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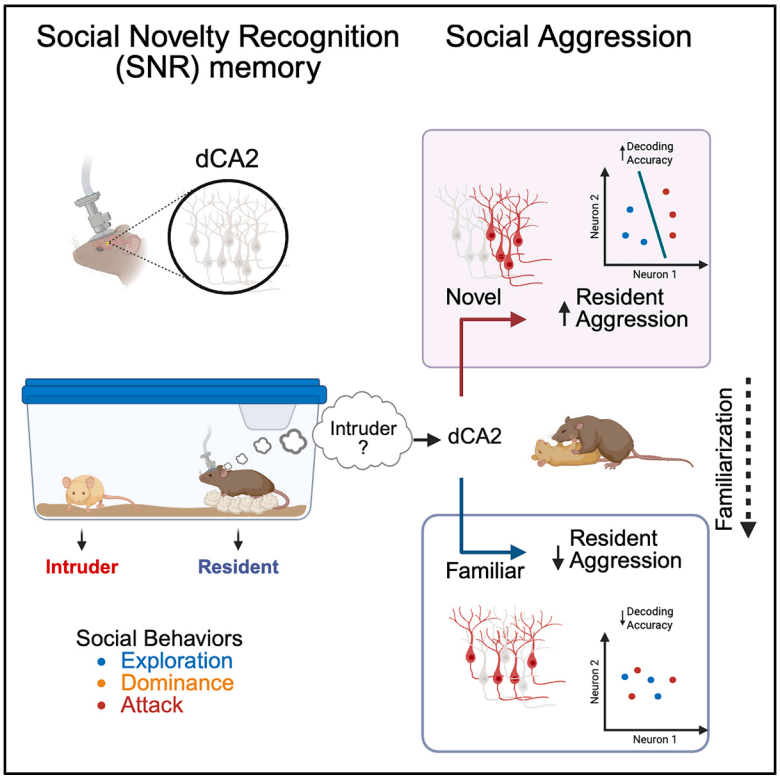
Modulation of aggression by social novelty recognition memory in the hippocampal CA2 region

Graphical abstract



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In brief

Villegas and Siegelbaum report that the dorsal CA2 region of the hippocampus acts to selectively increase aggression toward novel compared with familiar individuals. Calcium imaging from mice during aggressive behavior shows that dCA2 neurons encode aggression more strongly during attacks of novel compared with familiar intruders.

Highlights

- Miniscope imaging shows dCA2 encodes social behaviors toward novel and familiar intruders
- Social novelty increases dCA2 decoding accuracy of aggressive behaviors
- Chemogenetic silencing of dCA2 reduces aggression to novel but not familiar intruders
- dCA2 integrates social memory and aggression signals to promote adaptive social behaviors

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Modulation of aggression by social novelty recognition memory in the hippocampal CA2 region

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SUMMARY

Social aggression, a fundamental motivated behavior, is driven by external stimuli and internal states. Since early ethological studies, social novelty has been recognized as a key external trigger, with strangers eliciting increased aggression compared with familiar conspecifics. While progress has been made in identifying the neural bases of aggression and social novelty recognition (SNR) memory, the mechanisms linking social novelty detection to aggression remain unknown. Here, we report that the dorsal CA2 (dCA2) region of the hippocampus, previously implicated in both SNR memory and social aggression, plays a critical role in heightened aggression to novel conspecifics. Using calcium imaging during the resident-intruder test, we find that dCA2 neurons encode social behaviors—exploration, dominance, and aggression—with enhanced accuracy during interactions with novel compared with familiar conspecifics. Moreover, chemogenetic silencing of dCA2 pyramidal neurons preferentially suppresses aggression toward novel conspecifics. These results show a unified hippocampal mechanism; whereby social novelty tunes the representations of behavioral states to adaptively modulate aggression.

INTRODUCTION

Social aggression, an innate behavior important for survival, mating, and stability of social groups, is modulated by two experience-dependent processes. One is internal and based on the outcome of prior fights, with the experience of winning past fights leading to an increase in aggression.[1,2]The second is external, with an encounter with an unfamiliar conspecific—social novelty—causing an increase in aggression, a finding dating back to Darwin.[3–7] In contrast, social familiarity decreases the level of aggression in a social encounter.[3,5,8–10] Although recent progress has been made in understanding how experience in winning fights may enhance aggressiveness,[2] the neural mechanism by which the detection of social novelty, termed social novelty recognition (SNR) memory, leads to adaptive aggression is unclear.

To date, several brain regions have been identified as important for SNR memory, including the hippocampus,[11,12] nucleus accumbens,[13] supramammillary nucleus,[14] and medial prefrontal cortex.[15] A number of other brain regions have been shown to modulate aggression, including most notably the ventrolateral part of the ventral medial hypothalamus (VMHvl),[16] lateral septum,[17,18] amygdala,[19] and prefrontal cortex.[20] Studies over the past several years suggest that the dorsal CA2 (dCA2) subregion of the hippocampus may provide an important link between social memory and the control of aggression. Thus, dCA2 pyramidal neurons have been found to play crucial roles in the encoding, consolidation, and recall of SNR memory through a core dCA2—to—ventral CA1 circuit.[11,12,21,22] And other studies have demonstrated that dCA2 also serves to promote social aggression. Thus, arginine vasopressin inputs to dCA2 from the paraventricular nucleus of the hypothalamus enhance aggression by acting on the dCA2-enriched arginine vasopressin 1b receptor.[23–26] dCA2 activation promotes aggression through its projections to the lateral septum,[18] which disinhibits the VMHvl, a region known to promote aggression,[16] providing a top-down control of aggression via a social memory center. Moreover, fiber photometry measures of dCA2 calcium levels indicates that dCA2 activity is enhanced during aggressive encounters.[18] However, it is not known whether dCA2 acts to selectively promote aggression toward novel compared with familiar individuals or whether the neural mechanisms by which dCA2 contributes to SNR memory and social aggression are independent or causally linked functions.

Here, we test the hypothesis that dCA2 promotes aggression by providing a social novelty signal, enabling downstream circuits to increase aggression toward novel compared with familiar conspecifics. To investigate this idea, we compared the aggression of resident mice toward novel and familiar intruders using a modified

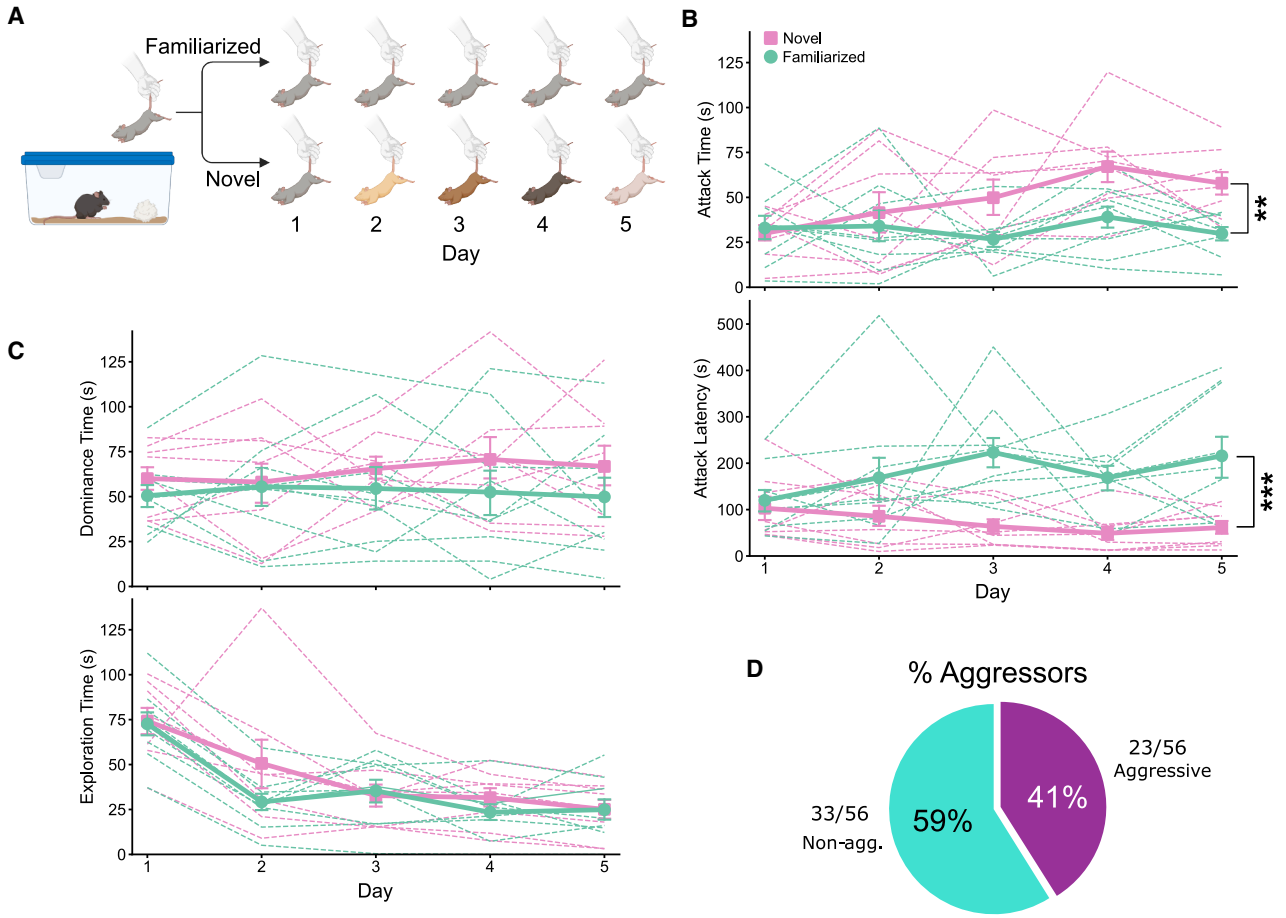

Figure 1. Experience-dependent increase in aggression toward novel compared with familiarized conspecifics

(A) Schematic and schedule of the 5-day modified R-I test for two groups of naive resident male mice, one that encounters a novel intruder each day and one that encounters the same, increasingly familiar intruder each day.

(B) Time spent in attack (top) and latency to first attack (bottom) for residents encountering the same intruder (green) or a novel intruder on each day (pink). Thick lines and error bars, represented as mean ± SE. Thin lines, data for individual residents. Results from a linear mixed model (LMM) show a significant effect of days on attack with the novel but not familiarized intruder (top panel). On days 4 and 5, the group with the novel intruder ($n = 8$) exhibited significantly greater attack compared with the group with the familiarized intruder ($n = 9$; day 4, $p = 0.026$; day 5, $p = 0.026$). There was also a difference in days 3–5 between groups in latency to first attack (LMM analysis) (bottom panel), with days 3 and 5 reaching statistical significance (day 3: $p = 0.008$; day 4: $p = 0.055$; day 5: $p = 0.01$).

(C) No difference in dominance (top) or exploration (bottom) across groups and days.

(D) Proportion of mice that were aggressive across all groups and used in the analysis (no significant difference in proportions across each group—see Figure S1F). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

longitudinal resident-intruder (R-I) test.[27] We then used calcium imaging and chemogenetic silencing of dCA2 pyramidal neurons in freely moving resident mice to assess the encoding of social exploration, dominance, and aggression behaviors during encounters with novel and familiar intruders in the R-I test, as well as the causal role of dCA2 in promoting aggression toward novel compared with familiar intruders.

RESULTS

Experience-dependent increase in aggression toward novel compared with familiarized conspecifics

We designed a modified version of the R-I test in which groups of residents (56 C57BL/6J male mice) encountered either the same initially novel intruder over 5 consecutive days or a different novel intruder each day, in one 10-min session per day (Figure 1A). For each session, we measured the time the resident spent engaged in: (1) neutral social exploration of the intruder, (2) social dominance (e.g., mounting, chasing, intensive grooming of the intruder), and (3) aggression (defined as one or more biting attacks). We limited our analysis to the ~40% of residents that attacked the intruder on the first and/or second day of the test (Figure 1D). Importantly, the proportion of aggressive residents did not vary across the randomly assigned group (Figure S1F).

We found a significant increase in aggressive behavior in the cohort that encountered a novel intruder on each day compared with the cohort that encountered the same intruder (Figure 1B). Residents that encountered a novel intruder each day displayed

escalating levels of aggression, with increasing total attack duration correlated with decreased latency to the first attack (Figure S1E). In contrast, mice that encountered the same increasingly familiar intruder each day showed stable levels of aggression. By days 4 and 5, differences in attack duration between the groups reached statistical significance. We attribute the relatively stable levels of aggression toward the familiarized intruder to the competing effects of experience in winning fights, which increases aggression, vs. the increasing familiarity of the intruder, which decreases aggression. Both groups of mice also showed a significant decrease in social exploration after initial contact on day 1 (Figure 1C, bottom). There was no significant difference between groups in time spent engaged in dominance or exploration behaviors during the 5 days of the test, although there was a trend for increasing time spent in dominance behavior with the group that encountered a novel intruder each day (Figure 1C, top).

To control for differences in the number of R-I sessions experienced by the intruders in the familiarized group (five sessions total) compared with the novel group (one session per intruder), we assessed aggression in a third group of residents that encountered a novel intruder each day that had undergone the same number of prior R-I tests as did the group of familiarized intruders, but with different intruders (Figures S1A–S1D). The residents showed a similar increase in aggression to these experienced intruders as to the naive intruders, indicating that it was the novelty of the intruders and not their prior experience that led to increased aggression. Of note, the subjects that had undergone the R-I test still were able to show significant levels of SNR memory (Figure S2), indicating that the R-I test itself did not impair SNR so that the increased aggressiveness toward the novel intruder could depend on circuits important for social memory.

Single-cell activity in dCA2 differentially responds to novel and familiar intruders

Given the known role of dCA2 pyramidal neurons in encoding social novelty[28,29] and promoting aggression,[18] we hypothesized that dCA2 neural activity encodes the behavioral state of the resident during the R-I test in a manner that is modulated by the novelty or familiarity of the intruder. To explore this possibility, we expressed the genetically encoded calcium indicator GCaMP6f or GCaMP8s in dCA2 pyramidal neurons using injections of Cre-independent AAV2/5 vectors, taking advantage of the natural tropism of this serotype to selectively infect dCA2 pyramidal neurons.[28,30] Post-hoc staining with the CA2 marker PCP4 revealed a strong overlap with GCaMP expression in dCA2 pyramidal neurons (Figures 2B and S3). We next performed microendoscopic calcium imaging 5–6 weeks after viral injection to monitor dCA2 pyramidal neuron activity in freely moving mice during performance of a modified version of the longitudinal R-I test, starting with 5 days of exposure to an initially novel intruder (familiarization protocol).

To separate the effects on aggression of the social novelty of the intruder from the fighting experience of the resident, we first imaged from the resident as it encountered an increasingly familiar intruder during 5 days of R-I testing. Despite being tethered to the implanted microendoscope, the resident still attacked the familiarized intruder (Figures 2A and S4), although the levels of attack were somewhat lower than observed with the non-tethered residents in Figure 1. We attribute a transient increase in aggression to the familiarized intruder on day 2 to be an effect of resident experience in fighting (see legend to Figure S4). We then explored the effect of social novelty by introducing a novel but experienced intruder on day 6. This led to a significant increase in aggression (Figure 2A), with an increase in both attack duration and number and a decrease in attack latency (Figures 2C, S4C, and S4D), providing further evidence that social novelty increases aggression.

This cohort showed a lower overall level of aggression during presentation of the familiarized intruder on days 1–5 compared with that of Figure 1. We posit that the lower aggression may be caused by the implantation of the GRIN lens and/or tethering of the resident to the microendoscope. In contrast to Figure 1, we also saw an increase in aggression on day 2 compared with day 1 of the test, which likely reflects the increased experience of the resident in winning a fight on day 1. Levels of attack then returned to a lower constant level on days 3–5 as the intruder became increasingly familiar to the resident (see discussion). Additionally, on days 3, 4, and 5, we observed significant differences in exploration and dominance behaviors toward the familiarized intruder compared with day 1 (Figures S4A and S4B).

We next asked whether and how dCA2 activity encodes different social behaviors, how these representations change over the course of 5-day presentation of the same intruder, and whether they differ during interactions with a familiar or novel intruder. We first investigated single-cell response profiles by comparing activity during social exploration, dominance, or attack with baseline levels of activity, defined as periods when the resident was not interacting with the intruder. Neuron responses were deemed activated or inhibited by a given behavior if the mean difference in activity relative to baseline was, respectively, significantly more positive or more negative than chance levels, defined as a difference that was greater or less than 95% of a null distribution consisting of 1,000 random assignments of the calcium traces to a behavior label ($\alpha = 0.05$). Neurons whose activity did not exceed the thresholds for any behavior were classified as non-responsive.

Based on an average of 586 neurons per day from 9 aggressive mice, a significant proportion of cells were either activated or inhibited during one of the 3 annotated behaviors compared with baseline (Figure 2E, and see Figure 2D for examples of activated response cells). Averaged across the 6 days of recordings, we found that 24.4%, 31.4%, and 12.5% of cells were activated during exploration, dominance, and attack, respectively (compared with chance levels of 5%). A somewhat smaller fraction of cells was inhibited during the behaviors, with 17.3% inhibited during exploration, 15.9% during dominance, and 5.8% during attack.

We found a number of changes in the fraction of behavior-responsive cells during familiarization with the same intruder on days 1–5 and during the encounter with a novel intruder on day 6. During social exploration, there was a marked decrease in the fraction of inhibited cells, from 24% on day 1 to 13% by day 3. The difference between day 1 was significant for days

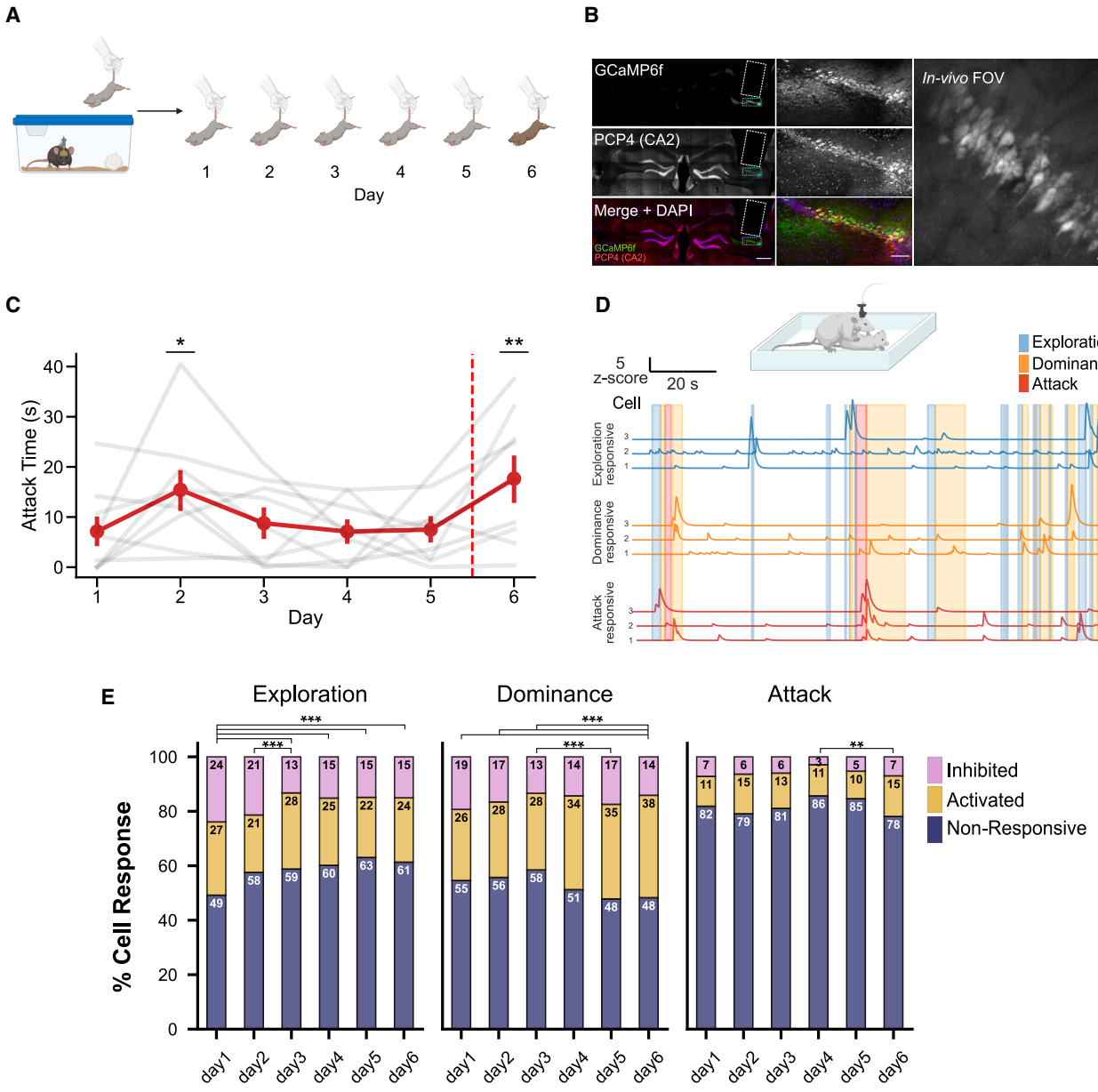

Figure 2. Social behavior encoding in resident dCA2 single cells during encounters with familiarized or novel intruders

(A) Protocol for imaging resident dCA2 calcium signals across 6-day R-I test. Exposure for 5 days to the same initially novel intruder followed by novel experienced intruder on day 6.

(B) Confocal image of a brain section from an example mouse showing co-localization of GCaMP6f (top) with CA2 marker PCP4 (middle). Scale bar, 500 μm. Middle, 20× magnification of bounding box in CA2 and corresponding markers. Right, *in vivo* one-photon image (maximum intensity projection) from representative field showing GCaMP6f fluorescence in dCA2 PN soma and dendrites. Scale bar, 100 μm.

(C) Time spent in attack for all imaged aggressive mice across days ($n = 9$). Note greater attack time on days 2 and 6 compared with day 1 (LMM, day 2, $p = 0.025$; day 6, $p = 0.004$).

(D) Normalized and background-subtracted calcium signals for three example cells during exploration (blue), dominance (orange), or attack (red) behaviors.

(E) Percent of dCA2 cells with increased (yellow), decreased (pink), or unchanged (blue) activity during exploration, dominance, or attack compared with baseline (non-social episodes). Magnitude of change for activated and inhibited cells was, respectively, greater or less than 97.5% of responses in 1,000 shuffled datasets. Total cells = 502, 646, 619, 482, 625, and 644 for days 1–6, respectively; $n = 6$, 9, 8, 7, 8, and 9 mice. Cell response distribution during attack differed between days 4 and 6 ($\chi^2 = 13.23$, $p = 0.0013$; $\alpha = (0.05)/15 = 0.0033$ using Bonferroni correction for multiple comparisons). Cell response distributions during exploration differed between day 1 and days 3, 4, 5, and 6 and between days 2 and 3. Cell response distributions during dominance differed between day 6 and days 1, 2, and 3 and between days 3 and 5. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3–6 and between days 2 and 3 (chi-squared test of independence, p value adjusted for 15 pairwise comparisons between days using a Bonferroni correction). In contrast, the proportion of activated cells during social exploration remained stable throughout the 6-day period (Figure 2E, left). For dominance behavior, we found a significant increase in the fraction of activated cells from days 1 to 3 (~27%) to day 6 (38%) and between days 3 and 5, with no change in the fraction of inhibited cells (Figure 2E, middle). Cell responses during attacks also evolved over the days, with a significant increase in both activated (from 11% to 15%) and inhibited cells (from 3% to 7%) between day 4 (familiarized intruder) and day 6 (novel intruder), with a similar trend that did not reach significance between day 5 (familiarized intruder) and day 6 (Figure 2E, right).

We found that some neurons responded (either activated or inhibited) to only one of the three behaviors, whereas most neurons responded to more than one behavior, although the extent of overlap did not differ from chance assuming random independent assignment of response profiles (Figures S5A and S5B). In contrast, when we examined separately the population of cells that were inhibited by one or more behaviors, we found that the fraction of cells that were inhibited by any two behaviors was greater than chance (Figures S5C and S5D, right). For the activated cell category on day 5, there were more cells activated by both dominance and exploration than chance (Figure S5C, left). On day 6, there was a significantly greater overlap in cells activated by dominance and exploration and in cells activated by both attack and dominance compared with chance ($\chi^2(1) = 4.9$, $p = 0.027$) (Figure S5D, left).

To test the hypothesis that dCA2 responds more vigorously to a novel intruder compared with a familiarized one, we focused our analysis on cell responses from days 4 to 5 (when exposed to a fully familiarized intruder) and compared them to responses on day 6 (when exposed to a novel intruder). We first compared the different types of cell responses (activated, inhibited, and non-responsive) for each behavior across the 3 days. We found a significant increase in the proportion of cells responding to attack from days 4 to 6 (Z-statistic = −3.23, $p = 0.0012$) and from days 5 to 6 (Z-statistic = −2.99, $p = 0.0028$). When analyzing each behavior by response type (activated or inhibited), we found a significant increase in the fraction of inhibited neurons from days 4 to 6 ($\chi^2(2) = 8.45$, $p = 0.0036$) and in activated neurons from days 5 to 6 ($\chi^2(2) = 6.31$, $p = 0.012$). In contrast, the fraction of cells responsive during exploration or dominance behaviors remained consistent across these days.

To control for the possibility that the differences in cell responses between days 5 and 6 were due to the difference in number of R-I sessions experienced by the resident and not the difference in familiarity of the intruder, we performed the same analysis comparing days 4 and 5 with the same familiarized intruder. We found no significant difference in responsive cells for any behavior category between these days ($\chi^2(2) = 4.1$, $p = 0.13$). Furthermore, there was no significant difference when activated and inhibited neuron categories were separately tested. The increase in the fraction of attack-activated dCA2 cells with a novel compared with familiar intruder suggests that dCA2 may contribute to the increased aggression toward a novel compared with familiar intruder, a hypothesis we explore further below.

Dynamics of dCA2 neuron calcium responses at the onset of resident behaviors

Our finding that a smaller fraction of cells was activated during attack compared with social exploration or dominance was somewhat unexpected given prior findings that population-wide Ca^{2+} signals measured by fiber photometry were larger during attack than dominance or exploration behaviors. However, a simple measure of cell proportions fails to provide information on the dynamics and magnitude of dCA2 cell responses at the onset of different behaviors. We therefore examined population response magnitude by aligning the activity of each cell to the start of a given behavior to determine the average responses of each cell for each behavior on a given day. Cells were then tracked over several days.[31] We first focus on responses during days 5 and 6 to compare activity levels of the same putative cells when a resident interacted with a familiarized (day 5) vs. novel intruder (day 6).

The subpopulation of activated cells (determined in Figure 2) showed a clear, time-dependent increase in the mean calcium signal aligned to the start of exploration, dominance, and attack behaviors, for both the familiarized intruder (day 5) and the novel intruder (day 6) (Figure 3A). For both days, the mean Z scored calcium responses were significantly greater than 0 measured 0–1 s after the onset of a behavior (Figure 3B, top) and significantly greater than baseline calcium measured 2–3 s prior to behavior onset (Figure 3B, bottom). Of note, the magnitude of the calcium transients for attack-activated cells was significantly greater than that observed for the dominance or exploration responsive cells (Figure 3B, right). There was no significant difference in dCA2 calcium responses during attack between days 5 and 6 (t-stat = 0.310, $p = 0.757$). However, the responses during exploration (t-stat = 2.351, $p = 0.019$), and dominance (t-stat = 2.430, $p = 0.015$), on day 6 were significantly lower than those on day 5, when measured as a difference from baseline (Figure 3B, bottom). The subpopulation of inhibited cells showed a significant decrease in calcium activity aligned to the onset of the three behaviors on both days 5 and 6 compared with baseline (Figure 3C). On both days the magnitude of the inhibitory response 0–1 s after behavior onset was significantly weaker during attack compared with exploration and dominance (Figure 3D, top). Furthermore, the magnitude of the inhibitory response to attack on day 6 was significantly greater than on day 5, with no difference in inhibitory responses to exploration or dominance (Figure 3D, bottom). Although our calcium measures during a given behavior could, in principle, be affected by differences in the prior behavior of a mouse during the baseline period, we found no difference in the sequence of behavioral transitions to an attack bout across days 4, 5, and 6 (Figure S6E).

Population vector analyses

Our results so far indicate that dCA2 neurons encode information about the behavioral state of the resident during interactions with the intruder. As dCA2 also encodes social identity[29,32] during social exploration, we examined whether dCA2 activity contained

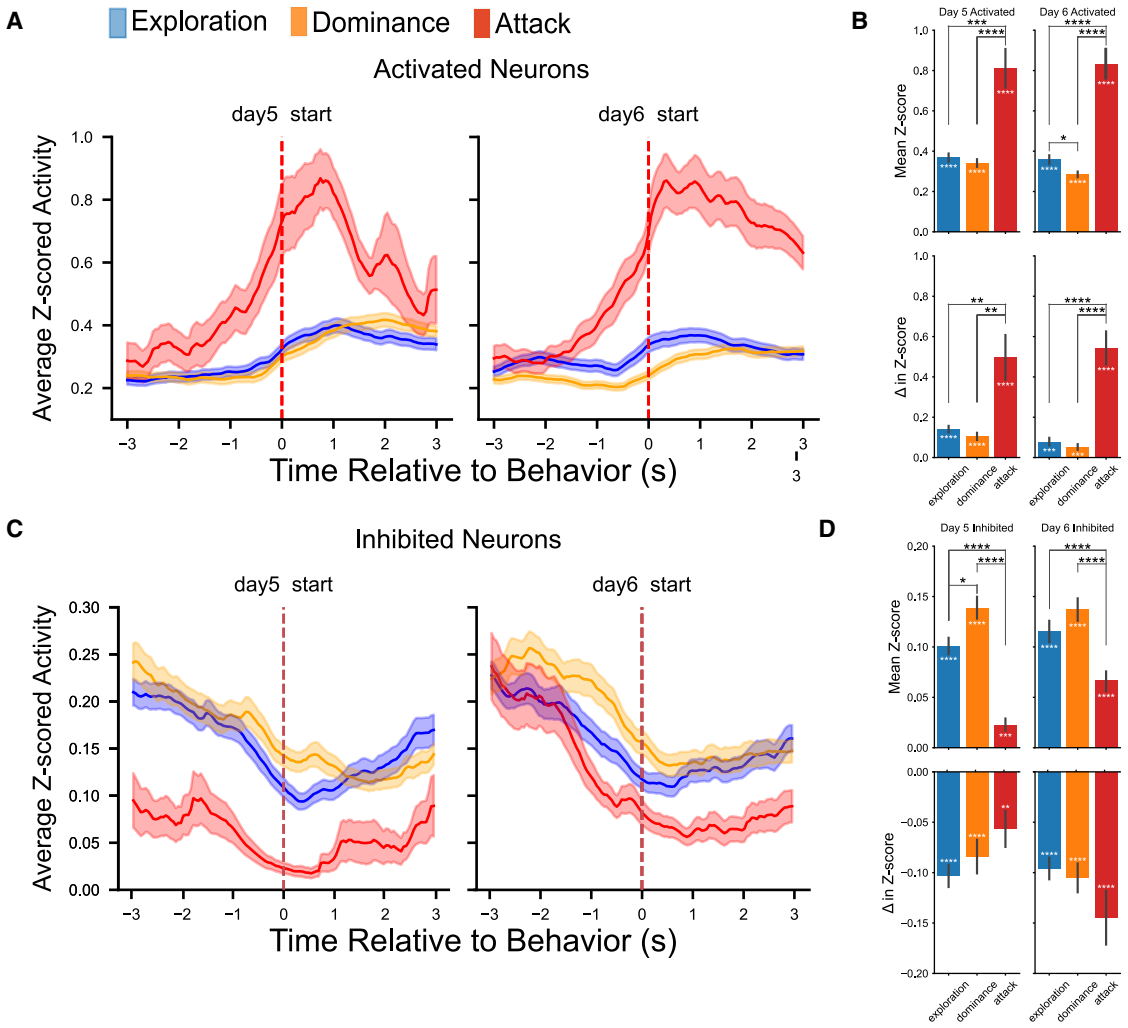

Figure 3. dCA2 neuron response dynamics during social encounters with familiar and novel intruders

(A) Mean ± SEM *Z* scored responses of behavior-activated neurons aligned to onset (dashed line) of attack (red), dominance (orange), and exploration (blue) with familiarized (day 5) and novel (day 6) intruder. Cell counts for days 5 and 6, respectively: exploration = 138, 152; dominance = 217, 242; attack = 58, 94.

(B) Top, Mean ± SEM Z scored calcium signals of activated neurons averaged 0–1 s after behavior onset. Bottom, Mean ± SEM Z score averaged 0 -1 s after behavior onset minus Z score averaged 2-3 s prior to behavior onset. Significance determined against null hypothesis (zero difference) using one-sided t test. Comparisons between each behavior within a day made using independent t test.

(C) Similar to (A) but for inhibited neurons. Cell counts for days 5 and 6, respectively: exploration: 93, 97; dominance: 109, 91; attack: 33, 45.

(D) Similar to (B) showing *Z* score responses for inhibited neurons. The same statistical test was applied as in (B). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

information about the identity of the intruder during the three classified behaviors in the R-I test.

We aligned the activity of each cell over a 500-ms window to the onset of a given behavior with the familiarized intruder tracked over days 4 and 5 and with the novel intruder on day 6. For display purposes, we first sorted the single-cell responses by their mean activity on day 6 (Figure 4A and see Figures S6A for alignment based on day 5). Next, we tested for any significant correlation in population activity between days using Spearman's rank-order correlation of population vectors, with a Bonferroni correction for multiple comparisons (Figure 4B). We evaluated the significance of all correlations by comparing the experimental value to the null distribution from random shuffling of cell identity across days (Figure S6D). We found that dCA2 activity during exploration and dominance behaviors was significantly correlated across each pair of days. However, the magnitude of the correlation was greater with the same intruder (days 4 vs. 5) compared with different intruders (days 4 vs. 6 and 5 vs. 6). We also found a significant correlation between activity during attack behaviors with the same intruder on days 4 vs. 5. In contrast, there was no significant correlation of activity during attack across either pair of days with different intruders.

The differences in cross-day correlations on days 4–6 might reflect differences in intruder identity or in levels of aggression. To distinguish these possibilities, we examined activity correlations across days 1–3, when the intruder was the same, but

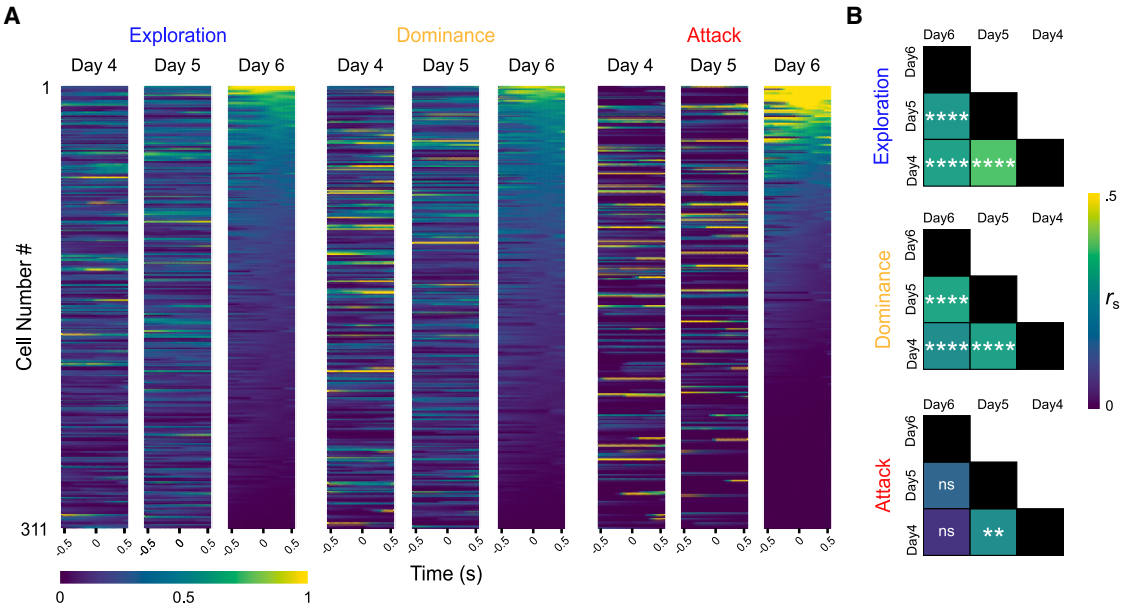

Figure 4. dCA2 activity correlation across days

(A) Average activity vectors for 311 neurons from $n = 6$ aggressive mice on days 4, 5, and 6, during 500-ms windows aligned to behavior onset. Each row is a neuron, sorted based on day 6 activity 0–500 ms after behavior onset.

(B) Pairwise correlation matrix across days. Significance based on Spearman's rank using $\alpha = (0.05)/3 = 0.0167$ (Bonferroni correction). Day 6 vs. 5; attack: $r_s = 0.166$, $p = 0.137$; exploration: $r_s = 0.266$, $p = 6.14e\text{-}06$; dominance: $r_s = 0.296$, $p = 3.62e\text{-}07$. Day 6 vs. 4; attack: $r_s = 0.076$, $p = 1$; exploration: $r_s = 0.288$, $p = 7.71e\text{-}07$; dominance: $r_s = 0.244$, $p = 4.36e\text{-}05$. Day 5 vs. 4; attack: $r_s = 0.254$, $p = 0.009$; exploration: $r_s = 0.363$, $p = 1.34e\text{-}10$; dominance: $r_s = 0.285$, $p = 1.04e\text{-}06$. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

levels of aggression differed (Figure 2C). Despite the differences in aggression, we found significant correlations in population vectors recorded during each behavior (social exploration, dominance, or attack) across all pairs of days (Figures S6B and S6C), suggesting that the differences in correlations across days 4 vs. 5 compared with days 4 or 5 vs. 6 reflect differences in dCA2 activity driven by intruder identity.

Assessing population-based decoding of social behaviors with novel compared with familiar intruders

The above single-cell analysis depends on arbitrary activity thresholds for response classification and does not account for the potential importance of weakly selective or mixed selectivity neurons or their correlations. We thus next determined whether and how dCA2 population activity encodes exploration, dominance, and attack behaviors during interactions with novel and familiar animals using a linear classifier to decode the three possible pairs of behaviors. We trained a support vector machine (SVM) classifier with a linear kernel to distinguish between the three behavioral dichotomies (exploration vs. dominance, exploration vs. attack, and dominance vs. attack) based on the aligned calcium signals from each subject across the 6 days of the R-I test (Figure 5A). We then tested the accuracy of the classifier on decoding behaviors for a subset of calcium activity data not used for training. Because the mice engaged in behaviors for differing amounts of time, we subsampled the data to equalize the time spent in each behavior for a given dichotomy for a given subject mouse on a given day. We calculated chance levels of decoding by random shuffling of the calcium signals relative to the behaviors.

We found that the classifiers accurately decoded all behavioral dichotomies significantly above chance levels on each day of the test (Figure 5B). There were no obvious trends for changes in decoding performance across days. However, the decoding results of Figure 5B are not strictly comparable across days and subjects due to differences in the time spent engaged in the different behaviors and because we did not compare the activity of the same neurons across the different days.

To enable comparisons across days and animals, we longitudinally registered the same neurons[31] in a given animal over triplets of successive days. We confined the registration to 3-day periods because of dropout of sequentially registered neurons over longer time frames. We also balanced the data by analyzing identical-sized blocks of data during each of the three behaviors across days and animals. This enabled us to directly compare SVM classifier decoding accuracies. We tracked neurons over days 3–5 and days 4–6. The first triplet of days allowed us to compare decoding accuracy across days when the subject was engaged in stable levels of aggression with the highly familiarized intruder (see Figures 2C and S4). The second triplet allowed us to compare decoding accuracy across days during interactions with the familiarized vs. novel intruder. We combined training data on days 4 and 5 to provide a sufficient amount on attack data to match the increased time spent in attack with the novel mouse on day 6. We then compared decoder performance on day 3 to days 4/5 ($n = 6$; Figure 6B), to compare decoding across days with the same familiarized intruder, and on days 4/5 to day 6 ($n = 8$; Figure 6A), to compare

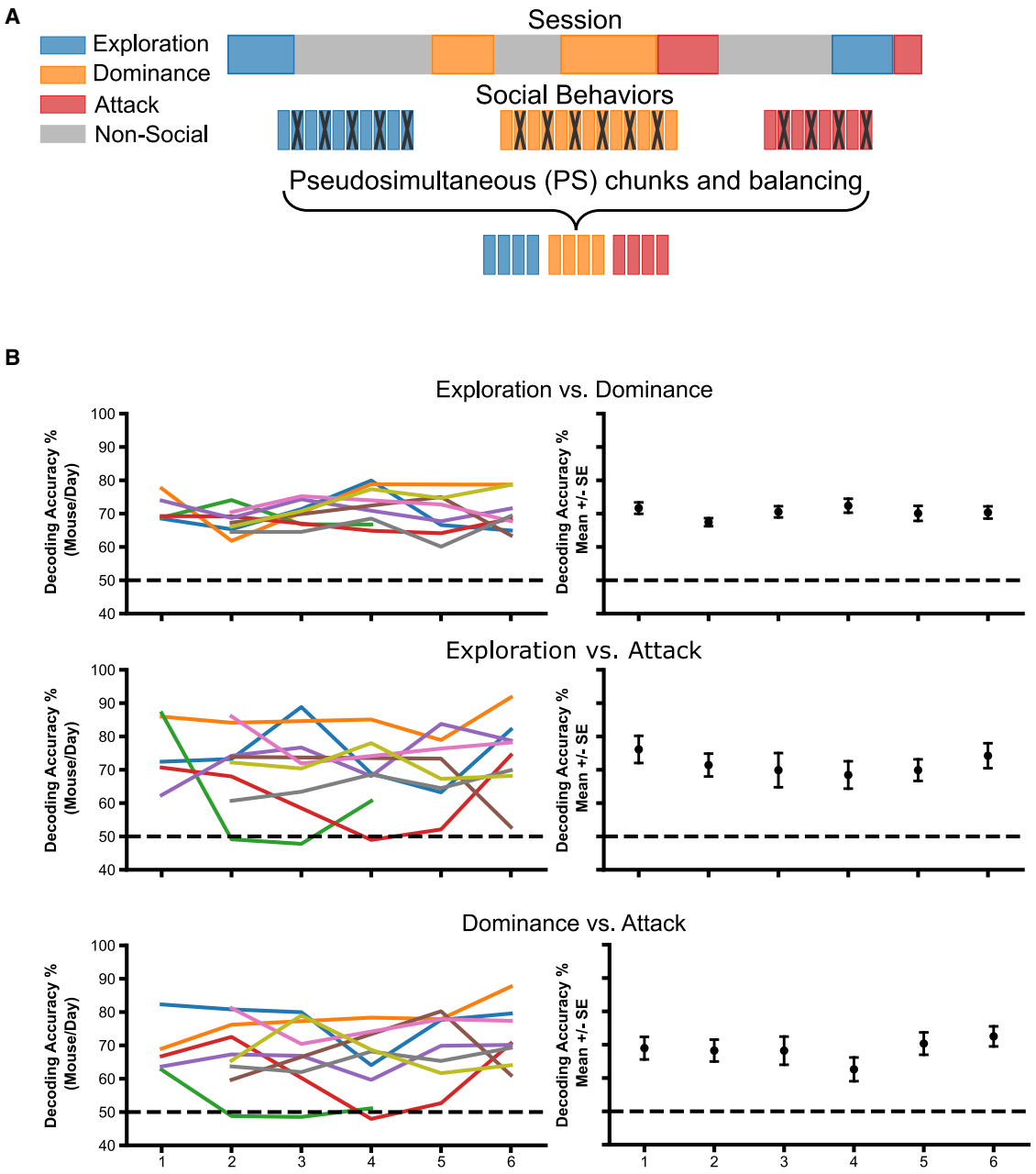

Figure 5. Decoding social behaviors during interactions with familiar and novel intruder

(A) Schematic of decoding approach. Data grouped by behavior and divided into 500 ms bins for analysis (see methods).

(B) Left, decoding accuracy across days for indicated behavior dichotomies using all available neurons for each mouse on each day. Right, mean ± SEM decoding accuracy for mice with sufficient attack bouts. Total cells = 455, 646, 307, 420, 482, 625, and 605 for days 1–6, respectively; n = 5, 9, 6, 7, 8, and 8 mice. There was no significant difference in decoding accuracy across days.

decoding of behaviors during interactions with the familiar vs. novel intruders.

Decoding performance did not differ significantly across days 3–5 during interactions with the same familiarized intruder. In contrast, in eight of the nine residents examined, decoding performance for all three behavioral dichotomies was greater on day 6, during interactions with the novel intruder, compared with days 4/5, during interactions with the familiar intruder. Interestingly, the one resident mouse that showed a decrease in decoding accuracy with the novel intruder showed an anomalous decrease in attack duration to the novel compared with familiarized intruder. When we excluded this aberrant resident from the analysis, we found a significant increase in decoding accuracy with the novel intruder for all three dichotomies compared with

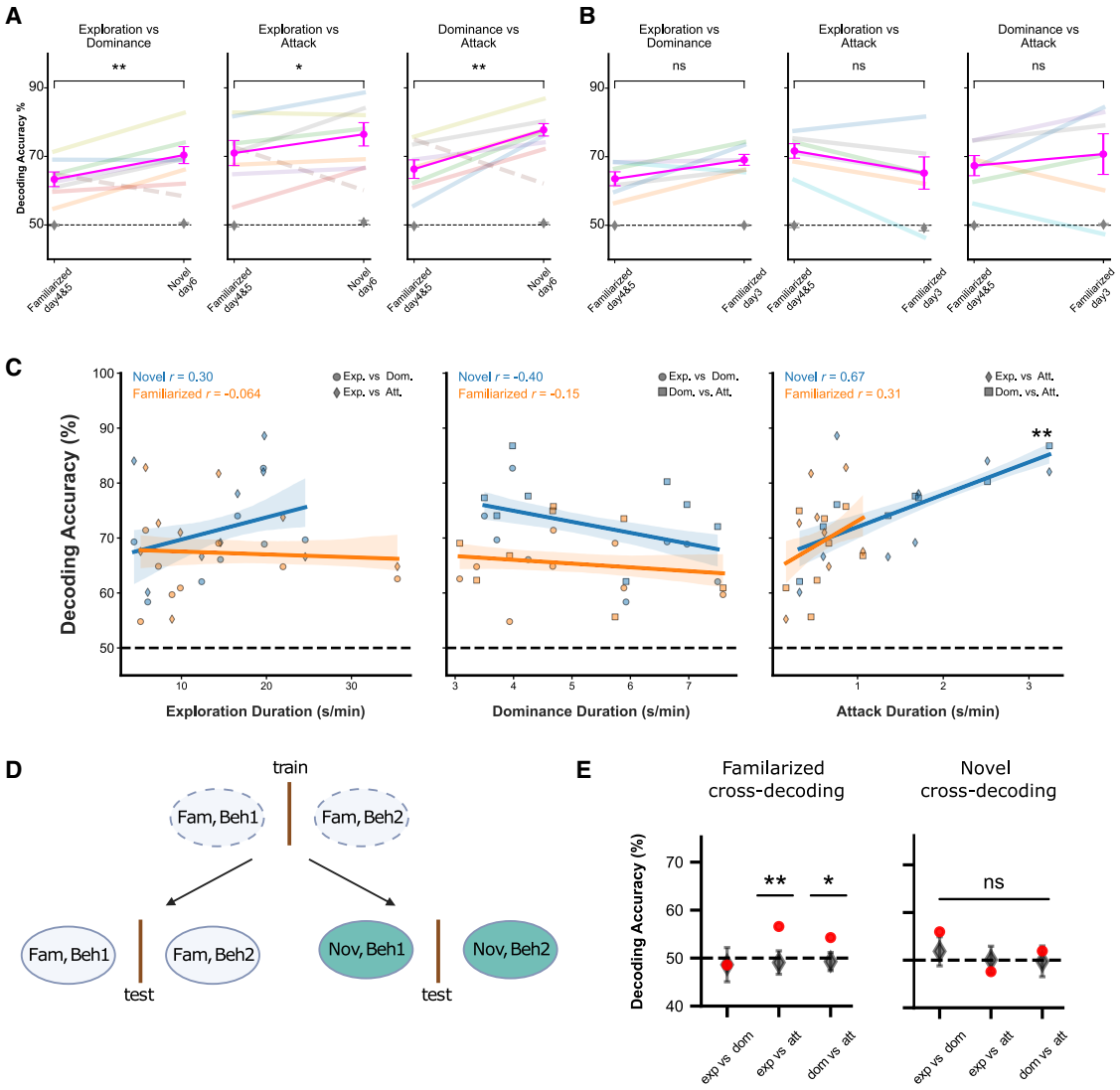

Figure 6. Decoding accuracy with familiar and novel intruders

(A) Decoding accuracy based on activity of same cells tracked across 3 days was significantly greater with novel intruder on day 6 compared with familiarized intruder using combined data from days 4 and 5. Exploration vs. dominance: t-stat = 4.24, p = 0.005; exploration vs. attack: t-stat = 2.77, p = 0.032; and dominance vs. attack: t-stat = 5.91, p = 0.00104 (paired t tests; n = 7; data from aberrant mouse that showed lower attack to the novel vs. familiarized intruder was excluded from analysis).

(B) No significant difference in decoding accuracies during interaction with familiar intruder on day 3 compared with combined data with same intruder on days 4 and 5. Exploration vs. dominance: t-stat = 2.22, p = 0.077; exploration vs. attack: t-stat = 2.32, p = 0.067; and dominance vs. attack: t-stat = 0.79, p = 0.46 (paired t tests; n = 6).

(C) Decoding accuracy for indicated behavior dichotomies plotted against duration a resident was engaged in exploration (left), dominance (middle), or attack (right) of familiarized (orange) or novel (blue) intruders. Duration normalized by total time spent in the arena (min) across days of R-I test. Lines show linear fit with Pearson's correlation. Left, no significant correlation of exploration decoding accuracy and exploration time, with novel (r = 0.30, p = 0.26) or familiarized (r = −0.064; p = 0.814) intruder. Middle, no significant correlation of dominance decoding accuracy and dominance time for novel (r = −0.40, p = 0.126) or familiarized (r = −0.15, p = 0.573) intruder. Right, significant correlation of attack decoding and attack time with novel (r = 0.67, p = 0.005) but not familiarized (r = 0.31, p = 0.24) intruder.

(D) Schema for cross-day decoding of behavioral dichotomies from same tracked neurons.

(E) Left, decoding accuracy for familiarized intruder with linear classifier trained on day 3 and tested on days 4/5 (and vice versa). Cross-day mean decoding accuracy for: exploration vs. dominance = 0.485; p = 0.491; exploration vs. attack = 0.565, p = 0.001; dominance vs. attack = 0.542, p = 0.0039. Right, decoding accuracy with classifier trained during interactions with familiar intruder (days 4/5) and tested during interactions with novel intruder (day 6), and vice versa. Decoding accuracy: exploration vs. dominance = 0.559, p = 0.089; exploration vs. attack = 0.475, p = 0.194; dominance vs. attack = 0.519, p = 0.251. Red circles show the average decoding performance from 20 cross-validations. Black circles and error bars show mean ±2 SD of shuffled data. p values are given by Z score of performance values compared with the null model distributions (one-sided t test). Decoding performances from n = 399 neurons and 8 mice. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

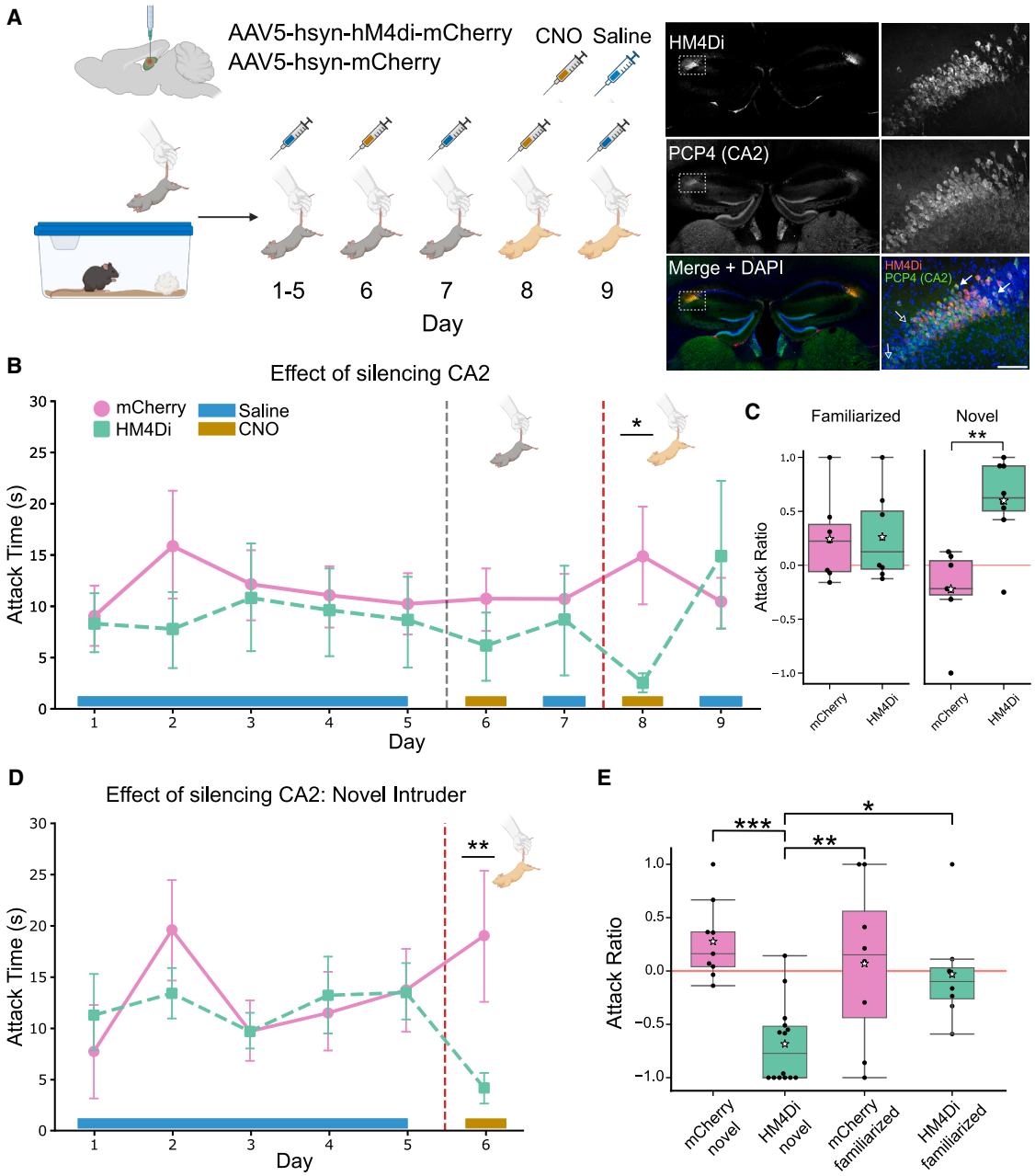

Figure 7. Silencing dCA2 attenuates aggression to novel but not familiarized conspecifics

(A) Schematic of 9-day R-I test. Mice bilaterally injected in dCA2 with hsyn-AAV5-hM4D(Gi)-mCherry (experimental) or hsyn-AAV5-mCherry (control). Both groups were injected intraperitoneally with saline (days 1–5, 7, and 9) or CNO (days 6 and 8) 30 min prior to R-I test with familiarized (days 1–7) or novel (days 8 and 9) intruder. Right panel: left side, coronal sections showing dCA2 expression of hM4Di-mCherry (top), CA2 marker PCP4 (middle), and merged image co-stained with DAPI (bottom). Right side, 20× magnification of area outlined to left. Scale bar, 100 μm. Merged image: filled arrows indicate cells that co-stained with PCP4 and mCherry; empty arrows denote cells expressing PCP4 alone.

(B) Time spent in attack for mice in experimental and control conditions across 9 days. Experimental (n = 8, days 1–9) and control (n = 8, days 1–6; n = 7, days 7–9) mice injected with CNO prior to R-I test on day 6 showed no differences in attack time to familiarized intruder (LMM fit). Injection of CNO on day 8 significantly decreased attack time to a novel intruder (day 8) in experimental compared with control groups (LMM interaction effect, p = 0.022).

(C) Effect of CA2 silencing measured by comparing attack times in presence and absence of CNO with familiarized intruders (left panel: days 6 vs. 7) and novel intruders (right panel: days 8 vs. 9). Effect of CNO measured by "attack ratio" = (attack duration without CNO – attack duration with CNO)/(total attack time on both days). Experimental (hM4Di-mCherry) showed significantly less attack (attack ratio > 0) than control (mCherry) group with novel (t = 4.044, p = 0.00139) but not familiarized (t = 0.088, p = 0.931) intruder (paired t test).

(D) dCA2 silencing in experimental group (n = 14 mice) reduced attack of novel intruder performed on day 6 compared with control group (n = 9 mice) based on LMM (day 6: p = 0.002).

(legend continued on next page)

the decoding accuracy with the familiarized intruder (Figure 6A). The results did not depend on the combining of attack data from days 4 to 5 since, when we limited our analysis to day 4 or 5, selecting the day with maximal attack time, we again found a significantly greater decoding accuracy with the novel compared with familiar intruder (Figures S7B and S7D). When we included data from the aberrant resident, we still observed a significantly greater decoding accuracy with the novel compared with familiarized intruders when data from days 4 to 5 were combined, although the difference was not significant when the 2 days were analyzed separately (Figures S7A and S7C).

To determine whether the increased decoding accuracy was driven by the increase in aggression or the novelty of the intruder, we compared decoding accuracies when the same familiarized intruder was encountered on day 2, when aggression was high, and on day 3, when aggression decreased. However, we found no significant difference in accuracies across the 2 days (Figure S7F), suggesting that the enhanced decoding accuracy is not driven by differences in aggression alone but may reflect an influence of social novelty.

Finally, we compared decoding accuracy on day 1, when the intruder was novel, but aggression was low (presumably due to the lack of aggressive experience by the resident), with decoding accuracy on day 2, when the intruder was familiar and aggression was higher (presumably due to the increased experience in attack of the intruder by the resident). However, there was no significant difference in decoding performance across these first 2 days (Figure S7G), may reflect offsetting effects of the decrease in novelty and increase in aggression on day 2 compared with day 1.

Decoding accuracy with novel conspecifics correlated with social aggression

We next asked whether the strength with which dCA2 representations encoded a particular behavior might be related to the extent to which a resident engaged in that behavior. Indeed, we found a significant positive correlation between the duration of attack and the accuracy of decoding attack behavior (relative to exploration or dominance) for novel but not familiar intruders, with or without grouping days 4 and 5 (Figures 6C and S7E). The correlation was specific for attack behavior as there was no significant correlation between attack decoding accuracy and the duration of exploration or dominance behaviors, for either novel or familiar intruders (Figure 6C, left-middle).

Finally, we investigated the stability of social behavior representations in dCA2 across 2 days of interaction, either with the same familiarized intruder or with a familiarized and a novel intruder, using neurons tracked across sessions. Separate linear classifiers were trained to discriminate social behavior dichotomies on a familiarized day (either days 4 or 5 based on maximal attack time). We then tested decoding accuracy on a different familiarized day (day 3) or a novel day (day 6) and then reversed the training/testing days to compute the average decoding score (Figure 6D). We found that cross-day decoding performance was significantly better than chance on attack-related dichotomies across the 2 days of interaction with the familiarized intruder (Figure 6E, left panel). In contrast, the classifier failed to decode above chance accuracy behavioral dichotomies trained on the familiarized intruder and tested on the novel intruder, and vice versa (Figure 6E, right panel). The lower cross-day decoding across the familiar-novel days (4 or 5 vs. 6) compared with the familiar-familiar days (3 vs. 4 or 5) was not due to a longer time interval between training and testing days for the former as the maximal day of attack with the familiar intruder (selected for this analysis) occurred on day 5 with four out of six residents. These results agree with our single-cell correlation analysis (Figure 4) and indicate that familiarized intruders are encoded through a stable representation while the neural code for social behaviors of a familiar and novel conspecific are more distinct.

The behavioral role of dCA2 in promoting aggression toward novel conspecifics

The above results suggest that dCA2 pyramidal neurons may enhance aggression toward a novel intruder by detecting the novelty of the intruder. This hypothesis predicts that silencing dCA2 should cause a more profound inhibition of attack of a novel compared with a familiar intruder. To test this idea, we examined the effect of chemogenetic inactivation of dCA2 using the R-I protocol with both familiar and novel intruders (Figure 7A, left).

We expressed the inhibitory hM4Di DREADD tagged with mCherry (hM4Di-mCherry) in dCA2 of wild-type C57Bl/6J mice using injections of AAV2/5 vectors because this serotype preferentially infects dCA2 pyramidal neurons in a Cre-independent manner.[28,30] Control mice were injected with AAV2/5 to express mCherry. Two and a half weeks after AAV2/5 injection, R-I testing was performed over a period of several days. All residents were injected with saline 30 min prior to an R-I test on days 1–5 with the same initially novel intruder. Only mice that displayed one or more biting attacks within the first 2 days of the test were included in further analyses. Consequently, due to the variability in aggressive responses, the final number of mice retained for each experimental condition (control vs. CA2-silenced groups) differed. Post-hoc staining with the dCA2 pyramidal neuron marker PCP4 revealed near complete overlap with hM4Di expression (Figure 7A, right; and see Figures S3A and S3B).

On day 6 of the R-I protocol with the now familiarized intruder, coinciding with three weeks following AAV injection, the two cohorts were intraperitoneally injected with the iDREADD agonist CNO. After a 30-min habituation, residents were exposed to the same familiarized intruder they encountered on days 1–5. Interestingly, we found no significant difference in the time spent in attack between groups, although there was a trend for

(E) Differential effect of CA2 silencing on attack of novel (left two bars, data from D) vs. familiar (right two bars, data from B) intruders. Attack ratios calculated from difference in attack times of familiar intruder on day 5 (no CNO) and novel (D) or familiar (B) intruder on day 6 in presence of CNO, divided by total attack on 2 days. One-way ANOVA, $F(3, 35) = 8.428$, $p = 0.00024$. Attack ratio of experimental group with novel intruder differed significantly from control group with novel intruder ($p = 0.0003$), control group with familiarized intruder ($p = 0.0075$), and experimental group with familiarized intruder ($p = 0.024$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

decreased attack in the iDREADD group. We then repeated the R-I test following saline injection on day 7, a time when CNO from the previous day is no longer effective,[21] and observed normal levels of aggression in both groups of residents (Figure 7C, left panel).

Next, on day 8, we determined the effect of silencing dCA2 on aggression toward a novel intruder. In stark contrast to the lack of effect on aggression toward the familiar intruder on day 6, dCA2 silencing produced a marked, significant decrease in attack of the novel intruder on day 8 compared with the mCherry control group. The decrease in aggression to the novel animal upon dCA2 silencing was reversible as attack duration to the same intruder presented on day 8 increased significantly to control levels when dCA2 was active (with saline injection) on day 9 (Figures 7B and 7C, right panel, and S8, right panel). The difference in efficacy by which dCA2 silencing decreased aggression to a novel compared with familiar intruder was not due to the difference in the order of presentation of the intruders as we observed a near complete suppression of aggression with CA2 silencing relative to the mCherry control group when the novel intruder was presented on day 6 (Figures 7D and S8D). Moreover, the decrease in attack time upon dCA2 silencing relative to the control group with the novel intruder was significantly greater than the decrease with a familiar intruder (Figures 7E and S8D). Interestingly, whereas silencing dCA2 decreased aggression to the novel intruder on day 6, the time spent in exploration toward the intruder significantly increased, with no change in dominance, suggesting a shift in the probability of behavioral responses (Figures S8A and S8B).

DISCUSSION

In this study, we provide, to our knowledge, the first neural mechanism for the long-standing finding that social novelty is a powerful driver of social aggression.[3,8,9] Our results using both calcium imaging and chemogenetic silencing support the hypothesis that the mnemonic[11,12,21,23] and aggression-promoting[18,24] actions of dCA2 are manifestations of a unitary function by which dCA2 provides a neural signal of social novelty that specifically increases aggression toward a novel intruder. This hypothesis was initially based on three complementary sets of findings. First, prior studies[2,8–10,33] and our present results (Figures 1, 2, and 7) show an increased probability of attack toward a novel relative to a familiar intruder. Second, dCA2 activity is required for SNR memory.[11,12] And third, electrophysiological[28,34] and cellular calcium imaging[29] studies demonstrate that dCA2 neural activity discriminates a novel and familiar conspecific, with a higher rate of spike firing around a novel compared with familiar conspecific.[28,35]

The importance of dCA2 in enhancing aggression to novel conspecifics

The hypothesis that the role of dCA2 in SNR memory and aggression are closely related is nicely illustrated in our chemogenetic silencing results of Figure 7B. Here, when dCA2 was silenced during an initial encounter with a novel intruder on day 6, not only was aggression suppressed during that encounter but, when the same novel intruder was re-introduced the following day after dCA2 had recovered from silencing, the intruder provoked a high level of aggression, as if the resident encountered the intruder for the first time. This is consistent with the idea that dCA2 activity was required to enable the memory of a prior encounter with the novel intruder.

Our data also support the view that social aggression is enhanced both by the novelty of the intruder and by the experience of the resident in winning bouts of aggression. These dual effects can explain why levels of aggression to a series of novel intruders increase on successive days of testing, whereas aggression to the same, increasing familiar intruder is constant (Figure 1). The dual effects of experience may also explain why, in some cohorts, aggression to the same novel intruder on day 2 can be greater than it was on day 1 (Figure 2). The latter finding suggests that the effect of experience in fighting to enhance aggression can outweigh the effect of loss of social novelty to decrease aggression. The effect of fighting experience may predominate when levels of aggression are initially low, as observed with the mice tethered for calcium imaging of Figure 2. Because of these dual, competing effects of prior experience, we have carefully based our conclusions of the effect of social novelty only after residents had gained sufficient experience in winning bouts of attack to stabilize levels of aggressiveness.

The encoding by dCA2 of behavioral state and social identity during the R-I test

Our calcium imaging experiments support the idea that dCA2 activity influences aggression through two related processes, the detection of intruder novelty and the differential encoding of behavioral state. Thus, we found that the activity of a significant fraction of dCA2 neurons responded relative to baseline to behavioral state (exploration, dominance, or attack). Moreover, dCA2 population activity decoded pairs of behavioral state significantly above chance levels, either with a novel or familiar intruder. At the same time our data indicate that dCA2 provides a stronger representation of behavioral state with novel compared with familiar intruders, with decoding accuracy of attack compared with exploration or dominance behaviors significantly greater during interactions with a novel compared with familiar intruder. Moreover, the accuracy of attack decoding was significantly correlated with time spent in attack of the novel but not the familiarized intruder.

Our findings at cellular resolution are in broad agreement with fiberphotometry recordings from dCA2 during the R-I test by Leroy et al.,[18] who found a greater elevation in mean population calcium levels during bouts attack compared with exploration or dominance. Surprisingly, we found that a greater proportion of dCA2 cells responded significantly above baseline during exploration and dominance behaviors compared with attack. However, when we separated the entire population into activated and inhibited response types and aligned the responses to the onset of a given behavior, we found that neurons encoding attack responded with a larger increase in Ca^{2+} levels at the onset of attack relative to onset of exploration or dominance behaviors, consistent with the results of Leroy et al.[18]

Two potential mechanisms may account for how dCA2 activity in response to a novel animal may increase aggression. First, enhanced firing of dCA2 pyramidal neurons in the presence of a novel animal would lead to increased activation of the dorsal lateral septum, which Leroy et al. found mediates the action of CA1 to enhance aggression by disinhibiting the VMHvl subnucleus of the hypothalamus. The second mechanism follows from recent findings that dCA2 encodes novel and familiar individuals in different geometric arrangements in neural activity space, which provides a generalized or abstract readout of social novelty vs. familiarity.[29] This implies that a neuron receiving appropriately weighted synaptic inputs from dCA2, for example, in dorsal lateral septum, could selectively fire in response to a novel vs. familiar intruder, thereby triggering aggression to a novel social stimulus.

Together, our findings provide a neural mechanism by which SNR memory and social aggression interact as complementary processes within dCA2 to modulate aggression. Social novelty is integrated with social aggression signals in dCA2, which are then translated into an adaptive aggression response dependent on context, experience, and internal state.

Limitations of the study

The R-I test, while effective for assessing aggression, has inherent limitations for evaluating social memory. Specifically, while we compared responses to familiar vs. novel animals across days, it is challenging to establish a neutral baseline for social memory within the same group, as internal states differ across testing sessions. Moreover, social exploration in the R-I test does not reliably serve as a measure of memory, as it is influenced by factors such as aggression and dominance.[27]

Additionally, our study focused exclusively on male mice, leaving open the question of whether dCA2-dependent social novelty similarly drives aggression in females. While reactive aggression in females is typically observed during specific states such as lactation,[36] future studies exploring maternal aggression or the effects of social novelty during pregnancy, such as phenomena related to pregnancy termination (the Bruce effect), could reveal neural mechanisms of social novelty-induced aggression independent of sex.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Andres Villegas (av2686@columbia.edu).

Materials availability

This study did not generate new materials.

Data and code availability

- All animal data reported in this paper will be made available upon request by the lead contact.
- All original code generated in the analysis of this study has been deposited online at Zenodo.com and is available as of the date of publication. The DOI is listed in the key resources table.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

A.V. and S.A.S. conceived the project and designed the experiments. A.V. collected and analyzed the data with guidance from S.A.S. The data were interpreted by both A.V. and S.A.S., who also co-wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-PCP4	Sigma-Aldrich	Cat#HPA005792; RRID: AB_1855086
Mouse polyclonal anti-RFP	Invitrogen	Cat#MA5-15257; RRID: AB_10999796
Bacterial and virus strains		
AAV2/5.hSyn.GcaMP6f.WPRE	Addgene (Douglas Kim & GENIE Project)	RRID: Addgene_100837
AAV2/5-hSyn-hM4D(Gi)-mCherry	Addgene (Bryan Roth)	RRID: Addgene_50475
AAV2/5-hSyn-mCherry	Signagen	Cat# SL116104
AAV2/5.hSyn.jGCaMP8s.WPRE	GENIE Project	RRID: Addgene_162374
Deposited data		
Custom code for data analysis	This study	Zenodo Data: https://doi.org/10.5281/zenodo.15368747
Experimental models: Organisms/strains		
C57BL/6J	The Jackson Labratory	RRID: IMSR_JAX:000664
Software and algorithms		
ANY-maze	Stoelting Co.	https://www.anymaze.co.uk
BORIS	Friard and Gamba	https://www.boris.unito.it/
CaImAn	Giovannucci et al.	https://github.com/flatironinstitute/CaImAn
Decodanda	Lorenzo Posani	https://github.com/lposani/decodanda
Fiji	Schindelin et al.	https://fiji.sc/
Inscopix Data Acquisition & Analysis Software	Inscopix	https://www.inscopix.com/
Mesmerize	Kolar et al.	https://github.com/kushalkolar/MESmerize

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

All mouse procedures were performed in accordance with the NIH guidelines and with the approval of the Columbia University Institutional Animal Care and Use Committee. All mice used as residents in the resident intruder test were males on a C57Bl/6J background (The Jackson Laboratory). We used male BALB/cJ (The Jackson Laboratory) mice as intruders. Behavioral tests were performed on sexually naive male mice only. All mice were maintained on a 12-h light–dark cycle with *ad libitum* access to food and water. We used mice between 2 and 4 months old.

METHOD DETAILS

Stereotaxic viral delivery

For imaging experiments, a volume of 200 nL AAV2/5.hsyn.GcaMP6f.WPRE.SV40 virus or AAV2/5.syn.jGCaMP8s.WPRE (respectively: titer: $1.3*10^{12}$ gc/mL; a gift from Douglas Kim & GENIE Project: Addgene viral prep # 100837-AAV5; http://n2t.net/addgene:100837; RRID:Addgene_100837; titer: $2*10^{12}$ gc/ml, gift from GENIE Project Addgene plasmid # 162374, virus packaged AAV2/5 in house; http://n2t.net/addgene:162374; RRID:Addgene_162374) was injected at a rate of 150 nL/min into the right hemisphere above dorsal hippocampal CA2 using stereotactic coordinates: (AP) −2.0 mm, (ML) +1.8 mm, DV -1.2 mm (from dura) all from bregma. The pipette was retracted after 5 min.

In pharmacogenetic silencing experiments, we injected AAV2/5-hSyn-hM4D(Gi)-mCherry (titer: $1.05*10^{12}$ gc/ml; a gift from Bryan Roth (Addgene viral prep # 50475-AAV5; http://n2t.net/addgene:50475; RRID:Addgene_50475) or AAV2/5-hsyn-mCherry (titer: $2*10^{12}$ gc/ml) was bilaterally injected (200 nL per site) to express the inhibitory Designer Receptors Exclusively Activated by Designer Drugs (iDREADD) hM4Di or a fluorescent control in dCA2 using the same coordinates as above.

GRIN lens implantation

Three weeks following viral injection, a 1.2 mm diameter circular craniotomy was centered at the following coordinates: AP -2.0 mm, ML +2.25 mm. We inserted a GRIN lens (Inscopix, 1.0 mm diameter, 4.0 mm length) into the craniotomy at a depth of −1.4 to −1.5 mm relative to bregma at a 10° angle from the midline, so that the lens was parallel to the dCA2 cell body layer. The Inscopix Proview system imaged cells during implantation to adjust the position of the lens to optimize visible fluorescence. Kwik-sil was placed around the craniotomy and the lens secured in place using Metabond dental cement. The top of the Proview lens cuff was filled with lens paper and Kwik-cast to protect the lens. Mice were housed in pairs separated by a divider for one week before a plastic baseplate was placed over the lens and secured with Metabond dental cement. The baseplate and microscope were placed over the lens and the position was adjusted until cells were maximally in focus. After 1 week of recovery, mice were separated into their own home cages in preparation of acclimation to imaging setup.

Immunofluorescent labeling and imaging

We perfused mice at the end of the experiments using saline followed by 4% PFA in ice-cold PBS. Brains were extracted and incubated in 4% PFA overnight. After 1 h washing in 0.3% glycine in PBS, 60-μm sections were prepared in coronal orientation using a Leica VT1000S vibratome. Sections were permeabilized and blocked for 1 h with 5% goat serum and 0.4% Triton X- in PBS at room temperature. Sections were incubated overnight at 4°C in 0.1% Triton X- in PBS plus 5% goat serum. The following day, slices were washed three times for 10 min each in PBS and incubated with secondary antibodies for two hours. DAPI (ThermoFisher Scientific, #D1306) staining was applied at 1:4000 for 10 min in PBS at room temperature prior to mounting. Slices were mounted using Fluoromount (Sigma-Aldrich) and imaged using Zeiss LSM 700 confocal microscope.

For mCherry and PCP4 labeling, the first incubation was performed with mouse anti-RFP and rabbit anti-PCP4(respectively: 1:500, Invitrogen, # 710530 and 1:300; Sigma-Aldrich, #HPA005792, RRID: AB_1855086). The secondary incubation was performed with anti-mouse conjugated to Alexa 488 and anti-rabbit IgG2a conjugated to Alexa 568 (respectively: 1:500; #A21131, RRID: AB_2535771 and 1:500; #A11011, RRID: AB_143157).

For PCP4 labeling, the first incubation was performed with rabbit anti-PCP4 (1:300; rabbit anti-pcp4 #HPA005792, Sigma-Aldrich). The secondary incubation was performed with anti-rabbit conjugated to Alexa 568 (1:500; #A11011, RRID: AB_143157).

Social aggression

The resident–intruder paradigm was used to assess social aggression as previously described.[27] Subject male mice (residents) were individually housed for a minimum of 1 week, with a cage change no less than 1 week before the encounter with a novel intruder. Stimulus mice (male BALB/cJ intruders) were group housed and used for sequential encounters. Feeding and water apparatuses were removed for habituation to allow unimpeded interaction and better recording during testing. Ten-minute presentations (or 15 min during calcium recording) of weight-matched intruders occurred in the home cages of the resident mice after 30-min habituation to the behavioral room.

Behavioral data was collected in a standard home cage arena measuring 18.5 cm × 38 cm, under red light illumination and sound attenuated conditions for later ethological analysis using Boris annotation software.[37] Videos were recorded perpendicular to the cage floor at 60 Hz using an Image source camera with a wide angle TPL 0420 6MP lens.

Ethological analysis of aggression was performed by a blinded observer. We measured: (1) the duration of attack; (2) the number of bites; (3) the duration of dominance displays; and (4) the duration of exploration displays. Operational definitions for these behaviors and their manual annotations are presented in Table S1.

Pharmacogenetic silencing of dorsal CA2

Mice were injected with rAAV expressing iDREADD or inert fluorescent control and returned to their home cage for recovery. After 10 days of recovery the mice were housed singly. Habituation consisted of 5 days of Intraperitoneal (IP) Injection of saline prior to the social aggression test. On day 6, mice were injected with clozapine N-oxide (CNO) (5 mg/kg or 10 mg/kg in saline) or vehicle (saline) 30 min before testing. Each dose of CNO was analyzed separately and no significant difference was found using independent samples t test between 5 and 10 mg/kg (t-stat = 0.83, $p = 0.42$) and so CNO data for both doses was combined.

A Linear Mixed Model (LMM) was employed to analyze the repeated measures R-I test data, with attack (or other social behavior) duration as the dependent variable. The model included fixed effects for Group (hM4Di: experimental or mCherry: control) and Day (1–6), their interaction, and a random intercept for each mouse to account for random variations between individual mice. The 'mCherry:control' group served as the reference category. The LMM was fitted using the Restricted Maximum Likelihood (REML) method. The analysis was conducted using the statsmodels package in Python. The model formula is as follows (using Attack as example):

$$Attack_{ijk} = \beta_0 + \beta_{Groupi} + \beta_{Dayj} + \beta_{Groupi * Dayj} + \mu_{mousek} + \epsilon_{ijk}$$

Here, $Attack_{ijk}$ denotes the measured time spent in attack for the ith group on the jth day for the kth mouse, while β_0 is the intercept of the model. $\beta_{Groupi} + \beta_{Dayj}$ represents the coefficients associated with different levels of the group variable or levels of day,

respectively. $\beta_{Groupi * Dayj}$ denotes the interaction effects between the 'Group' and 'Day' variables. μ_{mousek} accounts for intrinsic aggressive differences for each mouse k. ϵ_{ijk} is the residual error term for each observation.

Behavior with intruder experience

Mice were singly housed for 1 week prior to the start of the social aggression test. Subject (resident) mice were randomly assigned the condition of the corresponding intruder and aggression encounters were recorded for 10 min across 5 days. Three groups of residents were exposed to different types of intruders.

(1) The 'familiarized' group: These residents encountered the same intruder repeatedly throughout the experiment.
(2) The 'naive' group: This group was introduced to a new intruder that they had never encountered before.
(3) The 'experienced' group: Residents in this group faced a unique intruder who was both new to them and had equal experience interacting as an intruder to other aggressive residents in an equal number of R-I tests.

An LMM was used assessed the fixed effects of 'Group' ('novel naive', 'familiarized', and 'novel experienced') and 'Day' (1–5), with each mouse accounted for as a random intercept to account for individual variability. The 'familiarized' group served as the reference category. The analysis included data from 8, 9, and 13 mice in Groups naive, familiarized, and experienced, respectively. Significance of fixed effects and interactions was gauged using Wald tests with p-values reported.

Direct interaction test

This procedure was adapted from Meira et al.[21] On a separate day following the completion of the longitudinal R-I test (as discussed in the 'behavior calcium recordings' section), resident mice were place in a neutral cage, measuring 18.5 cm × 38 cm, with fresh bedding. Subject male mice were allowed to habituate to the arena for 30 min prior to presentation of a novel male juvenile (3–4 week old) stimulus mouse. Interactions lasted 5 min but importantly were consistent for the same subject mouse through trials. Videos were recorded using ANY-maze software for offline annotation using Boris. Exploration behaviors were manually annotated using operational definitions in Table S1.

In trial 1, resident mice were allowed to interact with the juvenile stimulus mouse for 5 min. After a 30-min intertrial interval, the same, now familiarized, juvenile was re-introduced into the cage for another 5-min social interaction in trial 2. Finally, after another 30-min habituation, a now novel but experienced stimulus juvenile mouse (i.e., presented to another subject mouse), was presented to the subject mouse for 5 min.

Behavior with microendoscopic calcium imaging

Prior to the first test, mice were handled and habituated for six days on the following schedule: day 1: handling; day 2: handling, exposure to test room for 30 min; day 3: handling, exposure to test room for 30 min, and insertion of dummy miniscope overnight; day 4: handling, exposure to test room for 30 min; day 5: handling, exposure to test room for 30 min, exposure to test arena in home cage for 10 min, scruffing/insertion of the microscope, and removal of dummy miniscope; day 6: handling, exposure to test room for 30 min, exposure to test arena in home cage for 10 min scruffing/insertion of the microscope. In each test, subject mice were first allowed to habituate for 5 min in their home cage and interact with an intruder for 15 min. The same intruder mouse was placed in the subject mouse home cage over 5 days. On day 6 a novel and experienced intruder was placed in the same subject mouse home cage. Intruder mice were weight- and sex-matched to subject mice. In each session, the subject mouse was free to explore the arena. Boris software[37] was used to manually score periods of interaction with the intruder consisting of non-overlapping exploration, dominance, or attack behaviors.

An LMM was used assessed the fixed effects of 'Day' (1–6), with each mouse accounted for as a random intercept to account for individual variability. 'Day 1' served as the reference category. The analysis included data from 9 mice. Significance of fixed effects and interactions were gauged using Wald tests with p-values reported.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data acquisition, preprocessing and motion-correction

On the day of the experiment, mice were moved to the behavior room. Mice were allowed to acclimate to the environment for 30 min. An nVista 3.0 Inscopix miniaturized microscope was inserted into the baseplate and used to record calcium fluorescence from dCA2 pyramidal neurons during social and non-social aggressive behaviors using Inscopix data acquisition software (20 frames per second, 50-ms exposure, 0.2–0.5 mW/mm2 EX-LED). The working distance between the microscope objective and the lens was adjusted to maximize cell focus, and this distance was maintained for all sessions. To align behavior and calcium videos, a 5V TTL pulse from an Ami-2 Optogenetic interface triggered calcium recordings through Anymaze software at the start of each trial along with a behavior video recording. Behavior recordings were collected at a rate of 60 Hz. The raw calcium videos were then run through Inscopix Data Analysis software for pre-processing as a concatenated habituation and test phase file. Videos were corrected for defective pixels and 4x spatially down-sampled. Background fluorescence was removed using a spatial band-pass filter and

fluorescence videos were motion-corrected using the Inscopix motion correction algorithm. The preprocessed and motion corrected tiff files were then exported for cell identification and signal demixing (CNMFe) using the CaImAn package.[31,38]

Segmentation and ROI identification

Cell regions-of-interest (ROIs) were identified using the Python CaImAn package for large-scale calcium imaging data. The spatial footprints and deconvolved signal for the active sources (ROIs) were extracted using CNMFe, and then the denoised, detrended temporal traces (over a 25 s window) and spatial footprints were exported. We used the Mesmerize package to evaluate individual ROIs and spatial footprints, and those with non-spherical or non-oval shapes caused by motion artifacts were excluded from analysis. Finally, the computed traces were normalized to obtain z-scores, dividing by the standard deviation of the raw fluorescence trace. The calcium data were aligned to the manually annotated behavioral movie based on the nearest time stamp, accounting for the time offset returned by anymaze relative to the onset of optical recording following an Ami-2 Optogenetic interface delivered pulse. We only included animals with stable window implants through all sessions.

Longitudinal registration

For day-to-day longitudinal cell registration, we used a pipeline reported earlier.[31] Briefly, the approach takes a set of spatial components and finds components present in all using an intersection over union metric and the Hungarian algorithm for optimization. Cells were registered across days 3,4 and 5 and separately for days 4,5, and 6 to maximize cell retention on all days and form meaningful comparisons between familiarized-to-familiarized and familiarized-to-novel intruder representations.

Cell response

Neurons were classified as responsive for a particular behavioral variable (e.g., exploration, dominance, or attack) by comparing the activity during a given social behavior to activity during periods of non-social interactions during each R-I test. To assess statistical significance of each cell response, we computed a chance distribution from temporally shifted behavior labels. Briefly, we employed a two-step procedure that maintained the neural calcium trace structure while destroying dependency with behavior labels, e.g., the animal's behavior vector and neural traces. The temporal sequence of the behavioral vector was reversed by reindexing with flipped indices (e.g., the last index of the session became the first datapoint and vice versa). Subsequently, the flipped behavior vector was subjected to a random shift, where the shift magnitude, denoted as n, was randomly chosen within the range $0 \leq n \leq$ the total length of the neural trace. For each shuffle the mean calcium rate was computed for time spent during a selected social behavior and non-social interaction, and the difference in mean calcium rates during these periods were recorded 1000 times, resulting in a null distribution. The true (non-shuffled) mean rate difference was compared to the shuffled distribution using a nonparametric two-sided permutation test. A neuron was deemed responsive for a behavior if the permutation test p-value was less than 0.05. If the rate difference was greater than 0, the cell was classified as 'activated'; if the rate difference was less than 0, the neuron was classified as inhibited. A neuron that was not responsive for any behavior was considered 'non-responsive'.

Cell response dynamics

Mean ± SEM *z-scored* responses of behavior activated neurons aligned to onset of attack bouts, dominance bouts and exploration bouts during interaction with familiarized (day 5) and novel (day 6) were calculated from neurons identified as response selective (activated or inhibited) by the approach in section 'cell response'. We identified neuron trials with elevated baseline activity using Median Absolute Deviation (MAD) filtering. A strict 5.0 × MAD threshold was used to exclude trials where the mean pre-event activity (-3s–0s) exceeded 5.0 × MAD above the median baseline.

Neural data correlation across behavior days

Neurons were tracked across the last 3 sequential days (4, 5, and 6) of the resident intruder test for direct comparisons (see section on longitudinal registration). The activity of each neuron ($n = 311$) was averaged over a 1-s interval, starting 500 milliseconds before and ending 500 milliseconds after the onset of each behavior. Spearman rank correlation (r_s) was used to assess significance across each pair of behaviors and corrected for multiple comparisons using the Bonferroni method. Shuffling approach: For each behavior across days, cell IDs were independently randomized to disrupt any inherent association between the day variable, while preserving the overall structure of the dataset. Following the shuffling procedure, correlation coefficients were calculated for each pair of days within each behavior category, mirroring the analysis conducted on the unshuffled data. This step was repeated for 1000 iterations, creating a null distribution of correlation coefficients for each behavioral observation across days. The significance of the observed correlations was assessed by comparing them against the null distribution obtained from the shuffling process. A correlation value in the observed data was considered significantly different from chance if it fell outside the central 95% of the null distribution, corresponding to a p-value less than 0.05.

Data labeling for decoding

For each subject (resident) and session (day), we selected neural data corresponding to periods in which the subject was actively interacting with the intruder in periods defined as non-social behavior, exploration, dominance, or attack (operational definitions in Table S1). This resulted in 3 social behavior dichotomies [exploration vs. dominance, exploration vs. attack, and dominance vs.

attack] and 3 non-social behavior dichotomies. We then divided the conditions into binary dichotomies (class 0 and class 1) according to the variable we wished to decode.

Cross-validation and pseudo-simultaneous population activity

Briefly, we divided data from each class of conditions (0 and 1) into training and test pseudo-trials, which each trial defined by a bout of interaction, with bout duration lasting from the beginning to end of a given interaction. Bout durations lasting longer than 500 ms were split into multiple 500-ms-long pseudo-trials. To avoid temporal artifacts, present in consecutive time bins through autocorrelation of calcium signals, contiguous trials were discarded prior to train/test splitting. We randomly selected 75% of pseudo-trials for training a classifier and the remaining 25% were used for testing decoding performance. The training dataset was then used to train an SVM linear classifier, which was tested on the testing dataset to assess the decoding performance. The whole procedure, from training-testing division to performance assessment, was repeated for $k = 10$ times to implement a k-fold cross-validation scheme, taking the mean score (μ) as the estimated performance value of the decoding procedure. Decoding performances obtained by the cross-validated procedure were tested against a null model where the labels (0 and 1 as defined above) of pseudo-trials were randomly shuffled. After each shuffling, the same cross-validation procedure was repeated, obtaining a null-model value for decoding performance. We repeated the shuffling n times to obtain a distribution of null model performance values, yielding a mean null decoding performance ($<\mu>$) and standard deviation of the null distribution (σ). The p value was then derived from the Z score of the performance computed on data compared to the distribution of n null-model values: $z = [\mu - <\mu>]/\sigma$.

Cross-day decoding performance

Cross-day decoding for behavioral dichotomies was performed as previously described. A separate linear SVM classifier was trained/tested on each behavior dichotomy (exploration versus dominance versus attack) for identity. Identity was chosen in pairs (familiarized-familiarized vs. familiarized-novel). Cross validation and decoding performance were determined as defined in the ‘cross-validation and pseudo-simultaneous population activity’ section.