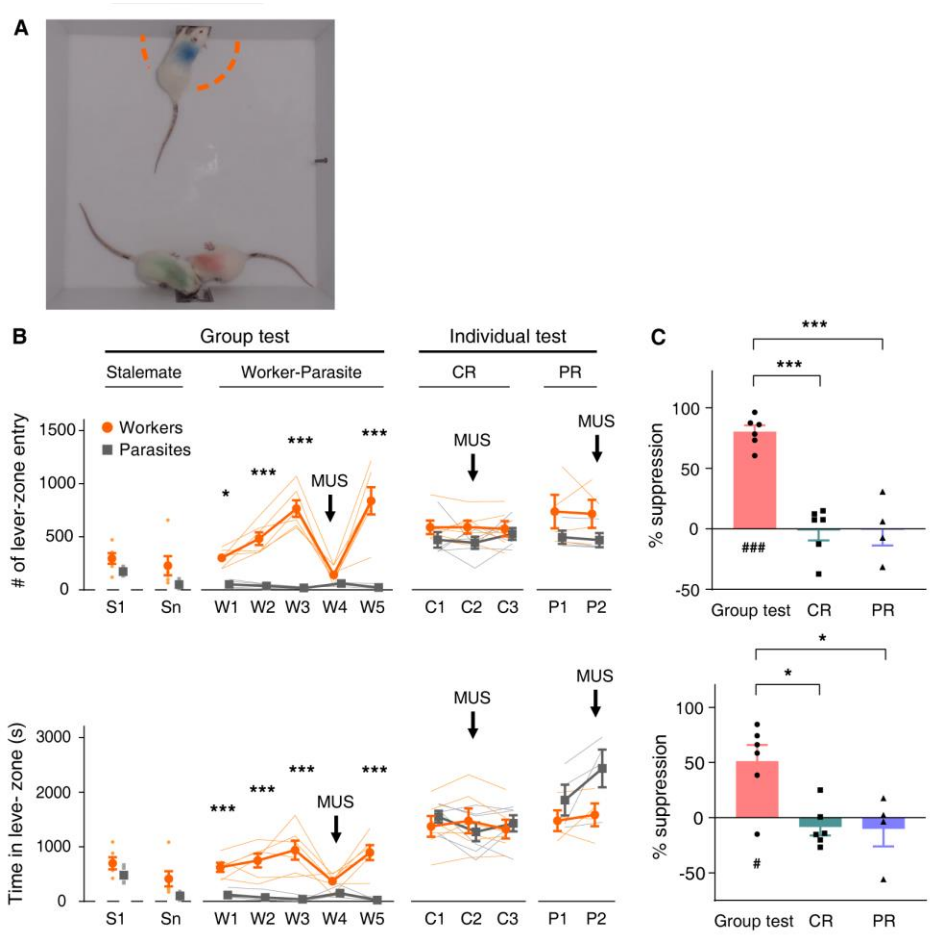


**Fig. S7. Muscimol effects on other measures of lever-press behavior.** (**A**) A photograph showing the demarcated lever zone boundary (orange dashed line). (**B**) Intra-ACC muscimol effects on the frequency of lever-zone entry (top) and the total time spent in the lever zone (bottom) across group and individual test sessions (same format as in Fig. 4B). The parasites' number of lever-zone entry and time in lever zone were averaged for each group. $*P < 0.05$, $***P < 0.001$ (workers versus parasites, post-hoc Sidak's test following two-way repeated measures ANOVA). (**C**) Comparison of muscimol effect on the worker's lever-zone entry frequency (top) and dwelling time (bottom) between the group and individual tests (% reduction from the previous session). The same format as in Fig. 4D. As shown, muscimol infusion significantly reduced the frequency of lever-zone entry and the amount of time spent in the lever zone during the group test, but not during the CR or PR test ($^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, significant difference from zero, one sample *t*-test). Also, the amount of muscimol effect on these measures (% suppression from the previous session) was significantly larger in the group than individual tests ($*P < 0.05$, $***P < 0.001$, significant difference between behavioral tests, one-way ANOVA followed by post-hoc Sidak's test).

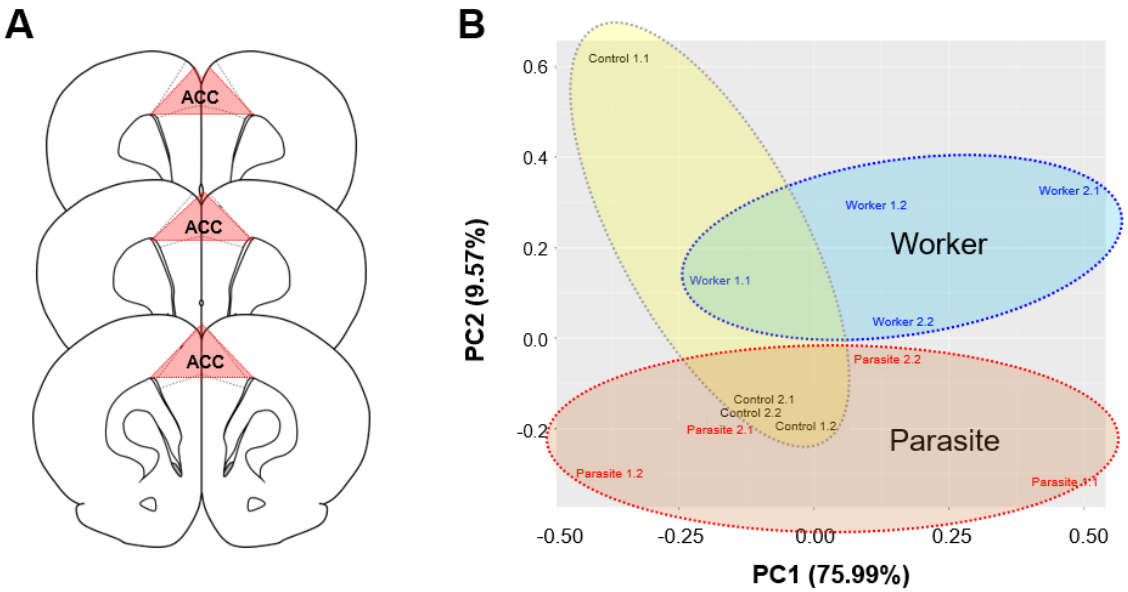


**Fig. S8. Distinct mRNA expressions in the ACC of worker versus parasite animals. (A)** Schematic for ACC dissection from sample coronal sections. **(B)** Principle component analysis (PCA) of rat ACC transcriptome.

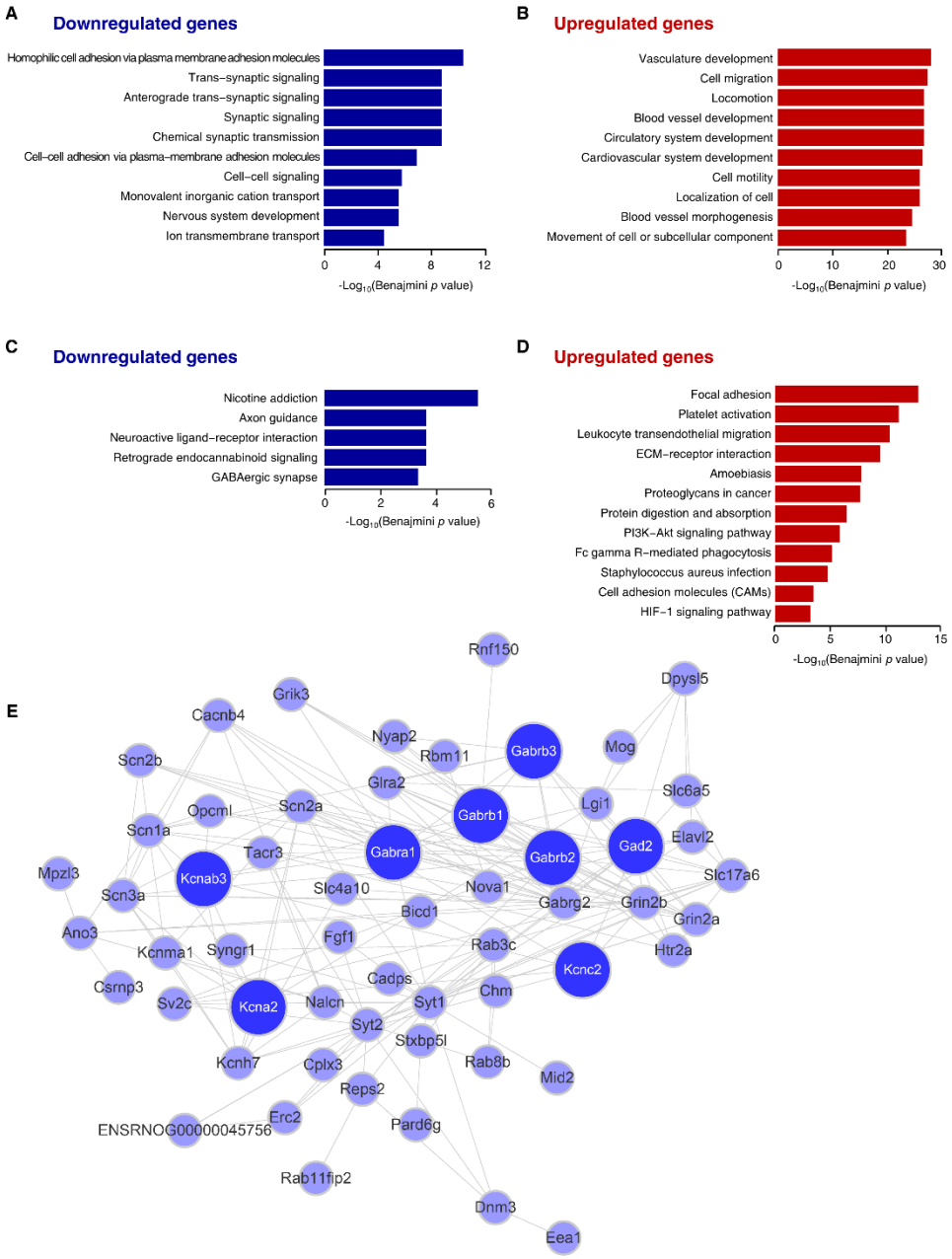


**Fig. S9. Co-expression sub-network of GABA- and $K^+$ channel-related genes.** (**A** and **B**) Gene ontology (GO) enrichment analysis of DEGs in the ACC of worker compared to parasite rats (**A**, downregulated and **B**, upregulated). The downregulated DEGs are mainly those involved in synaptic signaling. **(C** and **D)** Kyoto encyclopedia of genes (**C**, downregulated and **D**, upregulated) and genomes (KEGG) pathway analysis indicating responsive signaling pathways in the ACC of worker compared to parasite rats. **(E)** A co-expression sub-network of downregulated DEGs that are associated with GABAergic signaling and $K^+$ channels. Genes that are validated by RT-PCR are indicated as large blue circles. See also Supplementary Datasets S2 and S3.

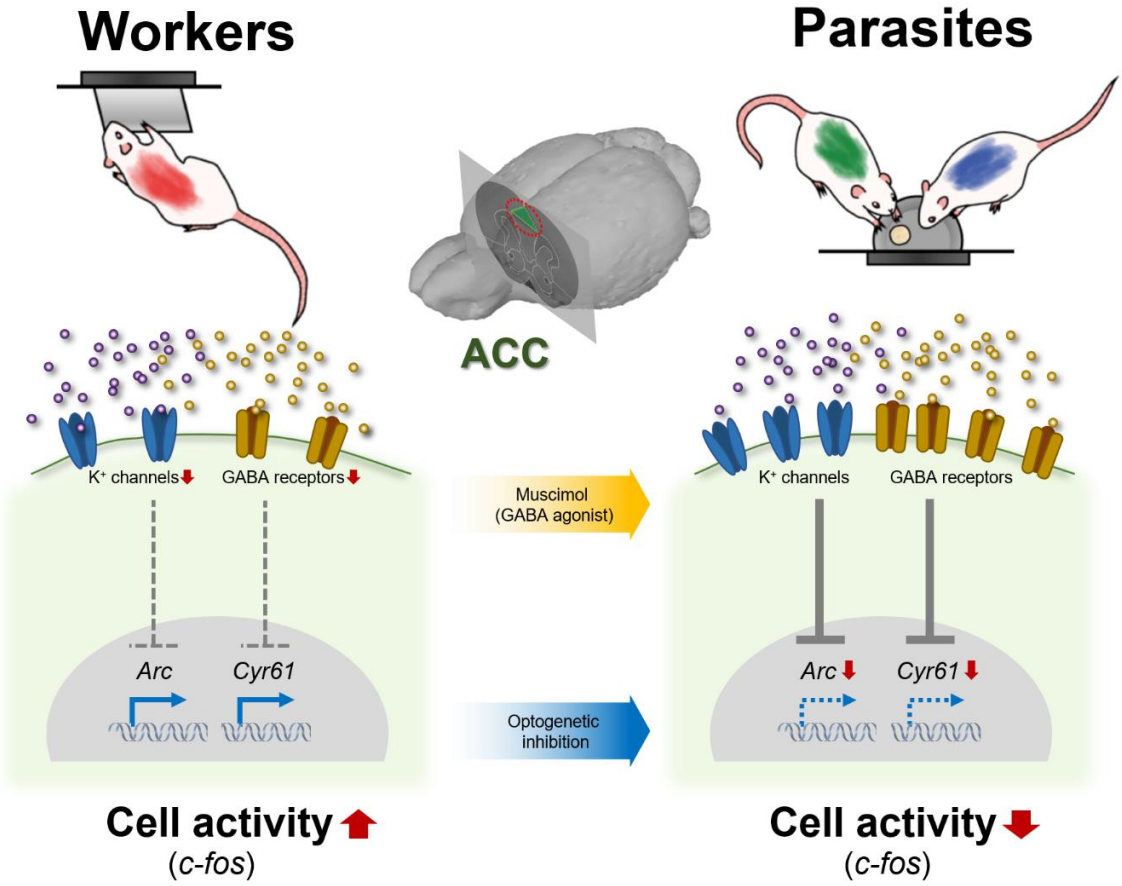


**Fig. S10. A putative molecular mechanism for worker-parasite separation in a social setting.** Expressions of GABA signaling-related proteins and potassium channels are reduced in the ACC of worker rats under a social dilemma setting. These changes will presumably decrease the inhibitory tone of the ACC neural network, and thereby increase its neuronal activity. This notion is supported by enhanced c-Fos expression in the worker's ACC. Importantly, suppression of the ACC neuronal activity using pharmacological (i.e., muscimol, a $GABA_A$ agonist) and optogenetic (i.e., eNpHR3.0) approaches abolished/reduced the worker's lever-press behavior strongly under social, but only weakly under individual, situations. This suggests a causal role of GABA signaling and its relevant ACC neural activity in the worker's effortful behavior under social dilemmas. Conversely, intact expressions of GABA-related proteins and $K^+$ channels contribute to maintaining the inhibitory tone and thereby dampening the parasite's ACC activity. In fact, the parasites showed reduced expression of $GABA_A$ antagonist/$K^+$ channel blocker-induced activity responsive factors, such as *Cyr61* and *Arc* (1, 2). This suggests that the parasite's ACC activity could be suppressed by the GABA- and $K^+$ channel-related inhibitory mechanisms. Three-dimensional visualizations of the rat brain was performed and modified using the 3d Brain Atlas Reconstructor (http://www.3dbar.org/) (3).

**Table S1. qRT-PCR Primers.**

| Target gene | Primer sequence (5′ – 3′) |
|---|---|
| *Gapdh* | GTGGACCTCATGGCCTACAT |
| *Gapdh*_rev | TGTGAGGGAGATGCTCAGTG |
| *Kcna2* | GACCCTCTTAGGAGACCCCA |
| *Kcna2*_rev | CACAGGTCGCCTCAACC |
| *Kcnb2* | CCTGGCCATCGTGTCTATCC |
| *Kcnb2*_rev | GCTCCGGAAGTGTGTTGAGA |
| *Kcnc2* | GAGTGACACATGTCTGGGC |
| *Kcnc2*_rev | CCGGAGGTGATAATGGCG |
| *Kcnab3* | GTCCTGGAGGCGGTAATGGAG |
| *Kcnab3*_rev | GGTTCCTGTATTTCATGCCAGTG |
| *Kcnh5* | CTGGAATATGGAGCAGCGGT |
| *Kcnh5*_rev | GCTCAAGGCCAACTGGT |
| *Kcnip4* | CGGTTTCCTCTACGCTCAGAA |
| *Kcnip4*_rev | GCAGGAGACGACGTTTTGG |
| *Gabra1* | GTCCATGATGGCTCAAACCG |
| *Gabra1*_rev | TCTTCATCACGGGCTTGTCC |
| *Gabrb1* | CCGCCGACTAAGTTGCATTC |
| *Gabrb1*_rev | TATGCTGGCGACATCGATCC |
| *Gabrb2* | ACCATCCTCTCCTGGGTCTC |
| *Gabrb2*_rev | TTGACATCCAGGCGCATCTT |
| *Gabrb3* | GCTACGACATTCGCCTGAGA |
| *Gabrb3*_rev | CAGCACTGTTCCATCAGGGT |
| *Gad2* | GTGTTCGATGGGAAGCCTCA |
| *Gad2*_rev | TGAGTTGCTGCAGGGTTTGA |
| *Erbb4* | GTACGAGCCTGCCCTAGTTC |
| *Erbb4*_rev | GTCCCCATGAATGCCAGTGA |
| *Cyr61* | CACGGAACCTCGAGTCCTTT |
| *Cyr61*_rev | GGGACCAGGACGTAGTCTGA |
| *Arc* | CTCAGACCATCACAGAACACCT |
| *Arc*_rev | TTCACGCTGGCTTGTCTTCA |
| *Klf4* | GCCCTTCGGTCATCAGTGTT |
| *Klf4*_rev | CTCAAGTGGGCCTCTAGGGA |
| *Adamts1* | CAATGCCGCTCTCACCCTTA |
| *Adamts1*_rev | CTCACGCCATTAAAGCTGGC |
| *Slc2a1* | GCTGTGGCTGGCTTCTCTAA |
| *Slc2a1*_rev | CCGGAAGCGATCTCATCGAA |

## SI References


1. G. E. Hardingham, Y. Fukunaga, H. Bading, Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5, 405-414 (2002).
2. Y. Chen, Y. Wang, Z. Modrusan, M. Sheng, J. S. Kaminker, Regulation of neuronal gene expression and survival by basal NMDA receptor activity: a role for histone deacetylase 4. J Neurosci 34, 15327-15339 (2014).
3. P. Majka, E. Kublik, G. Furga, D. K. Wojcik, Common atlas format and 3D brain atlas reconstructor: infrastructure for constructing 3D brain atlases. Neuroinformatics 10, 181-197 (2012).

**Legends for supplementary movies**

**Movie S1.**
Representative lever-pressing behavior of a rat during an individual test day 3 (prior to group testing).

**Movie S2.**
Representative behaviors exhibited by animals during group test day 1 (an early phase prior to stalemate).

**Movie S3.**
Representative behaviors shown by animals during group test day 4 (stalemate phase).

**Movie S4.**
Representative group behaviors on the first day that a worker-parasite relationship emerged.

**Movie S5.**
Representative group behaviors on the third day of worker-parasite relationship. (Note: movies S1-S5 are from the same group of animals).

**Movie S6.**
Representative group behaviors of cannulated animals when the worker rat received intra-ACC muscimol.

**Movie S7.**
Representative group behaviors of cannulated animals the next day when the worker rat received intra-ACC ACSF.

**Movie S8.**
Representative lever-pressing behavior of a worker rat with intra-ACC muscimol during an individual test day. (Note: movies S6-S8 are from the same group of animals).

**Legends for supplementary datasets**

**Dataset S1.**
Genes with differential expressions in the ACC between worker versus parasite rats.

**Dataset S2**
Gene ontology (GO) enrichment analysis in the ACC of worker rats, compared to parasite rats.

**Dataset S3**
Kyoto encyclopedia of genes and genomes (KEGG) pathways in the ACC of worker rats, compared to parasite rats.