

Valence and Salience Encoding in the Central Amygdala

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This **useful** work reveals differential activity to food and shock outcomes in central amygdala GABAergic neurons. **Solid** evidence supports claims of unconditioned stimulus activity that changes with learning. However, the evidence regarding claims related to valence or salience signaling in these neurons is **inadequate**. This work will be of interest to neuroscientists studying sensory processing and learning in the amygdala.

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Abstract

The central amygdala (CeA) has emerged as an important brain region for regulating both negative (fear and anxiety) and positive (reward) affective behaviors. The CeA has been proposed to encode affective information in the form of valence (whether the stimulus is good or bad) or salience (how significant is the stimulus), but the extent to which these two types of stimulus representation occur in the CeA is not known. Here, we used single cell calcium imaging in mice during appetitive and aversive conditioning and found that majority of CeA neurons (~65%) encode the valence of the unconditioned stimulus (US) with a smaller subset of cells (~15%) encoding the salience of the US. Valence and salience encoding of the conditioned stimulus (CS) was also observed, albeit to a lesser extent. These findings show that the CeA is a site of convergence for encoding oppositely valenced US information.

Introduction

The CeA is comprised of multiple genetically distinct cell types that contribute to both appetitive and aversive processes (Cai et al., 2014 ; Douglass et al., 2017 ; Fadok et al., 2018 ; Hardaway et al., 2019 ; Haubensak et al., 2010 ; Isosaka et al., 2015 ; Kim et al., 2017 ; Li et al., 2013 ; McCullough et al., 2018 ; Warlow et al., 2020 ; Yu et al., 2017 .

Subsets of cells in the CeA encode conditioned and/or unconditioned fear stimulus information (Cicocchi et al., 2010 [↗](#); Duvarci et al., 2011 [↗](#); Fadok et al., 2017 [↗](#); Li et al., 2013 [↗](#); Sanford et al., 2017 [↗](#); Yang et al., 2023 [↗](#); Yu et al., 2017 [↗](#)), as well as appetitive information (Douglass et al., 2017 [↗](#); Hardaway et al., 2019 [↗](#); Yang et al., 2023 [↗](#)). Plasticity within neurons of the CeA (Fu & Shinnick-Gallagher, 2005 [↗](#)) contributes to both fear-related learning (Li et al., 2013 [↗](#); Penzo et al., 2014 [↗](#); Sanford et al., 2017 [↗](#)) and reward-associated learning (Yang et al., 2023 [↗](#)). Cells implicated in fear processing have also been shown to regulate consummatory behaviors (Cai et al., 2014 [↗](#); Douglass et al., 2017 [↗](#); Yang et al., 2023 [↗](#); Yu et al., 2017 [↗](#)), suggesting that subsets of CeA neurons encode either incentive salience (the significance of the stimulus regardless of valence) or valence (whether it is a positive or negative outcome).

Specific subsets of neurons within the CeA have been shown to contribute to multiple aspects of appetitive and aversive behaviors (Balleine & Killcross, 2006 [↗](#); Kong & Zweifel, 2021 [↗](#); Pignatelli & Beyeler, 2019 [↗](#)) and it was recently shown that subpopulations of CeA neurons provide scalar signals to oppositely valenced stimuli to promote learning (Yang et al., 2023 [↗](#)). However, the full extent to which the CeA encodes the salience or valence of positive and negative affective information remains unresolved.

To address the extent of valence and salience encoding within the CeA, we used single cell calcium imaging to longitudinally track neurons during Pavlovian reward and Pavlovian fear conditioning. We found that the majority of CeA neurons are responsive to either the reward or the fear US. Although a subset of cells displays salience-like encoding of the US, most CeA neurons were selectively responsive to the valence of the US. We observed six distinct types of valence encoding that was either selective for one or the other USs or oppositely encoded US information. Salience and valence encoding was also observed in response to a reward- or fear-associated CS, but the magnitude of these responses and the number of cells responding to the CSs were considerably smaller than the responses observed to the USs. These data indicate that the CeA encodes multiple aspects of positive and negative reinforcing stimuli, but the prevailing responses relate to the encoding of the valence of the US.

Results

To monitor calcium dynamics in CeA neurons during positive and negative reinforcement, we selectively expressed GCaMP6m in GABAergic neurons of Vgat-Cre mice (4 males and 6 females) and implanted a gradient-index (GRIN) lens in the CeA (**Figure 1A** [↗](#) and Figure 1—figure supplement 1). Mice were conditioned in both Pavlovian appetitive and Pavlovian fear paradigms, with the conditioning order counterbalanced (**Figure 1B** [↗](#)). During appetitive learning, mice demonstrated a significant increase in time spent near the food hopper during the food-predicting CS (CS^{Food}), reduced latency to retrieve the food pellet, and an increased success rate in procuring the reward (**Figure 1C** [↗](#)). During fear conditioning, the animals learned that the shock-predicting CS (CS^{Shock}) was followed by an impending foot shock and displayed increased freezing behavior to the CS^{Shock} as trials progressed (**Figure 1D** [↗](#)).

During conditioning, we observed responsive and non-responsive cells to both the appetitive and aversive US (**Figure 2A** [↗](#)). To resolve event-related calcium signals, we developed a variation of the ‘circular shift’ method²⁷ to stringently define cells with statistically significant responses (see Methods for detailed description). In short, we used circular shifting to generate artificial “null” calcium traces resembling a sample from a hypothetical population of neuron traces unrelated to the exact time of behavior events (Step 1, **Figure 2B** [↗](#)). Wilcoxon rank sum tests (comparing pre-stimulus vs. post-stimulus activity) were performed on recorded traces and “null” traces and summed across trials (Step 2 and Step 3, **Figure 2C,D** [↗](#)) to create a single test statistic for each

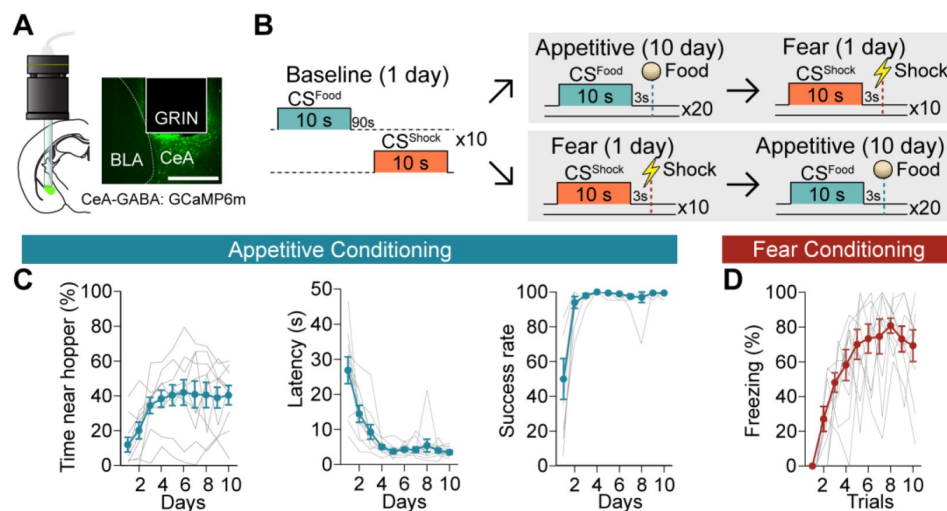


Figure 1

Acquisition of calcium signals during Pavlovian appetitive and fear conditioning.

A, CeA-GABAergic neurons were labelled with GCaMP6m, and their activity was recorded using a miniature microscope (Inscopix) via an implanted GRIN lens (left). A representative image of GCaMP6m expression and lens placement is shown on the right. Scale bar, 0.5 mm. **B**, Behavioral paradigm of the baseline, appetitive, and fear conditioning. To counterbalance the order of the two valence conditioning paradigms, there were two groups: appetitive → fear (5 mice) and fear → appetitive (5 mice) after the baseline session. **C**, Time spent near the food hopper during CS^{Food} (left, day: $F = 7.422$, $P = 0.0005$), latency to procure a pellet (middle, day: $F = 20.41$, $P < 0.0001$), and success rate (right, day: $F = 14.69$, $P = 0.0017$) over 10 days of appetitive learning (mean $\pm$ s.e.m., grey lines represent individual data, $n = 10$). **D**, Freezing behavior during CS^{Shock} (trial: $F = 13.49$, $P < 0.0001$) throughout 10 trials of fear conditioning (mean $\pm$ s.e.m., grey lines represent individual data, $n = 10$). Detailed information about statistical results is provided in Supplementary Table 1.

neuron. P-values were obtained by comparing the summed Wilcoxon rank sum test statistics from actual data against the summed Wilcoxon rank sum test statistics from the artificially generated null distribution (Step 4, **Figure 2E** [↗](#)).

During Pavlovian appetitive conditioning, alignment to the cue presentation revealed no responsive cells to the CS^{Food} or food delivery on day 1 (**Figure 3A** [↗](#), top). However, on day 10, numerous cells were identified that were responsive to food delivery with either increases or decreases in calcium signals (**Figure 3A** [↗](#), bottom).

Analysis of responses aligned to food retrieval (head entry) revealed responsive cells on both day 1 and day 10 (**Figure 3B** [↗](#)). The number of cells showing increased calcium to head entry and the magnitude of the increase was larger on day 10 compared to day 1 (**Figure 3B-D** [↗](#)). Of the 872 cells recorded on day 10 of conditioning, a small proportion of cells were responsive to CS^{Food} presentation, showing similar proportions of cells with increased or decreased calcium signals (**Figure 3E,F** [↗](#)). Of these CS^{Food} responsive cells, the majority were responsive to both the CS and the food delivery (Supplementary Data Figure 2A,B). In contrast to the CS^{Food} responsive cells, a large number (64%) of CeA neurons were responsive to food delivery, with the largest proportion of cells showing excitatory responses (40%) compared to inhibited responses (24%) (**Figure 3G,H** [↗](#)).

Additional analysis of CeA neuron responses on day 10 of Pavlovian appetitive conditioning revealed that there was a trend toward decreased responsiveness to the CS^{Food} late in conditioning compared to early in the excited neurons and a significant increase in the inhibitory response in late conditioning compared to early (Figure 3—figure supplement 1C,D). In response to food delivery, no differences were observed in food-excited neurons; however, a small but significant difference was observed in food-inhibited cells with an increase in the magnitude of the inhibited response late in conditioning compared to early (Figure 3—figure supplement 1E,F).

During Pavlovian fear conditioning, alignment of calcium signals to the CS^{Shock} revealed that the greatest responses were to the US presentation (**Figure 4A** [↗](#)). Of the 519 cells recorded, a small percentage were either excited or inhibited by the CS^{Shock} (**Figure 4B,C** [↗](#)). A large number of cells (42%) were responsive to the shock US with the largest proportion showing excitations (30%) compared to inhibitions (12%) (**Figure 4D,E** [↗](#)). Of the CS^{Shock} responsive cells, an approximately equal number were responsive to the CS^{Shock} only or the CS^{Shock} and US^{Shock} (Figure 4—figure supplement 1A,B). In contrast to the CS^{Food} responsive cells during day 10 of Pavlovian appetitive conditioning, we did not observe any changes in the CS^{Shock} responsive cells during early compared to late conditioning (Figure 4—figure supplement 1C,D). However, shock-excited CeA neurons showed a significant attenuation of the excited response in late conditioning compared to early conditioning (Figure 4—figure supplement 1E). No changes were observed in shock-inhibited cells (Figure 4—figure supplement 1F).

Qualitatively, food-excited and shock-excited neurons exhibited distinctive properties. Food-excited neurons showed more sustained activity in response to food, and their time to peak activity occurred significantly later than that of shock-excited neurons, likely reflecting the longer time needed to consume food compared to the passive delivery of the shock (Figure 5—figure supplement 1A). Additionally, the maximum Z-score of food-excited neurons was larger than that of shock-excited neurons, indicating that food was more salient for the CeA (Figure 5—figure supplement 1B).

To categorize cells as salience or valence encoding, we identified 303 neurons that appeared during both appetitive and fear conditioning (**Figure 5A** [↗](#)). Salience-encoding neurons (see Methods for detailed definition) were defined as those showing both excited or inhibited responses to food and shock, and comprised 15% of the total neurons (**Figure 5B,C** [↗](#)). In contrast, valence-encoding neurons were defined as those displaying differential activity to food and shock

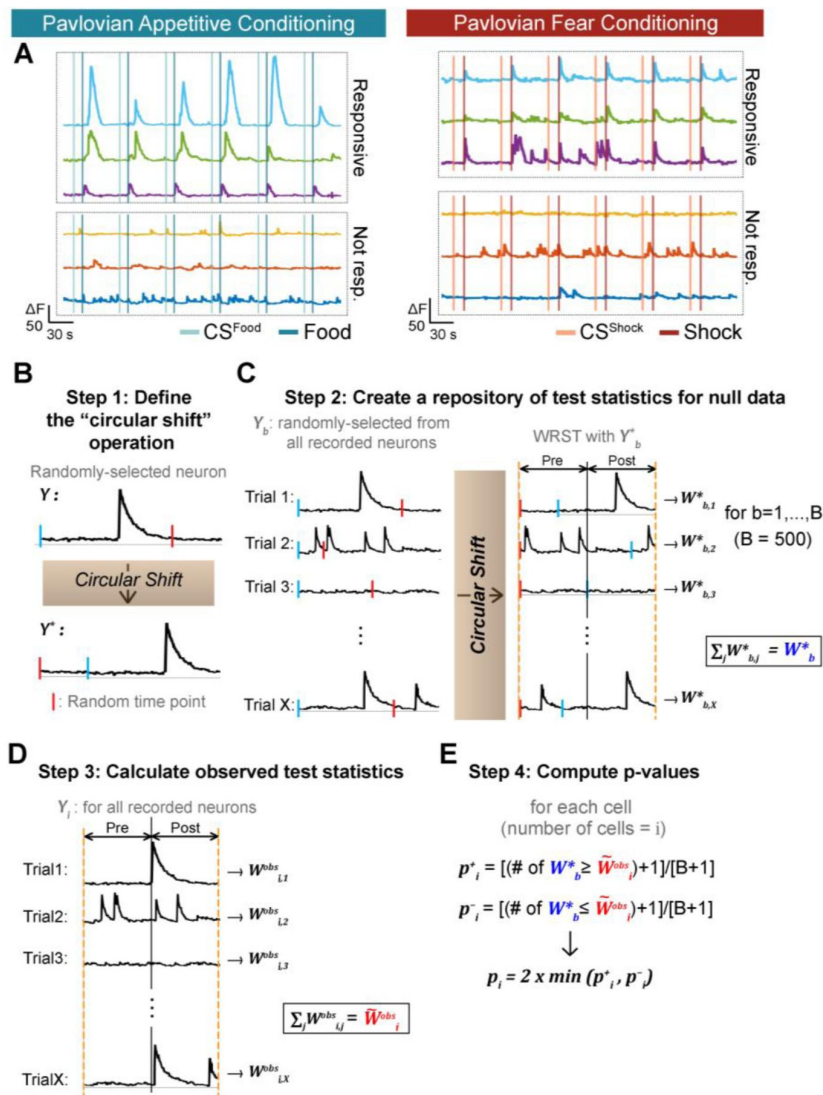


Figure 2

Determination of CeA responses to appetitive and fearful stimuli.

A. Left: Example traces of CeA neurons that were responsive (upper three traces) and not responsive to food reward (bottom three traces). Right: Example traces of CeA neurons that were responsive (upper three traces) and not responsive to foot shock (bottom three traces). **B.** Step 1: Defining the "circular shift" operation. A neuron is randomly selected from the recorded neuron pool in our current study (denoted as Y). The calcium trace of the selected neuron is subjected to a circular shift operation, wherein it is moved from a randomly chosen time point (red bar). This operation results in a new calcium trace, as shown by the movement of the blue bar in **Figure 2B**; the traces that initially start with blue bars become red bar starting traces. **C.** Step 2: Creating a repository of test statistics for null data. A neuron is selected at random; then, the activity in each of its trials (20 trials for appetitive or 10 trials for fear conditioning, X = the number of trials) undergoes 'circular shifting', where a random point (red bar) is chosen within the circular shifting range. Then, on each trial, the Wilcoxon rank sum test between pre-stimulus vs. post-stimulus periods is computed. The final test statistic W_b^* representing a summary of all trials is obtained by summing the individual Wilcoxon rank sum test statistics over the trials with $W_b^* = \sum_j W_{b,j}^*$. Step 2 is repeated B times (we used $B = 500$), generating a null distribution of summed statistics $\{W_b^*\}_{b=1}^B$. **D.** Step 3: calculating observed test statistics. Each observed neuron receives a summary test statistic $\bar{W}_i^{obs} = \sum_j W_{i,j}^{obs}$ obtained by summing the Wilcoxon rank sum test statistics from the individual trials. **E.** Step 4: Computing p-values. A p-value for the i^{th} neuron comes from a comparison of $\bar{W}_i^{obs}$ with $\{W_b^*\}_{b=1}^B$. For the i^{th} neuron, p_i^+ (the number of $W_b^* \geq \bar{W}_i^{obs} + 1$ divided by $B + 1$) and p_i^- (the number of $W_b^* \leq \bar{W}_i^{obs} + 1$ divided by $B + 1$) are calculated. The final p-value for the i^{th} neuron (denoted as p_i) is 2 times the lesser of p_i^+ and p_i^- . A detailed description of this statistical analysis is provided in Methods.

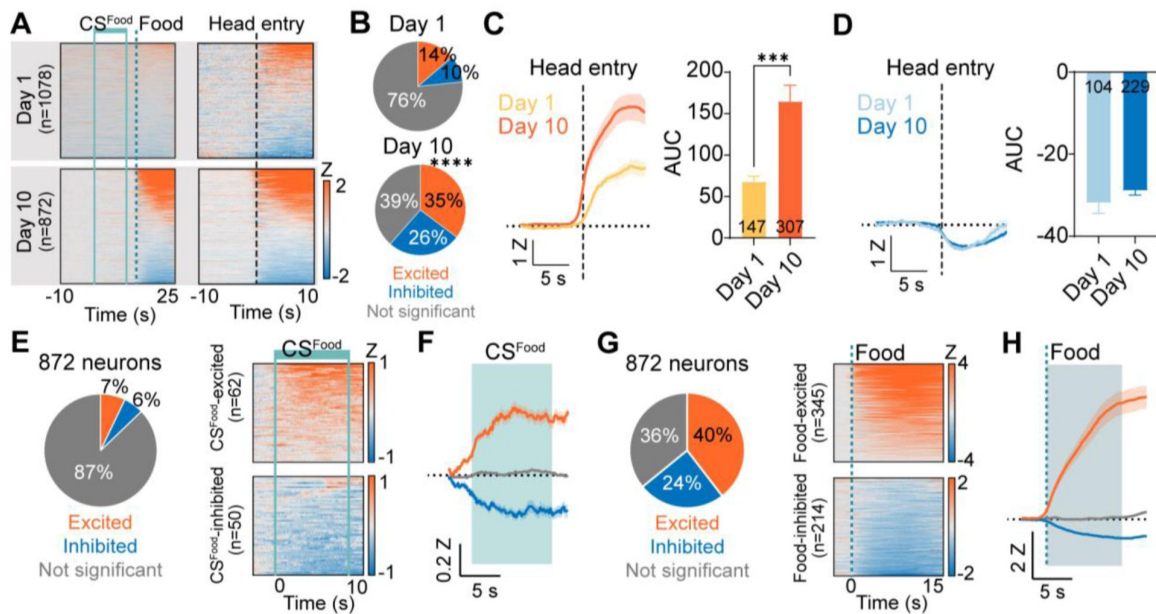


Figure 3

Responses of CeA neurons during Pavlovian appetitive conditioning.

A, Top: Heat maps of CeA neurons aligned to CS^{Food} onset (top left, 1078 neurons) and head entry (top right) from day 1 of Pavlovian appetitive conditioning. Bottom: Heat maps of CeA neurons aligned to CS^{Food} onset (bottom left, 872 neurons) and head entry (bottom right) from day 10 of Pavlovian appetitive conditioning. Solid mint lines indicate CS^{Food}, and a dotted darker mint line represents food delivery. Black dotted line represents the first head entry after food delivery. **B**, Proportion of head entry responsive neurons between day 1 (top) and day 10 (bottom). **C**, Left: The average Z-scored activity of head entry-excited neurons on day 1 (147 neurons) and day 10 (307 neurons) of appetitive conditioning during -10 s to 10 s after the first head entry of each reward delivery. The dark lines and shaded areas indicate the mean and standard error of the mean (s.e.m.). Right: Average area under the curve (AUC) for the Z-scored activity after head entry on day 1 compared to day 10 of appetitive conditioning (0-5 s after head entry, mean ± s.e.m.). **D**, Left: The average Z-scored activity of head entry-inhibited neurons on day 1 (104 neurons) and day 10 (229 neurons) of appetitive conditioning during -10 s to 10 s after the first head entry of each reward delivery. Right: Average AUC for the Z-scored activity after head entry on day 1 compared to day 10 of appetitive conditioning (0-5 s after head entry). **E**, Left: Proportion of CS^{Food}-excited (orange), CS^{Food}-inhibited (blue), and not significant neurons (grey) on day 10 of appetitive learning (total 872 neurons). Right: Heat maps of CS^{Food}-excited neurons (top, n = 62) and CS^{Food}-inhibited neurons (bottom, n = 50) from all trials (20 trials). Solid mint lines indicate CS^{Food} onset and offset. **F**, The average Z-scored activity of each response type (orange, CS^{Food}-excited; blue, CS^{Food}-inhibited; grey, not significant). The dark lines and shaded areas represent the mean and s.e.m. The mint area represents 10 s of CS^{Food}. **G**, Left: Proportion of food-excited (orange), food-inhibited (blue), and not significant neurons (grey) on day 10 of appetitive learning (total 872 neurons). Right: Heat maps of food-excited neurons (top, n = 345) and food-inhibited neurons (bottom, n = 214) from all trials (20 trials). The dotted line indicates a food delivery. **H**, The average Z-scored activity of each response type (orange, food-excited; blue, food-inhibited; grey, not significant). The dark lines and shaded areas represent the mean and s.e.m. ***P < 0.001, ****P < 0.0001. Detailed information about statistical results is provided in Supplementary Table 1.

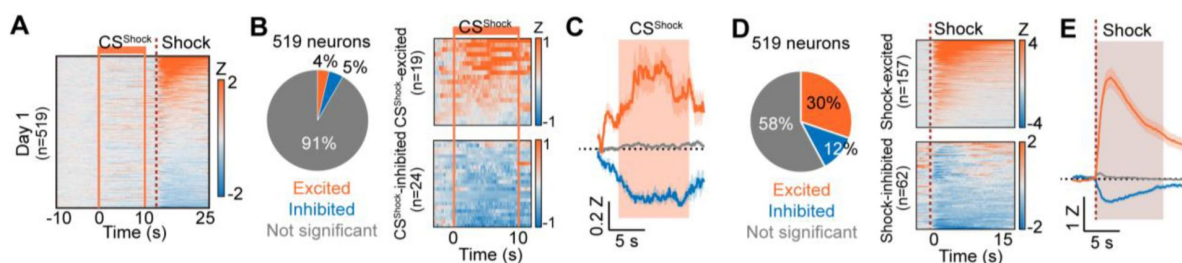


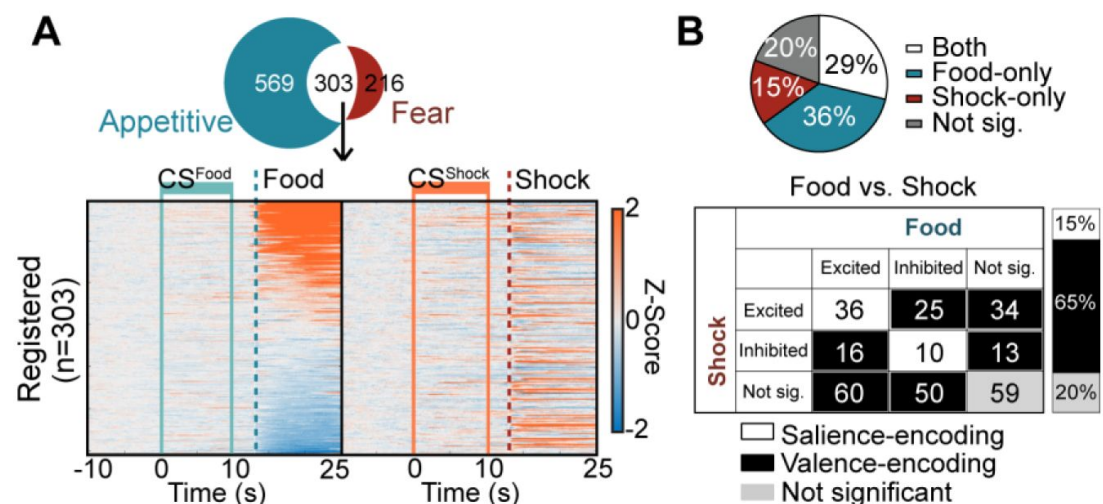
Figure 4

Responses of CeA neurons during Pavlovian fear conditioning.

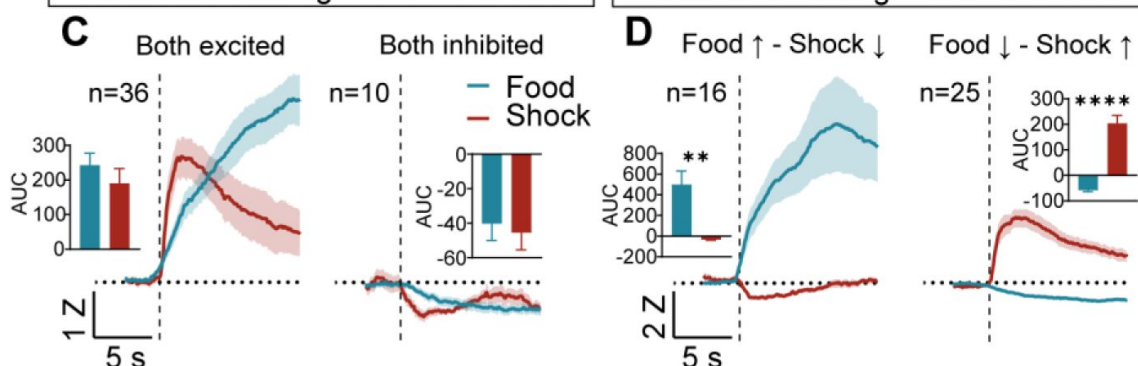
A, Heat maps of CeA neurons aligned to CS^{Shock} onset (519 neurons) from day 1 of Pavlovian fear conditioning. Solid orange lines indicate CS^{Shock} , and a dotted red line represents shock delivery. **B**, Left: Proportion of CS^{Shock} -excited (orange), CS^{Shock} -inhibited (blue), and not significant neurons (grey) on day 1 of fear conditioning (total 519 neurons). Right: Heat maps of CS^{Shock} -excited neurons (top, $n = 19$) and CS^{Shock} -inhibited neurons (bottom, $n = 24$) from all trials (10 trials). Solid orange lines indicate CS^{Shock} onset and offset. **C**, The average Z-scored activity of each response type (orange, CS^{Shock} -excited; blue, CS^{Shock} -inhibited; grey, not significant). The dark lines and shaded areas represent the mean and s.e.m. The orange area represents 10 s of CS^{Shock} . **D**, Left: Proportion of shock-excited (orange), shock-inhibited (blue), and not significant neurons (grey) on day 1 of fear conditioning (total 519 neurons). Right: Averaged heat maps of shock-excited neurons (top, $n = 157$) and shock-inhibited neurons (bottom, $n = 62$) from all trials (10 trials). A dotted red line represents shock delivery. **E**, The average Z-scored activity of each response type (orange, shock-excited; blue, shock-inhibited; grey, not significant). The dark lines and shaded areas represent the mean and s.e.m.

and represented 65% of the neurons (**Figure 5B-E** [↗](#)). Of these, 13.5% showed opposite responses to food or shock while the remaining 51% showed selective responses to one or the other USs (**Figure 5D,E** [↗](#)). The remaining 20% were categorized as non-responsive. These findings demonstrate that CeA neurons encode either the valence or the salience of the US, with the valence encoding being more prevalent.

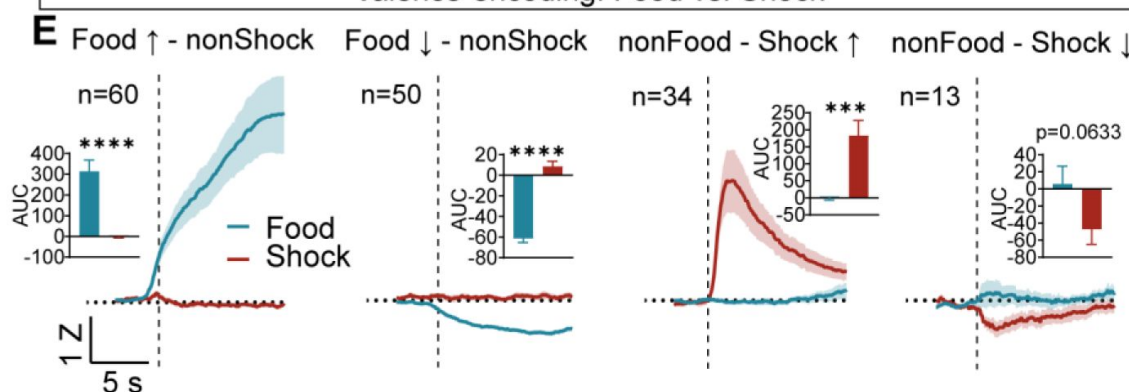
Appetitive vs. Fear conditioning: Food vs. Shock



Salience-encoding: Food vs. Shock



Valence-encoding: Food vs. Shock



Appetitive vs. Fear conditioning: CS^{Food} vs. CS^{Shock}



Figure 5

Salience and valence encoding in the CeA.

A, Among 872 neurons from appetitive conditioning and 519 neurons from fear conditioning, 303 neurons were registered during both learning. Heat maps of these 303 neurons during -10 s to 25 s after CS^{Food} onset from appetitive (left) and during -10 s to 25 s after CS^{Shock} onset from fear conditioning (right). All neurons are aligned to their activity to food. The same neuron is represented in the same row. Solid mint lines indicate CS^{Food} , and a dotted darker mint line represents food delivery, solid orange lines indicate CS^{Shock} , and a dotted red line represents shock delivery. **B**, Top: the proportion of significant neurons to food and shock (both; white), food-only (blue), shock-only (red), and not significant to both (grey). Bottom: detailed response types of all possible categories (excited, inhibited, not significant) for food vs. shock. White represents salience-encoding neurons showing the same response types to food and shock ($n = 46$, 15%), and black represents valence-encoding neurons showing different response types to food and shock ($n = 198$, 65%). Grey represents not significant neurons ($n = 59$, 20%). **C**, Salience-encoding neurons: the average Z-scored activity of neurons that were excited to both food and shock ($n = 36$, left) and inhibited to both food and shock ($n = 10$, right). Inserted bar graphs indicate average AUC of the Z-scored activity after food and shock delivery (0-10 s, mean $\pm$ s.e.m.). **D**, Valence-encoding neurons: the average Z-scored activity of neurons that were excited to food but inhibited to shock ($n = 16$, left) and inhibited to food but excited to shock ($n = 25$, right). Average AUC of the Z-scored activity after food and shock delivery are shown in inserted bar graphs (0-10 s, mean $\pm$ s.e.m.). **E**, The average Z-scored activity of neurons that were excited to food but not responsive to shock ($n = 60$, first), neurons that were inhibited to food but not responsive to shock ($n = 50$, second), neurons that were not responsive to food but excited to shock ($n = 34$, third), and neurons that were not responsive to food but inhibited to shock ($n = 13$, fourth). Inserted bar graphs indicate average AUC of the Z-scored activity after food and shock delivery (0-10 s, mean $\pm$ s.e.m.). The dark lines and shaded areas represent the mean and s.e.m. **F**, Proportion of significant neurons to CS^{Food} and CS^{Shock} (both; white), CS^{Food} -only (mint), CS^{Shock} -only (orange), and not significant to both (grey). **G**, Detailed response type of all possible categories (excited, inhibited, not significant) for CS^{Food} vs. CS^{Shock} . White represents salience-encoding neurons showing the same response types to CS^{Food} and CS^{Shock} ($n = 7$, 2%), and black represents valence-encoding neurons showing different response types to CS^{Food} and CS^{Shock} ($n = 57$, 19%). Grey represents not significant neurons ($n = 239$, 79%). $*P < 0.05$, $***P < 0.0001$. Detailed information about statistical results is provided in Supplementary Table 1.

To categorize salience- and valence-encoding neurons based on their responses to CS^{Food} and CS^{Shock} , we analyzed the 303 registered neurons recorded during both reward and fear conditioning sessions (**Figure 5F,G** and Figure 5—figure supplement 1C,D). Only 2% of neurons were classified as salience-encoding, while 19% were identified as valence-encoding neurons. Although representing a significantly smaller proportion of CS encoding neurons compared to the US encoding neurons (CS encoding neurons: salience = 7, valence = 57, not significant = 239; US encoding neurons: salience = 46, valence = 198, not significant = 59; $X^2 = 215.4$, $P < 0.0001$, $df = 2$), these findings demonstrate that encoding of the valence of the CS is also the predominant response type of the CeA neurons.

It is possible that the low number of cells responsive to the CS^{Shock} during conditioning reflects the identification of these cells during acquisition as opposed to fear memory retrieval. To address this, we analyzed CeA neuron responses to the CS^{Food} and CS^{Shock} twenty-four hours following fear conditioning (post-test) and compared these responses to a baseline pre-conditioning session. The number of responsive cells during the post-test was less than those observed during conditioning and did not differ from the proportion observed prior to conditioning (Figure 5—figure supplement 1E-J). Another possible reason for the fewer than expected CS^{Food} and CS^{Shock} responsive cells is the analysis we used to classify these neurons. To address this, we used a more conventional Wilcoxon sign rank test on each trial relative to the pre-CS period combined with distribution cutoff (Figure 5—figure supplement 2A-C).

The number of CeA neurons excited or inhibited by the food US or shock US was similar to what was identified using the modified wrapping method (Figure 5—figure supplement 2D-G). Likewise, the number of cells responsive to the CS^{Food} was similar (Figure 5—figure supplement 2J,K). CeA neurons excited or inhibited by the CS^{Shock} were more prevalent using the sign rank method compared to the wrapping method (10% excited and 13% inhibited vs. 4% excited and 5% inhibited) (Figure 5—figure supplement 2L,M). These marginal differences indicate that the classification method is not a major contributing factor to the observed results and confirm that CeA neurons as a whole are more responsive to the US than to the CS.

We next asked whether the order of valence conditioning (appetitive → fear or fear → appetitive) had any effect on the encoding of subsequent valence conditioning. Among the 303 registered neurons, 165 neurons belonged to the appetitive → fear group (Figure 5—figure supplement 3A), and 138 neurons were from the fear → appetitive group (Figure 5—figure supplement 3E). Our analysis revealed the following key findings: i) CeA neurons were more responsive to food compared to shock regardless of the conditioning order (Figure 5—figure supplement 3B,F), ii) the ratios of salience-, valence-encoding, and not significant neurons between the two groups were not significantly different (appetitive → fear group US: salience = 22, valence = 115, not significant = 28 vs. fear → appetitive group US: salience = 24, valence = 83, not significant = 31, $X^2 = 3.029$, $P = 0.2199$, $df = 2$, Figure 5—figure supplement 3B,F; appetitive → fear group CS: salience = 2, valence = 33, not significant = 109 vs. fear → appetitive group CS: salience = 4, valence = 35, not significant = 120, $X^2 = 0.5126$, $P = 0.7739$, $df = 2$, Figure 5—figure supplement 3C,G), and iii) the conditioning order did not preferentially recruit CeA neurons based on their topographical locations (Figure 5—figure supplement 3D,H), indicating that neurons encoding salience or valence were intermingled.

Finally, we investigated whether the responses to the USs or CSs were qualitatively biased by the initial valence conditioning experience. We found that the response to food before shock (appetitive → fear) was not significantly different from the response to food after the shock experience (fear → appetitive, Figure 5—figure supplement 3I). Similar results were observed for shock, CS^{Food}, and CS^{Shock} responses (Figure 5—figure supplement 3J-L), suggesting that the encoding of positive or negative stimuli in CeA neurons is not influenced by the previous experience with opposing stimuli.

Discussion

The CeA is a principally GABAergic structure, allowing us to image the bulk of CeA neurons through conditional expression of GCaMP in Vgat-Cre mice. By imaging a total of 10 mice, we were able to achieve lens placements that largely covered the extent of the dorsal-ventral axis of the CeA. Interestingly, although the CeA is demarcated by functionally distinct subdivisions and genetically distinct cell types, we found that cells responsive to reward and/or fear were intermingled throughout the structure.

The CeA has been proposed to encode both salience and valence (Fadok et al., 2018; Kong & Zweifel, 2021; Pignatelli & Beyeler, 2019), which is supported by studies performing bulk or single cell imaging or recording in the CeA during appetitive or aversive behaviors (Ciocchi et al., 2010; Douglass et al., 2017; Duvarci et al., 2011; Fadok et al., 2017; Hardaway et al., 2019; Li et al., 2013; Sanford et al., 2017; Yang et al., 2023; Yu et al., 2017). The definition of salience or valence encoding is often poorly defined, and the terms are frequently used interchangeably. Moreover, with the exception of a recent study (Yang et al., 2023), analysis of specific cell types has not included both an appetitive and aversive stimulus to effectively resolve at the single cell level salience versus valence encoding. For salience encoding, we defined these cells as responding in the same direction to both the appetitive or aversive stimulus as described previously (Gao et al., 2020; Lin & Nicolelis, 2008; Steinberg et al., 2020; Zhu et al., 2018). Valence encoding cells were defined as cells responding in either the

opposite direction to positive or negative stimuli, or selectively responding to one or the other (Douglass et al., 2017 [DOI](#); Isosaka et al., 2015 [DOI](#); Yu et al., 2017 [DOI](#)). Although we observed numerous examples of distinct types of salience and valence encoding cells, the majority of neurons within the CeA were similar to what we previously defined as Type 1 valence neurons (Kong & Zweifel, 2021 [DOI](#)), i.e., they respond selectively to either the positive or the negative valence of the US.

Consistent with observations reported for learning-dependent changes in somatostatin expressing neurons of the CeA (Yang et al., 2023 [DOI](#)), we observed increased responsiveness in excited neurons and the number of excited neurons of the CeA to the food US on day 10 compared to day 1 of conditioning. Within the last session of Pavlovian appetitive conditioning, we observed enhanced responses in cells inhibited by the food US and CS^{Food} and diminished excitatory response to the CS^{Food}. These results may reflect satiety within the session. Indeed, neurons within the CeA have been shown to play important roles in the regulation of feeding cessation (Cai et al., 2014 [DOI](#)). We did not record calcium dynamics in CeA neurons across multiple days of fear conditioning; however, we did observe a within session decrease in the amplitude of fear US excited cells consistent with a desensitization of these neurons (Yu et al., 2017 [DOI](#)).

Numerous studies have identified CeA neurons that respond to the CS following conditioning (Cicchi et al., 2010 [DOI](#); Duvarci et al., 2011 [DOI](#); Fadok et al., 2017 [DOI](#); Li et al., 2013 [DOI](#); Sanford et al., 2017 [DOI](#); Yu et al., 2017 [DOI](#)). We also observed cells that responded to the CS; however, it was evident that these responses occurred in a much smaller number of cells compared to the US responding and with a smaller amplitude. This is consistent with what has been reported previously for specific cell types within the CeA (Yang et al., 2023 [DOI](#); Yu et al., 2017 [DOI](#)) and does not discount the previously defined importance of plasticity within these cells for fear-related learning (Li et al., 2013 [DOI](#); Penzo et al., 2014 [DOI](#); Sanford et al., 2017 [DOI](#)). Our findings do suggest however that the CeA as a whole is largely tuned to the valence of the US with approximately equal encoding of both positive and negative valences.

Methods

Animals

Male and female Vgat^{IRE5-Cre} (Slc32a1^{tm2(cre)Low}/J; JAX strain #: 016962) mice were group-housed on a 12h-light/12h-dark cycle (lights on 7 AM) with *ad libitum* access to food and water until surgery and behavioral experiment. All experiments were conducted during the light cycle and strictly followed the guidelines set by the University of Washington Animal Care and Use Committee, ensuring ethical treatment of the animals.

Surgery and Virus

Under isoflurane-induced anesthesia, 12-16 week old Vgat^{IRE5-Cre} mice at the time of surgery were placed in a stereotaxic instrument (Kopf) and injected with 0.5 μ l of AAV1-FLEX-GCaMP6m (produced in-house with a titer of $\sim 3 \times 10^{12}$ particles/ml (Gore et al., 2013 [DOI](#))). The injection was made in the right CeA (AP: -1.2 mm, ML: 2.9 mm, DV: -4.6 mm) at a rate of 0.25 μ l/min. Following virus injection, mice were implanted with a gradient-index lens (GRIN lens with baseplates attached, 0.6mm diameter, 7.3 length; Inscopix) at the virus injection site. The GRIN lens was fixed with black dental cement (Lang Dental) with anchoring screws (Antrin Miniature Specialties). Four weeks after the surgery, animals were placed on a standard food restriction schedule with free access to water until they reached $\sim 85\%$ of pre-operation weights. Behavioral experiments began 5 weeks after surgery.

Behavioral paradigms

Throughout the experimental sessions (baseline, appetitive Pavlovian conditioning, Pavlovian fear conditioning, post-learning test; **Figure 1b** [↗](#)), the mice maintained their body weights at ~ 85% of their pre-operation weights. All sessions were conducted in the same operant chamber (21.6 X 17.8 X 12.7 cm) equipped with a house light, a speaker, a metal bar for fear Pavlovian conditioning, and a food dispenser for appetitive Pavlovian conditioning (MedAssociates). The operant chamber was placed in a sound-attenuating box, and each session had distinct contextual cues based on its type. Random assignment of mice was done to either the appetitive → fear or fear → appetitive group, indicating the order of valence conditioning.

Baseline

Prior to any conditioning, we assessed the basal freezing behavior and calcium activity in response to CS^{Food} and CS^{Shock} were measured. Two CSs with different frequencies of CSs (either 4 kHz or 12 kHz, 10 s each; counter-balanced) were delivered 10 times, alternating with a 90 s inter-trial interval (ITI). The contextual cues for the baseline session consisted of white walls and floor.

Pavlovian appetitive Conditioning

During this session, CS^{Food} was paired with a pellet (20 mg sucrose pellet, Bioserv) for 20 trials per day, conducted over a period of 10 days. A 3-s delay was introduced between CS^{Food} and the presentation of the food pellet to distinguish the offset of CS^{Food} from the onset of food delivery. The ITI was set to 90 s. The floor of the chamber was covered with a white plastic panel, while the walls were kept transparent. The chamber was cleaned with a chlorine dioxide-based sterilant (Clidox-S) between each animal.

Pavlovian fear conditioning

In this session, CS^{Shock} was paired with a foot shock (0.5 mA) for 10 trials, conducted for 1 day. Similar to the appetitive conditioning, a 3-s delay was introduced between CS^{Shock} and the delivery of the foot shock to distinguish the offset of CS^{Shock} from the onset of shock. The contextual cues during this session included white walls, a grid floor for shock delivery, and a 1% acetic acid odor cue. The chamber was cleaned with 70% ethanol between each animal.

Pre-processing calcium imaging data

One week prior to the start of the experiment, imaging parameters such as focal planes and LED power were calibrated by screening calcium activity from CGaMP6m-expressing CeA GABAergic neurons. The screening session involved checking calcium signals using nVoke miniscope (Inscopix) at two focal planes (multiplane imaging) with LED power set at 40–70% depending on the GCaMP6m expression level. Calcium activity was acquired at 10 Hz per plane (20 Hz for alternating acquisition), and this final temporal resolution was used for the imaging data without further down-sampling. The fixed pre-set focal planes and LED power were maintained throughout the entire experiment to ensure consistency.

To prevent prolonged exposure to the LED light, the Med Associates chamber sent a TTL pulse to turn on the LED 30 s before the CS onset and turn it off 30 s after the CS onset. Trial-structured multiplane imaging data were pre-processed through sequential concatenation and spatial down-sampled (spatial factor 4) using Inscopix Data Processing Software (IDPS). The IDPS was then utilized for the subsequent data processing steps, including motion correction, cell identification, and cell registration between planes or days. The following steps were followed: 1. Motion correction was performed multiple times as necessary until the image stabilized. 2. The motion-corrected TIFF (tag image file format) file was reloaded for easier processing of multiplane registration. 3. Multiplane registration was conducted to eliminate imaging the same cells from

multiple planes (minimum spatial correlation = 0.5, temporal correlation = 0.99). 4. Cells were identified with a constrained non-negative matrix factorization algorithm for microendoscopic data (CNMF-E) (Zhou et al., 2018 [↗](#)). 5. Longitudinal registration was used for day 10 of appetitive and day 1 of aversive learning to compare how the same cell responded to appetitive vs. aversive stimuli.

Identifying significantly responsive neurons

We wish to identify neurons that are responsive to appetitive or fear stimuli (i.e. that display a difference in activity pre-versus post-stimulus). However, the usual p-values derived from statistical analyses such as t-tests or Wilcoxon rank sum tests assume independence of data points, which is violated for neuronal calcium transients due to an inherent dependency between adjacent transient values. Consequently, a p-value computed assuming independence will lead to highly inflated measures of significance in the case of dependent data, and thus inaccurate interpretation of the data. A related issue is discussed in the Harris' study along with suggested remedies (Harris, 2021 [↗](#)).

Therefore, to quantify the responsiveness of CeA neurons to appetitive vs. aversive stimuli, we rely on an alternative method for generating p-values based upon a variant of 'circular shifting' (Harris, 2021 [↗](#)). The null hypothesis for this statistical test is that the calcium transients are non-responsive to the stimuli (formally, pre-stimulus and post-stimulus data are drawn from the same distribution). To simulate hypothetical neuron data under the null hypothesis, we generated "null" calcium activity by circularly shifting the calcium trace of trials from randomly selected neurons (Step 1 in **Figure 2B** [↗](#), red bar). Thus, the "null" calcium traces mimic the hypothetical situation where transients in calcium traces are unrelated to the experimental variables, while preserving calcium dependency dynamics.

After generating "null" calcium activity as described above, we compared the test statistics based upon the observed data to the collection of test statistics obtained under the "null" calcium activity distribution; test statistics were obtained by summing Wilcoxon rank sum test (WRST) statistics across trials.

We note two substantial differences between our approach and the circular shifting approach of the Harris' study (Harris, 2021 [↗](#)): (i) We generate a null distribution by pooling from the entire population of recorded neurons to fully capture the heterogeneity of possible calcium transients under the null distribution. (ii) Our analysis relies on WRST statistics, which operate on ranks (rather than directly on the calcium transient values).

Details are provided in [Algorithm 1](#) [↗](#). Step 1 formally defines the circular shift operation. Step 2 involves repeatedly applying the circular shift operation to a randomly selected neuron, and then computing the resulting WRST; these results in a distribution of WRSTs generated under the null hypothesis. In Step 3, the WRST test statistics observed in the current study are computed, and in Step 4, they are compared with the null distribution generated in Step 2 to assess their statistical significance.

We now discuss [Algorithm 1](#) [↗](#) in greater detail. In [Algorithm 1](#) [↗](#), N is the number of timepoints for each neuron's transient (note that each neuron has been recorded for the same number of timepoints).

Algorithm 1:

Computing a p-value for a neuron's responsiveness

If testing for food and shock, consider only neural activity ranging from 30 seconds before CS to 30 seconds after CS.

Step 1: Define the “circular shift” operation

Define CircularShift(Y , N , s):

$Y^* \leftarrow [Y[(s+1):N], Y[1:s]]$
return(Y^*)

Step 2: Create a repository of test statistics for null data

For b in $\{1, 2, \dots, B\}$ do

$i \leftarrow$ random integer from 1 to the number of neurons

For j in $\{1, 2, \dots, \text{number of trials}\}$ do

$s \leftarrow$ random integer between 1 and N

$Y_{b,j}^* \leftarrow \text{CircularShift}(Y_{i,j}, N, s)$

$W_{b,j}^* \leftarrow$ Wilcoxon rank sum test of a difference in $Y_{b,j}^*$
(pre- vs. post-stimuli)

end for

$W_b^* \leftarrow \sum_x W_{b,j}^*$

end for

Step 3: Calculate observed test statistics

For i in $\{1, 2, \dots, \text{number of neurons}\}$ do

For j in $\{1, 2, \dots, \text{number of trials}\}$ do

$W_{i,j}^{obs} \leftarrow$ Wilcoxon rank sum test statistic with $Y_{i,j}$ (pre- vs. post-stimuli)

end for

$\bar{W}_i^{obs} \leftarrow \sum_j W_{i,j}^{obs}$

Step 4: Compute p-values

For i in $\{1, 2, \dots, \text{number of neurons}\}$ do

$p_i^+ \leftarrow [(\# \text{ of } W_b^* \geq \bar{W}_i^{obs}) + 1] / [B + 1]$

$p_i^- \leftarrow [(\# \text{ of } W_b^* \leq \bar{W}_i^{obs}) + 1] / [B + 1]$

$p_i \leftarrow 2 \times \min(p_i^+, p_i^-)$

end for

(Step 0): We restrict our attention to neural activity in a relevant time window. To test for CS, we consider timepoints ranging from -30 seconds to 10 seconds. To test for food and shock, we consider timepoints from -30 seconds to 30 seconds. Data outside of these windows is not used in the remaining analysis steps.

(Step 1): We formalize the “circular shift” operation described earlier.

(Step 2): We repeat the following procedure $B = 500$ times. We randomly select one neuron (Y_i) from all the neurons recorded during each session (appetitive: 872, aversive: 518, baseline: 925, post-test: 788), randomly select an integer s , and apply circular shifting by s to each trial for this neuron (this yields $Y_{b,j}^*$, which represents “null” neuronal activity, as shown in **Figure 2C** [↗](#)).

Next, for the j th trial, we compute the WRST ($W_{b,j}^*$) on the newly-generated “null” neuronal traces ($Y_{b,j}^*$) to compare pre-stimulus (CSs: -20 s to 0 s; food/shock: -5 s to 13 s) and post-stimulus (CSs: 0 s to 10 s; food: 13 s to 30 s; shock: 13 s to 18 s) activity (as illustrated in **Figure 2C** [↗](#)). Finally, we sum the WRST statistics across the trials ($\sum_j W_{b,j}^*$) and refer to this as W_b^* .

At the end of Step 2, we have B test statistics ($W_1^*, \dots, W_B^*$), each of which is obtained by summing the WRSTs of a different “null” neuron across trials. We set $B = 500$ to balance code runtime against having a rich enough null distribution of the test statistic.

(Step 3): For the j th trial of the i th recorded neuron, we compute the WRST between pre- vs. post-stimulus (w_{ij}^{obs}), and then sum these WRST statistics across the trials ($\bar{w}_i^{obs}$; **Figure 2D**). The testing window for pre-stimulus and post-stimulus remains the same as in Step 2.

(Step 4): We compare the i th neuron’s observed test statistic ($\bar{w}_i^{obs}$) to the null distribution ($W_b^*, b = 1, \dots, B$) generated in Step 2. The right-tailed p-value p_i^+ is calculated as (the number of $w_b^* \geq \bar{w}_i^{obs}$)+1, divided by $B+1$. Similarly, the left-tailed p-value p_i^- is calculated as (the number of $w_b^* \leq \bar{w}_i^{obs}$)+1, divided by $B+1$ (**Figure 2E**). We compute the left- and right-tailed p-values separately since $\bar{w}_i^{obs}$ can be either extremely large (indicating significant excitation) or small (indicating significant inhibition). For example, if $w_b^* \geq \bar{w}_i^{obs}$ for 10 of the “null” neurons, with $B=500$, then $p_i^+ = (10+1) / (500+1) = 0.022$ and $p_i^- = 0.978$. The two-sided p-value for this neuron is 2×0.022 (since p_i^+ is smaller than p_i^-), i.e. it equals 0.044. The significance level is set to 0.05. If the p-value of a neuron is smaller than 0.05, it is considered responsive (significantly excited).

Figure 5—figure supplement 2 displays the results obtained using the traditional WRST that relies on the theoretical null distribution, i.e., that assumes independence between time points, and then sets a threshold for the number of trials with a p-value below 0.05 in order to declare that a neuron is “responsive”. We see little difference between the two sets of results.

Classification of salience-encoding and valence-encoding neurons

With the neurons that appeared during both appetitive and fear conditioning, we conducted a direct comparison of how individual neurons respond to appetitive vs. aversive stimuli. After the new analysis confirmed ‘responsive neurons’ (either excited or inhibited) among the registered neurons, we further classified them into salience-encoding and valence-encoding neurons based on their response patterns. Salience-encoding neurons demonstrate significant responses to both appetitive and aversive stimuli in the same direction (both excited or both inhibited), reflecting the stimulus strength, which is the definition of salience (**Figure 5B** white, **Figure 5G** white, Figure 5—figure supplement 2I,O white) (Kong & Zweifel, 2021). On the other hand, valence-encoding neurons exhibit a more diverse range of response types. For instance, some neurons exclusively encode one of the stimuli by showing a significant response to only one type.

Alternatively, some neurons respond significantly to both stimuli but in opposite directions (e.g., excited to food and inhibited to shock, **Figure 5B** black, **Figure 5G** black, Figure 5—figure supplement 2I,O black). These response patterns correspond to the definition of valence, representing either positive (good) or negative (bad) valence (Kong & Zweifel, 2021). Neurons that showed no significant activity in response to both appetitive and aversive stimuli were categorized as not-significant neurons (**Figure 5B** grey, **Figure 5G** grey, Figure 5—figure supplement 2I,O grey).

Histology

At the conclusion of the experiment, the mice were administered an overdose of Beuthanasia and then perfused transcranially with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA). Following this, each mouse head with a GRIN lens was immersed in PFA at 4°C overnight for a week to preserve the lens track. The brains were subsequently extracted from the post-fixed heads, including the lens track, and cryoprotected in PBS containing 30% sucrose for 72 hours. Immunohistochemistry was performed on transverse sections (50 μ m) using a primary antibody (anti-GFP Chicken polyclonal, Abcam, ab13970) followed by a secondary antibody (Alexa Fluor 488,

JacksonImmuno, 703-545-155). The treated sections were then mounted on slides and coverslipped with DAPI Fluoromount-G (Southern Biotech). The expression of GCaMP6m and the location of the lens were examined using a Keyence Fluorescence Microscope (Keyence).

Statistical analysis

The responsiveness of CeA neurons to both appetitive and aversive stimuli was quantified using the previously outlined **Algorithm 1** [↗](#), implemented in the R programming language. For the determination of statistical significance in behavioral results and area under curve data, we employed One-way repeated measured ANOVA, One-way ANOVA (with Tukey *post hoc*), paired t-test, and unpaired t-test using Prism software. A comprehensive presentation of the statistical results is available in Supplementary Table 1. A significance level of $P < 0.05$ was set for all tests. Graphs were generated using GraphPad Prism (version 10) and customized MATLAB codes.

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Additional information

Author contributions

M-S.K and L.S.Z. conceptualized the study and designed experiments. M-S.K. performed experiments and analyzed data with assistance from E.A. and D.M.W. M-S.K., E.A., and D.M.W. wrote analysis code. E.A. and D.M.W. designed analysis and wrote code for cell activation designations. M-S.K., L.S.Z., E.A., and D.M.W. wrote manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

Data and code availability

All data and codes will be made available through online repositories prior to final acceptance.

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Reviewer #1 (Public review):

From the Reviewing Editor:

Four reviewers have assessed your manuscript on valence and salience signaling in the central amygdala. There was universal agreement that the question being asked by the experiment is important. There was consensus that the neural population being examined (GABA neurons) was important and the circular shift method for identifying task-responsive neurons was rigorous. Indeed, observing valenced outcome signaling in GABA neurons would considerably increase the role the central amygdala in valence. However, each reviewer brought up significant concerns about the design, analysis and interpretation of the results. Overall, these concerns limit the conclusions that can be drawn from the results. Addressing the concerns (described below) would work towards better answering the question at the outset of the experiment: how does the central amygdala represent salience vs valence.

A weakness noted by all reviewers was the use of the terms 'valence' and 'salience' as well as the experimental design used to reveal these signals. The two outcomes used emphasized non-overlapping sensory modalities and produced unrelated behavioral responses. Within each modality there are no manipulations that would scale either the value of the valenced outcomes or the intensity of the salient outcomes. While the food outcomes were presented many times (20 times per session over 10 sessions of appetitive conditioning) the shock outcomes were presented many fewer times (10 times in a single session). The large difference in presentations is likely to further distinguish the two outcomes. Collectively, these experimental design decisions meant that any observed differences in central amygdala GABA neuron responding are unlikely to reflect valence, but likely to reflect one or more of the above features.

A second weakness noted by a majority of reviewers was a lack of cue-responsive unit and a lack of exploration of the diversity of response types, and the relationship cue and outcome firing. The lack of large numbers of neurons increasing firing to one or both cues is particularly surprising given the critical contribution of central amygdala GABA neurons to the acquisition of conditioned fear (which the authors measured) as well as to conditioned orienting (which the authors did not measure). Regression-like analyses would be a straightforward means of identifying neurons varying their firing in accordance with these or other behaviors. It was also noted that appetitive behavior was not measured in a rigorous way. Instead of measuring time near hopper, measures of licking would have been better. Further, measures of orienting behaviors such as startle were missing. The authors also missed an opportunity for clustering-like analyses which could have been used to reveal neurons uniquely signaling cues, outcomes or combinations of cues and outcomes. If the authors calcium imaging approach is not able to detect expected central amygdala cue responding, might it be missing other critical aspects of responding?

All reviewers point out that the evidence for salience encoding is even more limited than the evidence for valence. Although the specific concern for each reviewer varied, they all centered on an oversimplistic definition of salience. Salience ought to scale with the absolute value and intensity of the stimulus. Salience cannot simply be responding in the same direction. Further, even though the authors observed subsets of central amygdala neurons increasing or decreasing activity to both outcomes - the outcomes can readily be distinguished based on the temporal profile of responding.

Additional concerns are raised by each reviewer. Our consensus is that this study sought to answer an important question - whether central amygdala signal salience or valence in cue-outcome learning. However, the experimental design, analyses, and interpretations do not permit a rigorous and definitive answer to that question. Such an answer would require additional experiments whose designs would address the significant concerns described here. Fully addressing the concerns of each reviewer would result in a re-evaluation of the findings. For example, experimental design better revealing valence and salience, and analyses describing diversity of neuronal responding and relationship to behavior would likely make the results Important or even Fundamental.

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Reviewer #2 (Public review):

In this article, Kong and authors sought to determine the encoding properties of central amygdala (CeA) neurons in response to oppositely valenced stimuli and cues predicting those stimuli. The amygdala and its subregional components have historically been understood to be regions that encode associative information, including valence stimuli. The authors performed calcium imaging of GABA-ergic CeA neurons in freely-moving mice conditioned in Pavlovian appetitive and fear paradigms, and showed that CeA neurons are responsive to both appetitive and aversive unconditioned and conditioned stimuli. They used a variant of a previously published 'circular shifting' technique (Harris, 2021), which allowed them to delineate between excited/non-responsive/inhibited neurons. While there is considerable overlap of CeA neurons responding to both unconditioned stimuli (in this case, food and shock, deemed "salience-encoding" neurons), there are considerably fewer CeA neurons that respond to both conditioned stimuli that predict the food and shock. The authors finally demonstrated that there are no differences in the order of Pavlovian paradigms (fear - shock vs. shock - fear), which is an interesting result, and convincingly presented given their counterbalanced experimental design.

In total, I find the presented study useful in understanding the dynamics of CeA neurons during a Pavlovian learning paradigm. There are many strengths of this study, including the important question and clear presentation, the circular shifting analysis was convincing to me, and the manuscript was well written. We hope the authors will find our comments constructive if they choose to revise their manuscript.

While the experiments and data are of value, I do not agree with the authors interpretation of their data, and take issue with the way they used the terms "salience" and "valence" (and would encourage them to check out Namburi et al., NPP, 2016) regarding the operational definitions of salience and valence which differ from my reading of the literature. To be fair, a recent study from another group that reports experiments/findings which are very similar to the ones in the present study (Yang et al., 2023, describing valence coding in the CeA using a similar approach) also uses the terms valence and salience in a rather liberal way that I would also have issues with (see below). Either new experiments or revised claims would be needed here, and more balanced discussion on this topic would be nice to see, and I felt that there were some aspects of novelty in this study that could be better highlighted (see below).

One noteworthy point of alarm is that it seems as if two data panels including heatmaps are duplicated (perhaps that panel G of Figure 5-figure supplement 2 is a cut and paste error? It is duplicated from panel E and does not match the associated histogram).

Major concerns:

(1) The authors wish to make claims about salience and valence. This is my biggest gripe, so I will start here.

(1a) Valence scales for positive and negative stimuli and as stated in Namburi et al., NPP, 2016 where we operationalize "valence" as having different responses for positive and negative values and no response for stimuli that are not motivational significant (neutral cues that do not predict an outcome). The threshold for claiming salience, which we define as scaling with the absolute value of the stimulus, and not responding to a neutral stimulus (Namburi et al., NPP, 2016; Tye, Neuron, 2018; Li et al., Nature, 2022) would require the lack of response to a neutral cue.

(1b) The other major issue is that the authors choose to make claims about the neural responses to the USs rather than the CSs. However, being shocked and receiving sucrose also would have very different sensorimotor representations, and any differences in responses could be attributed to those confounds rather than valence or salience. They could make claims regarding salience or valence with respect to the differences in the CSs but they should restrict analysis to the period prior to the US delivery.

(1c) The third obstacle to using the terms "salience" or "valence" is the lack of scaling, which is perhaps a bigger ask. At minimum either the scaling or the neutral cue would be needed to make claims about valence or salience encoding. Perhaps the authors disagree - that is fine. But they should at least acknowledge that there is literature that would say otherwise.

(1d) In order to make claims about valence, the authors must take into account the sensory confound of the modality of the US (also mentioned in Namburi et al., 2016). The claim that these CeA neurons are indeed valence-encoding (based on their responses to the unconditioned stimuli) is confounded by the fact that the appetitive US (food) is a gustatory stimulus while the aversive US (shock) is a tactile stimulus.

(2) Much of the central findings in this manuscript have been previously described in the literature. Yang et al., 2023 for instance shows that the CeA encodes salience (as demonstrated by the scaled responses to the increased value of unconditioned stimuli, Figure 1 j-m), and that learning amplifies responsiveness to unconditioned stimuli (Figure 2). It is nice to see a reproduction of the finding that learning amplifies CeA responses, though one study is in SST::Cre and this one in VGAT::cre - perhaps highlighting this difference could maximize the collective utility for the scientific community?

(3) There is at least one instance of copy-paste error in the figures that raised alarm. In the supplementary information (Figure 5- figure supplement 2 E;G), the heat maps for food-responsive neurons and shock-responsive neurons are identical. While this almost certainly is a clerical error, the authors would benefit from carefully reviewing each figure to ensure that no data is incorrectly duplicated.

(4) The authors describe experiments to compare shock and reward learning; however, there are temporal differences in what they compare in Figure 5. The authors compare the 10th day of reward learning with the 1st day of fear conditioning, which effectively represent different points of learning and retrieval. At the end of reward conditioning, animals are utilizing a learned association to the cue, which demonstrates retrieval. On the day of fear conditioning, animals are still learning the cue at the beginning of the session, but they are not necessarily retrieving an association to a learned cue. The authors would benefit from recording at a later timepoint (to be consistent with reward learning- 10 days after fear conditioning), to more accurately compare these two timepoints. Or perhaps, it might be

easier to just make the comparison between Day 1 of reward learning and Day 1 of fear learning, since they must already have these data.

(5) The authors make a claim of valence encoding in their title and throughout the paper, which is not possible to make given their experimental design. However, they would greatly benefit from actually using a decoder to demonstrate their encoding claim (decoding performance for shock-food versus shuffled labels) and simply make claims about decoding food-predictive cues and shock-predictive cues. Interestingly, it seems like relatively few CeA neurons actually show differential responses to the food and shock CSs, and that is interesting in itself.

<https://doi.org/10.7554/eLife.101980.1.sa3>

Reviewer #3 (Public review):

Summary:

In their manuscript entitled Kong and colleagues investigate the role of distinct populations of neurons in the central amygdala (CeA) in encoding valence and salience during both appetitive and aversive conditioning. The study expands on the work of Yang et al. (2023), which specifically focused on somatostatin (SST) neurons of the CeA. Thus, this study broadens the scope to other neuronal subtypes, demonstrating that CeA neurons in general are predominantly tuned to valence representations rather than salience.

Strengths:

One of the key strengths of the study is its rigorous quantitative approach based on the "circular-shift method", which carefully assesses correlations between neural activity and behavior-related variables. The authors' findings that neuronal responses to the unconditioned stimulus (US) change with learning are consistent with previous studies (Yang et al., 2023). They also show that the encoding of positive and negative valence is not influenced by prior training order, indicating that prior experience does not affect how these neurons process valence.

Weaknesses:

However, there are limitations to the analysis, including the lack of population-based analyses, such as clustering approaches. The authors do not employ hierarchical clustering or other methods to extract meaning from the diversity of neuronal responses they recorded. Clustering-based approaches could provide deeper insights into how different subpopulations of neurons contribute to emotional processing. Without these methods, the study may miss patterns of functional specialization within the neuronal populations that could be crucial for understanding how valence and salience are encoded at the population level.

Furthermore, while salience encoding is inferred based on responses to stimuli of opposite valence, the study does not test whether these neuronal responses scale with stimulus intensity—a hallmark of classical salience encoding. This limits the conclusions that can be drawn about salience encoding specifically.

In sum, while the study makes valuable contributions to our understanding of CeA function, the lack of clustering-based population analyses and the absence of intensity scaling in the assessment of salience encoding are notable limitations.

<https://doi.org/10.7554/eLife.101980.1.sa2>

Reviewer #4 (Public review):**Summary:**

The authors have performed endoscopic calcium recordings of individual CeA neuron responses to food and shock, as well as to cues predicting food and shock. They claim that a majority of neurons encode valence, with a substantial minority encoding salience.

Strengths:

The use of endoscopic imaging is valuable, as it provides the ability to resolve signals from single cells, while also being able to track these cells across time. The recordings appear well-executed, and employ a sophisticated circular shifting analysis to avoid statistical errors caused by correlations between neighboring image pixels.

Weaknesses:

My main critique is that the authors didn't fully test whether neurons encode valence. While it is true that they found CeA neurons responding to stimuli that have positive or negative value, this by itself doesn't indicate that valence is the primary driver of neural activity. For example, they report that a majority of CeA neurons respond selectively to either the positive or negative US, and that this is evidence for "type I" valence encoding. However, it could also be the case that these neurons simply discriminate between motivationally relevant stimuli in a manner unrelated to valence per se. A simple test of this would be to check if neural responses generalize across more than one type of appetitive or aversive stimulus, but this was not done. The closest the authors came was to note that a small number of neurons respond to CS cues, of which some respond to the corresponding US in the same direction. This is relegated to the supplemental figures (3 and 4), and it is not noted whether the same-direction CS-US neurons are also valence-encoding with respect to different USs. For example, are the neurons excited by CS-food and US-food also inhibited by shock? If so, that would go a long way toward classifying at least a few neurons as truly encoding valence in a generalizable way.

A second and related critique is that, although the authors correctly point out that definitions of salience and valence are sometimes confused in the existing literature, they then go on themselves to use the terms very loosely. For example, the authors define these terms in such a way that every neuron that responds to at least one stimulus is either salience or valence-encoding. This seems far too broad, as it makes essentially unfalsifiable their assertion that the CeA encodes some mixture of salience and valence. I already noted above that simply having different responses to food and shock does not qualify as valence-encoding. It also seems to me that having same-direction responses to these two stimuli similarly does not qualify a neuron as encoding salience. Many authors define salience as being related to the ability of a stimulus to attract attention (which is itself a complex topic). However, the current paper does not acknowledge whether they are using this, or any other definition of salience, nor is this explicitly tested, e.g. by comparing neural response magnitudes to any measure of attention.

The impression I get from the authors' data is that CeA neurons respond to motivationally relevant stimuli, but in a way that is possibly more complex than what the authors currently imply. At the same time, they appear to have collected a large and high-quality dataset that could profitably be made available for additional analyses by themselves and/or others.

Lastly, the use of 10 daily sessions of training with 20 trials each seems rather low to me. In our hands, Pavlovian training in mice requires considerably more trials in order to effectively elicit responses to the CS. I wonder if the relatively sparse training might explain the relative lack of CS responses?

Author response:

Reviewer #1 (Public review):

From the Reviewing Editor:

Four reviewers have assessed your manuscript on valence and salience signaling in the central amygdala. There was universal agreement that the question being asked by the experiment is important. There was consensus that the neural population being examined (GABA neurons) was important and the circular shift method for identifying task-responsive neurons was rigorous. Indeed, observing valenced outcome signaling in GABA neurons would considerably increase the role the central amygdala in valence. However, each reviewer brought up significant concerns about the design, analysis and interpretation of the results. Overall, these concerns limit the conclusions that can be drawn from the results. Addressing the concerns (described below) would work towards better answering the question at the outset of the experiment: how does the central amygdala represent salience vs valence.

A weakness noted by all reviewers was the use of the terms 'valence' and 'salience' as well as the experimental design used to reveal these signals. The two outcomes used emphasized non-overlapping sensory modalities and produced unrelated behavioral responses. Within each modality there are no manipulations that would scale either the value of the valenced outcomes or the intensity of the salient outcomes. While the food outcomes were presented many times (20 times per session over 10 sessions of appetitive conditioning) the shock outcomes were presented many fewer times (10 times in a single session). The large difference in presentations is likely to further distinguish the two outcomes. Collectively, these experimental design decisions meant that any observed differences in central amygdala GABA neuron responding are unlikely to reflect valence, but likely to reflect one or more of the above features.

We appreciate the reviewers' comments regarding the experimental design. When assessing fear versus reward, we chose stimuli that elicit known behavioral responses, freezing versus consumption. The use of stimuli of the same modality is unlikely to elicit easily definable fear or reward responses or to be precisely matched for sensory intensity. For example, sweet or bitter tastes can be used, but even these activate different taste receptors and vary in the duration of the activation of taste-specific signaling (e.g. how long the taste lingers in the mouth). The approach we employed is similar to that of Yang et al., 2023 (doi: 10.1038/s41586-023-05910-2) that used water reward and shock to characterize the response profiles of somatostatin neurons of the central amygdala. Similar to what was reported by Yang and colleagues we observed that the majority of CeA GABA neurons responded selectively to one unconditioned stimulus (~52%). We observed that 15% of neurons responded in the same direction, either activated or inhibited, by the food or shock US. These were defined as salience based on the definitions of Lin and Nicolelis, 2008 (doi: 10.1016/j.neuron.2008.04.031) in which basal forebrain neurons responded similarly to reward or punishment irrespective of valence. The designation of valence encoding based opposite responses to the food or shock is straightforward (~10% of cells); however, we agree that the designation of modality-specific encoding neurons as valence encoding is less straightforward.

A second weakness noted by a majority of reviewers was a lack of cue-responsive unit and a lack of exploration of the diversity of response types, and the relationship cue and outcome firing. The lack of large numbers of neurons increasing firing to one or both cues is particularly surprising given the critical contribution of central amygdala GABA

neurons to the acquisition of conditioned fear (which the authors measured) as well as to conditioned orienting (which the authors did not measure). Regression-like analyses would be a straightforward means of identifying neurons varying their firing in accordance with these or other behaviors. It was also noted that appetitive behavior was not measured in a rigorous way. Instead of measuring time near hopper, measures of licking would have been better. Further, measures of orienting behaviors such as startle were missing.

The authors also missed an opportunity for clustering-like analyses which could have been used to reveal neurons uniquely signaling cues, outcomes or combinations of cues and outcomes. If the authors calcium imaging approach is not able to detect expected central amygdala cue responding, might it be missing other critical aspects of responding?

As stated in the manuscript, we were surprised by the relatively low number of cue responsive cells; however, when using a less stringent statistical method (Figure 5 - Supplement 2), we observed 13% of neurons responded to the food associated cue and 23% responded to the shock associated cue. The differences are therefore likely a reflection of the rigor of the statistical measure to define the responsive units. The number of CS responsive units is less than reported in the CeAl by Cioocchi et al., 2010 (doi: 10.1038/nature09559) who observed 30% activated by the CS and 25% inhibited, but is not that dissimilar from the results of Duvarci et al., 2011 (doi: 10.1523/JNEUROSCI.4985-10.2011) who observed 11% activated in the CeAl and 25% inhibited by the CS. These numbers are also consistent with previous single cell calcium imaging of cell types in the CeA. For example, Yang et al., 2023 (doi: 10.1038/s41586-023-05910-2) observed that 13% of somatostatin neurons responded to a reward CS and 8% responded to a shock CS. Yu et al., 2017 (doi: 10.1038/s41593-017-0009-9) observed 26.5% of PKCdelta neurons responded to the shock CS. It should also be noted that our analysis was not restricted to the CeAl. Finally, Food learning was assessed in an operant chamber in freely moving mice with reward pellet delivery. Because liquids were not used for the reward US, licking is not a metric that can be used.

All reviewers point out that the evidence for salience encoding is even more limited than the evidence for valence. Although the specific concern for each reviewer varied, they all centered on an oversimplistic definition of salience. Salience ought to scale with the absolute value and intensity of the stimulus. Salience cannot simply be responding in the same direction. Further, even though the authors observed subsets of central amygdala neurons increasing or decreasing activity to both outcomes - the outcomes can readily be distinguished based on the temporal profile of responding.

We thank the reviewers for their comments relating to the definition of salience and valence encoding by central amygdala neurons. We have addressed each of the concerns below.

Additional concerns are raised by each reviewer. Our consensus is that this study sought to answer an important question - whether central amygdala signal salience or valence in cue-outcome learning. However, the experimental design, analyses, and interpretations do not permit a rigorous and definitive answer to that question. Such an answer would require additional experiments whose designs would address the significant concerns described here. Fully addressing the concerns of each reviewer would result in a re-evaluation of the findings. For example, experimental design better revealing valence and salience, and analyses describing diversity of neuronal responding and relationship to behavior would likely make the results Important or even Fundamental.

We appreciate the reviewers' comments and have addressed each concern below.

Reviewer #2 (Public review):

In this article, Kong and authors sought to determine the encoding properties of central amygdala (CeA) neurons in response to oppositely valenced stimuli and cues predicting those stimuli. The amygdala and its subregional components have historically been understood to be regions that encode associative information, including valence stimuli. The authors performed calcium imaging of GABA-ergic CeA neurons in freely-moving mice conditioned in Pavlovian appetitive and fear paradigms, and showed that CeA neurons are responsive to both appetitive and aversive unconditioned and conditioned stimuli. They used a variant of a previously published 'circular shifting' technique (Harris, 2021), which allowed them to delineate between excited/non-responsive/inhibited neurons. While there is considerable overlap of CeA neurons responding to both unconditioned stimuli (in this case, food and shock, deemed "salience-encoding" neurons), there are considerably fewer CeA neurons that respond to both conditioned stimuli that predict the food and shock. The authors finally demonstrated that there are no differences in the order of Pavlovian paradigms (fear - shock vs. shock - fear), which is an interesting result, and convincingly presented given their counterbalanced experimental design.

In total, I find the presented study useful in understanding the dynamics of CeA neurons during a Pavlovian learning paradigm. There are many strengths of this study, including the important question and clear presentation, the circular shifting analysis was convincing to me, and the manuscript was well written. We hope the authors will find our comments constructive if they choose to revise their manuscript.

While the experiments and data are of value, I do not agree with the authors interpretation of their data, and take issue with the way they used the terms "salience" and "valence" (and would encourage them to check out Namburi et al., NPP, 2016) regarding the operational definitions of salience and valence which differ from my reading of the literature. To be fair, a recent study from another group that reports experiments/findings which are very similar to the ones in the present study (Yang et al., 2023, describing valence coding in the CeA using a similar approach) also uses the terms valence and salience in a rather liberal way that I would also have issues with (see below). Either new experiments or revised claims would be needed here, and more balanced discussion on this topic would be nice to see, and I felt that there were some aspects of novelty in this study that could be better highlighted (see below).

One noteworthy point of alarm is that it seems as if two data panels including heatmaps are duplicated (perhaps that panel G of Figure 5-figure supplement 2 is a cut and paste error? It is duplicated from panel E and does not match the associated histogram).

We thank the reviewer for their insightful comments and assessment of the manuscript.

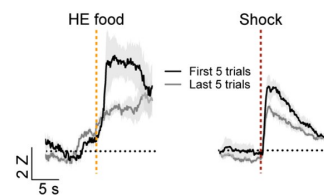
Major concerns:

(1) The authors wish to make claims about salience and valence. This is my biggest gripe, so I will start here.

(1a) Valence scales for positive and negative stimuli and as stated in Namburi et al., NPP, 2016 where we operationalize "valence" as having different responses for positive and negative values and no response for stimuli that are not motivational significant (neutral cues that do not predict an outcome). The threshold for claiming salience, which we define as scaling with the absolute value of the stimulus, and not responding to a neutral stimulus (Namburi et al., NPP, 2016; Tye, Neuron, 2018; Li et al., Nature, 2022) would require the lack of response to a neutral cue.

We appreciate the reviewer's comment on the definitions of salience and valence and agree that there is not a consistent classification of these response types in the field. As stated above, we used the designation of salience encoding if the cells respond in the same direction to different stimuli regardless of the valence of the stimulus similar to what was described previously (Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031). Similar definitions of salience have also been reported elsewhere (for examples see: Stephenson-Jones et al., 2020, doi: 10.1016/j.neuron.2019.12.006, Zhu et al., 2018 doi: 10.1126/science.aat0481, and Comoli et al., 2003, doi: 10.1038/nn1113P). Per the suggestion of the reviewer, we longitudinally tracked cells on the first day of Pavlovian reward conditioning the fear conditioning day. Although there were considerably fewer head entries on the first day of reward conditioning, we were able to identify 10 cells that were activated by both the food US and shock US. We compared the responses to the first five head entries and last head entries and the first 5 shocks and last five shocks. Consistent with what has been reported for salience encoding neurons in the basal forebrain (Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031), we observed that the responses were highest when the US was most unexpected and decreased in later trials.

Author response image 1.



(1b) The other major issue is that the authors choose to make claims about the neural responses to the USs rather than the CSs. However, being shocked and receiving sucrose also would have very different sensorimotor representations, and any differences in responses could be attributed to those confounds rather than valence or salience. They could make claims regarding salience or valence with respect to the differences in the CSs but they should restrict analysis to the period prior to the US delivery.

Perhaps the reviewer missed this, but analysis of valence and salience encoding to the different CSs are presented in Figure 5G, Figure 5 -Supplement 1 C-D, and Figure 5 -Supplement 2 N-O. Analysis of CS responsiveness to CSFood and CSShock were analyzed during the conditioning sessions Figure 3E-F, Figure 4B-C, Figure 5 – Supplement 2J-O and Figure 5 – Supplement 3K-L, and during recall probe tests for both CSFood and CSShock, Figure 5 – Supplement 1C-J.

(1c) The third obstacle to using the terms "salience" or "valence" is the lack of scaling, which is perhaps a bigger ask. At minimum either the scaling or the neutral cue would be needed to make claims about valence or salience encoding. Perhaps the authors disagree - that is fine. But they should at least acknowledge that there is literature that would say otherwise.

(1d) In order to make claims about valence, the authors must take into account the sensory confound of the modality of the US (also mentioned in Namburi et al., 2016). The claim that these CeA neurons are indeed valence-encoding (based on their responses to the unconditioned stimuli) is confounded by the fact that the appetitive US (food) is a gustatory stimulus while the aversive US (shock) is a tactile stimulus.

We provided the same analysis for the US and CS. The US responses were larger and more prevalent, but similar types of encoding were observed for the CS. We agree that the food

reward and the shock are very different sensory modalities. As stated above, the use of stimuli of the same modality is unlikely to elicit easily definable fear or reward responses or to be precisely matched for sensory intensity. We agree that the definition of cells that respond to only one stimulus is difficult to define in terms of valence encoding, as opposed to being specific for the sensory modality and without scaling of the stimulus it is difficult to fully address this issue. It should be noted however, that if the cells in the CeA were exclusively tuned to stimuli of different sensory modalities, we would expect to see a similar number of cells responding to the CS tones (auditory) as respond to the food (taste) and shock (somatosensory) but we do not. Of the cells tracked longitudinally 80% responded to the USs, with 65% of cells responding to food (activated or inhibited) and 44% responding to shock (activated or inhibited).

(2) Much of the central findings in this manuscript have been previously described in the literature. Yang et al., 2023 for instance shows that the CeA encodes salience (as demonstrated by the scaled responses to the increased value of unconditioned stimuli, Figure 1 j-m), and that learning amplifies responsiveness to unconditioned stimuli (Figure 2). It is nice to see a reproduction of the finding that learning amplifies CeA responses, though one study is in SST::Cre and this one in VGAT::cre - perhaps highlighting this difference could maximize the collective utility for the scientific community?

We agree that the analysis performed here is similar to what was conducted by Yang et al., 2023. With the major difference being the types of neurons sampled. Yang et al., imaged only somatostatin neurons were as we recorded all GABAergic cell types within the CeA. Moreover, because we imaged from 10 mice, we sampled neurons that ostensibly covered the entire dorsal to ventral extent of the CeA (Figure 1 – Supplement 1). Remarkably, we found that the vast majority of CeA neurons (80%) are responsive to food or shock. Within this 80% there are 8 distinct response profiles consistent with the heterogeneity of cell types within the CeA based on connectivity, electrophysiological properties, and gene expression. Moreover, we did not find any spatial distinction between food or shock responsive cells, with the responsive cell types being intermingled throughout the dorsal to ventral axis (Figure 5 – Supplement 3).

(3) There is at least one instance of copy-paste error in the figures that raised alarm. In the supplementary information (Figure 5- figure supplement 2 E;G), the heat maps for food-responsive neurons and shock-responsive neurons are identical. While this almost certainly is a clerical error, the authors would benefit from carefully reviewing each figure to ensure that no data is incorrectly duplicated.

We thank the reviewer for catching this error. It has been corrected.

(4) The authors describe experiments to compare shock and reward learning; however, there are temporal differences in what they compare in Figure 5. The authors compare the 10th day of reward learning with the 1st day of fear conditioning, which effectively represent different points of learning and retrieval. At the end of reward conditioning, animals are utilizing a learned association to the cue, which demonstrates retrieval. On the day of fear conditioning, animals are still learning the cue at the beginning of the session, but they are not necessarily retrieving an association to a learned cue. The authors would benefit from recording at a later timepoint (to be consistent with reward learning- 10 days after fear conditioning), to more accurately compare these two timepoints. Or perhaps, it might be easier to just make the comparison between Day 1 of reward learning and Day 1 of fear learning, since they must already have these data.

We agree that there are temporal differences between the food and shock US deliveries. This is likely a reflection of the fact that the shock delivery is passive and easily resolved based on

the time of the US delivery, whereas the food responses are variable because they are dependent upon the consumption of the sucrose pellet. Because of these differences the kinetics of the responses cannot be accurately compared. This is why we restricted our analysis to whether the cells were food or shock responsive. Aside from reporting the temporal differences in the signals did not draw major conclusions about the differences in kinetics. In our experimental design we counterbalanced the animals that received fear conditioning first then food conditioning, or food conditioning then fear conditioning to ensure that order effects did not influence the outcome of the study. It is widely known that Pavlovian fear conditioning can facilitate the acquisition of conditioned stimulus responses with just a single day of conditioning. In contrast, Pavlovian reward conditioning generally progresses more slowly. Because of this we restricted our analysis to the last day of reward conditioning to the first and only day of fear conditioning. However, as stated above, we compared the responses of neurons defined as salience during day 1 of reward conditioning and fear conditioning. As would be predicted based on previous definitions of salience encoding (Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031), we observed that the responses were highest when the US was most unexpected

(5) The authors make a claim of valence encoding in their title and throughout the paper, which is not possible to make given their experimental design. However, they would greatly benefit from actually using a decoder to demonstrate their encoding claim (decoding performance for shock-food versus shuffled labels) and simply make claims about decoding food-predictive cues and shock-predictive cues. Interestingly, it seems like relatively few CeA neurons actually show differential responses to the food and shock CSs, and that is interesting in itself.

As stated above, valence and salience encoding were defined similar to what has been previously reported (Li et al., 2019, doi: 10.7554/eLife.41223; Yang et al., 2023, doi: 10.1038/s41586-023-05910-2; Huang et al., 2024, doi: 10.1038/s41586-024-07819; Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031; Stephenson-Jones et al., 2020, doi: 10.1016/j.neuron.2019.12.006; Zhu et al., 2018, doi: 10.1126/science.aat0481; and Comoli et al., 2003, doi: 10.1038/nn1113P). Interestingly, many of these studies did not vary the US intensity.

Reviewer #3 (Public review):

Summary:

In their manuscript entitled Kong and colleagues investigate the role of distinct populations of neurons in the central amygdala (CeA) in encoding valence and salience during both appetitive and aversive conditioning. The study expands on the work of Yang et al. (2023), which specifically focused on somatostatin (SST) neurons of the CeA. Thus, this study broadens the scope to other neuronal subtypes, demonstrating that CeA neurons in general are predominantly tuned to valence representations rather than salience.

We thank the reviewer for their insightful comments and assessment of the manuscript.

Strengths:

One of the key strengths of the study is its rigorous quantitative approach based on the "circular-shift method", which carefully assesses correlations between neural activity and behavior-related variables. The authors' findings that neuronal responses to the unconditioned stimulus (US) change with learning are consistent with previous studies (Yang et al., 2023). They also show that the encoding of positive and negative valence is not influenced by prior training order, indicating that prior experience does not affect how these neurons process valence.

Weaknesses:

However, there are limitations to the analysis, including the lack of population-based analyses, such as clustering approaches. The authors do not employ hierarchical clustering or other methods to extract meaning from the diversity of neuronal responses they recorded. Clustering-based approaches could provide deeper insights into how different subpopulations of neurons contribute to emotional processing. Without these methods, the study may miss patterns of functional specialization within the neuronal populations that could be crucial for understanding how valence and salience are encoded at the population level.

We appreciate the reviewer's comments regarding clustering-based approaches. In order to classify cells as responsive to the US or CS we chose to develop a statistically rigorous method for classifying cell response types. Using this approach, we were able to define cell responses to the US and CS. Importantly, we identified 8 distinct response types to the USs. It is not clear how additional clustering analysis would improve cell classifications.

Furthermore, while salience encoding is inferred based on responses to stimuli of opposite valence, the study does not test whether these neuronal responses scale with stimulus intensity—a hallmark of classical salience encoding. This limits the conclusions that can be drawn about salience encoding specifically.

As stated above, we used salience classifications similar to those previously described (Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031; Stephenson-Jones et al., 2020, doi: 10.1016/j.neuron.2019.12.006; Zhu et al., 2018, doi: 10.1126/science.aat0481; and Comoli et al., 2003, doi: 10.1038/nn1113P). We agree that varying the stimulus intensity would provide a more rigorous assessment of salience encoding; however, several of the studies mentioned above classify cells as salience encoding without varying stimulus intensity. Additionally, the inclusion of recordings with varying US intensities on top of the Pavlovian reward and fear conditioning would further decrease the number of cells that can be longitudinally tracked and would likely decrease the number of cells that could be classified.

In sum, while the study makes valuable contributions to our understanding of CeA function, the lack of clustering-based population analyses and the absence of intensity scaling in the assessment of salience encoding are notable limitations.

Reviewer #4 (Public review):

Summary:

The authors have performed endoscopic calcium recordings of individual CeA neuron responses to food and shock, as well as to cues predicting food and shock. They claim that a majority of neurons encode valence, with a substantial minority encoding salience.

Strengths:

The use of endoscopic imaging is valuable, as it provides the ability to resolve signals from single cells, while also being able to track these cells across time. The recordings appear well-executed, and employ a sophisticated circular shifting analysis to avoid statistical errors caused by correlations between neighboring image pixels.

Weaknesses:

My main critique is that the authors didn't fully test whether neurons encode valence. While it is true that they found CeA neurons responding to stimuli that have positive or negative value, this by itself doesn't indicate that valence is the primary driver of neural

activity. For example, they report that a majority of CeA neurons respond selectively to either the positive or negative US, and that this is evidence for "type I" valence encoding. However, it could also be the case that these neurons simply discriminate between motivationally relevant stimuli in a manner unrelated to valence per se. A simple test of this would be to check if neural responses generalize across more than one type of appetitive or aversive stimulus, but this was not done. The closest the authors came was to note that a small number of neurons respond to CS cues, of which some respond to the corresponding US in the same direction. This is relegated to the supplemental figures (3 and 4), and it is not noted whether the the same-direction CS-US neurons are also valence-encoding with respect to different USs. For example, are the neurons excited by CS-food and US-food also inhibited by shock? If so, that would go a long way toward classifying at least a few neurons as truly encoding valence in a generalizable way.

As stated above, valence and salience encoding were defined similar to what has been previously reported (Li et al., 2019, doi: 10.7554/eLife.41223; Yang et al., 2023, doi: 10.1038/s41586-023-05910-2; Huang et al., 2024, doi: 10.1038/s41586-024-07819; Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031; Stephenson-Jones et al., 2020, doi: 10.1016/j.neuron.2019.12.006; Zhu et al., 2018, doi: 10.1126/science.aat0481; and Comoli et al., 2003, doi: 10.1038/nn1113P). As reported in Figure 5 and Figure 5 – Supplement 3, ~29% of CeA neurons responded to both food and shock USs (15% in the same direction and 13.5% in the opposite direction). In contrast, only 6 of 303 cells responded to both the CSfood and CSshock, all in the same direction.

A second and related critique is that, although the authors correctly point out that definitions of salience and valence are sometimes confused in the existing literature, they then go on themselves to use the terms very loosely. For example, the authors define these terms in such a way that every neuron that responds to at least one stimulus is either salience or valence-encoding. This seems far too broad, as it makes essentially unfalsifiable their assertion that the CeA encodes some mixture of salience and valence. I already noted above that simply having different responses to food and shock does not qualify as valence-encoding. It also seems to me that having same-direction responses to these two stimuli similarly does not qualify a neuron as encoding salience. Many authors define salience as being related to the ability of a stimulus to attract attention (which is itself a complex topic). However, the current paper does not acknowledge whether they are using this, or any other definition of salience, nor is this explicitly tested, e.g. by comparing neural response magnitudes to any measure of attention.

As stated in response to reviewer 2, we longitudinally tracked cells on the first day of Pavlovian reward conditioning the fear conditioning day. Although there were considerably fewer head entries on the first day of reward conditioning, we were able to identify 10 cells that were activated by both the food US and shock US. We compared the responses to the first five head entries and last head entries and the first 5 shocks and last five shocks. Consistent with what has been reported for salience encoding neurons in the basal forebrain (Lin and Nicolelis, 2008, doi: 10.1016/j.neuron.2008.04.031), we observed that the responses were highest when the US was most unexpected and decreased in later trials.

The impression I get from the authors' data is that CeA neurons respond to motivationally relevant stimuli, but in a way that is possibly more complex than what the authors currently imply. At the same time, they appear to have collected a large and high-quality dataset that could profitably be made available for additional analyses by themselves and/or others.

Lastly, the use of 10 daily sessions of training with 20 trials each seems rather low to me. In our hands, Pavlovian training in mice requires considerably more trials in order to

effectively elicit responses to the CS. I wonder if the relatively sparse training might explain the relative lack of CS responses?

It is possible that learning would have occurred more quickly if we had used greater than 20 trials per session. However, we routinely used 20-25 trials for Pavlovian reward conditioning (doi: 10.1073/pnas.1007827107; doi: 10.1523/JNEUROSCI.5532-12.2013; doi: 10.1016/j.neuron.2013.07.044; and doi: 10.1016/j.neuron.2019.11.024).

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