

Necessity and recruitment of cue-specific neuronal ensembles within the basolateral amygdala during appetitive reversal learning

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ABSTRACT

Through Pavlovian appetitive conditioning, environmental cues can become predictors of food availability. Over time, however, the food, and thus the value of the associated cues, can change based on environmental variations. This change in outcome necessitates updating of the value of the cue to appropriately alter behavioral responses to these cues. The basolateral amygdala (BLA) is critical in updating the outcomes of learned cues. However, it is unknown if the same BLA neuronal ensembles that are recruited in the initial associative memory are required when the new cue-outcome association is formed during reversal learning. The current study used the Daun02 inactivation method that enables selective targeting and disruption of activated neuronal ensembles in *Fos-lacZ* transgenic rats. Rats were implanted with bilateral cannulas that target the BLA and underwent appetitive discriminative conditioning in which rats had to discriminate between two auditory stimuli. One stimulus (CS+) co-terminated with food delivery, and the other stimulus was unrewarded (CS−; counter-balanced). Rats were then tested for CS+ or CS− memory retrieval and infused with either Daun02 or a vehicle solution into the BLA to inactivate either CS+ or CS− neuronal ensembles that were activated during that test. To assess if the same neuronal ensembles are necessary to update the value of the new association when the outcomes are changed, rats underwent reversal learning: the CS+ was no longer followed by food (reversal CS−, rCS−), and the CS− was now followed by food (reversal CS+, rCS+). The group that received Daun02 following CS+ session showed a decrease in conditioned responding and increased latency to the rCS− (previously CS+) during the first session of reversal learning, specifically during the first trial. This indicates that the neuronal ensemble that was activated during the recall of the CS+ memory was the same neuronal ensemble needed for learning the new outcome of the same CS, now rCS−. Additionally, the group that received Daun02 following CS− session was slower to respond to the rCS+ (previously CS−) during reversal learning. This indicates that the neuronal ensemble that was activated during the recall of the CS− memory was the same neuronal ensemble needed for learning the new outcome of the same CS. These results demonstrate that different neuronal ensembles within the BLA mediate memory recall of CS+ and CS− cues and reactivation of each cue-specific neuronal ensemble is necessary to update the value of that specific cue to respond appropriately during reversal learning. These results also indicate substantial plasticity within the BLA for behavioral flexibility as both groups eventually showed similar terminal levels of reversal learning.

1. Introduction

Environmental cues can become strongly associated with food if they frequently occur together, and subsequent presentation of these learned cues can lead to food procurement and consumption without hunger (Birch, McPhee, Sullivan, & Johnson, 1989; Holland & Petrovich, 2005; Petrovich, 2013; Petrovich & Gallagher, 2003; Saper, Chou, & Elmquist, 2002; Weingarten, 1983). However, the outcomes, and thus the values, of associated cues are not always static and can change based on

environmental variations. This change in the outcome of a learned cue requires updating the value of the cue to produce appropriate behavioral responses.

The basolateral nucleus of the amygdala (BLA) is a critical forebrain region necessary for associative conditioning and is an early processor of appetitive learning (Cole, Powell, & Petrovich, 2013; Piette, Baez-Santiago, Reid, Katz, & Moran, 2012). The BLA is critically involved in appropriate behavioral responding when the values of learned appetitive cues are changed (Corbit & Balleine, 2005; Nomura, Izaki,

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Takita, Tanaka, & Hori, 2004; Schoenbaum, Chiba, & Gallagher, 1999; Tye, Cone, Schairer, & Janak, 2010; Fisher, Pajser, & Pickens, 2020) or additional cues are incorporated to update the value of learned appetitive cues (Blundell, Hall, & Killcross, 2001; Everitt, Cardinal, Parkinson, & Robbins, 2003; Hatfield, Han, Conley, Gallagher, & Holland, 1996; Holland et al., 2002, 2001; Holland & Petrovich, 2005; Ishikawa, Ambroggi, Nicola, & Fields, 2008; Petrovich, 2013; Setlow, Gallagher, & Holland, 2002; Wassum & Izquierdo, 2015; Hoang & Sharpe, 2021). *In vivo* recording studies have shown that BLA neurons respond to appetitive cues, but then change their response profiles when cue outcomes are reversed (Schoenbaum et al., 1999) and when the reward is omitted (Tye et al., 2010). These studies suggest that the BLA neurons can alter their response to food predictive cues when the outcome changes. It is unknown, however, if the same or different BLA neuronal ensembles that are activated during the recall of learned cues are necessary for updating the values of these cues to form a new association when the outcomes change.

To this end, we used the Daun02 chemogenetic inactivation method (Cruz et al., 2013; Koya et al., 2009) to target BLA neuronal ensembles that are selectively activated by either the CS+ or CS− to determine if these specific neuronal ensembles are necessary to update the new value of the CSs during reversal learning. Specifically, *Fos-lacZ* transgenic rats underwent discriminative conditioning and were then infused with Daun02 or a vehicle solution into the BLA after presentation of either the CS+ or CS− to inactivate the responsive neuronal ensembles. Afterwards, rats underwent either one or fifteen sessions of reversal learning to observe how BLA neuronal ensemble inactivation affected conditioned responding to the initial memory recall of the CSs and during complete reversal learning, respectively. We hypothesized that separate BLA neuronal ensembles are activated during CS+ and CS− memory recall, and inactivating the neuronal ensembles that respond to a particular CS will only alter the memory of that CS and not the other CS. We also predicted that neuronal ensembles that are activated by a particular CS would be necessary to learn the new associations to the same CS when the outcome is changed during reversal learning.

2. Materials and methods

Rats underwent surgery for implantation of bilateral cannulas aimed at the basolateral amygdala (BLA). After recovery, rats underwent ten sessions of discriminative conditioning followed by reversal learning as previously described (Cole, Stone, & Petrovich, 2017; Keefer & Petrovich, 2020). Briefly, each session included 6 presentations of an auditory CS+ paired with the delivery of two food pellets (US) and 6 presentations of a separate, distinct auditory CS− presented alone. Following successful discrimination, rats underwent a brief induction session, which involved 6 presentations of either the CS+ or CS−, followed by infusion of Daun02 or vehicle ninety minutes after the beginning of the session. This induction session ensured selective activation of one CS neuronal ensemble without activating neurons recruited by the other CS or to the US. Next, rats underwent reversal learning where the outcomes of the CSs were reversed. Half of the rats were perfused 90 min after the cessation of the first reversal session (R1) for histological verification of β -gal decrease in Daun02 infused rats (See Supplemental Materials and Methods). The other half received 15 reversal sessions to observe value updating after Daun02 inactivation. The primary measures of learning were the percentage of time rats expressed food cup behavior during the CSs and latency to approach the food cup during the CSs. Brain tissue was processed for double-label fluorescence immunohistochemistry for Fos and β -gal detection (see Supplemental Materials and Methods for details). Notably, we found >65% of β -Gal neurons were also Fos-positive, comparable to prior studies (Bossert et al., 2011; Fanous et al., 2012; Funk et al., 2016).

3. Results

3.1. Histology

Location of injector tips were within or directly above the BLA as shown in Fig. 1. Final group numbers based on proper cannula placements were CS+ Daun02 ($n = 12$ total; $n = 6$ for Reversal Session 1 [R1]; $n = 6$ for Reversal Session 15 [R15]), CS− Daun02 ($n = 11$ total; $n = 5$ for R1; $n = 6$ for R15), and Vehicle ($n = 19$ total; $n = 10$ for R1; $n = 9$ for R15). To verify Daun02 inactivation methodology, the number of β -Gal-labeled neurons was compared between drug treatment groups. Our *a priori* hypothesis was a decrease in the number of β -Gal-labeled neurons in rats that received the Daun02 compared to Vehicle, specifically in the groups that received only 1 session of reversal learning (Fig. 1B, C), and not 15 sessions. This was statistically confirmed (R1: $t(19) = -2.459$, $p = 0.02$; R15: $t(19) = -0.442$, $p > 0.5$). No difference was found between CS+ and CS− Daun02 treated groups (CS+ Daun02: 59.5 ± 3.7 ; CS− Daun02: 51.0 ± 7.2 ; $t(9) = 0.986$, $p > 0.3$). Additionally, no difference was found between the R15 groups, suggesting that potentially 1) additional ensembles were activated as new learning occurred to compensate for the initial neuronal inactivation or 2) the BLA became less involved in extended learning as seen in the decreased β -Gal-labeled neurons from R1 Vehicle group to R15 Vehicle group.

3.2. Discriminative conditioning and induction session

All groups successfully discriminated between the CS+ and CS−, as shown by higher conditioned responding and faster latencies to the CS+ compared to the CS− during the tenth training session (Supplementary Fig. 1 A-C). These results were expected since no drug was given during training, and group allocation was based on drug treatment after the induction session. A group (CS+ Daun02, CS− Daun02, Vehicle) \times CS (CS+, CS−) repeated measures ANOVAs showed an effect of CS Elevation on food cup behavior ($F(1,39) = 331.00$, $p < 0.001$) and an effect of CS on latency ($F(1,39) = 110.85$, $p < 0.001$), but no Group effects or interactions (F 's < 0.5). After discriminative conditioning, rats underwent an induction session with presentation of either the CS+ or CS− (not both) to induce Fos in the BLA in response to the respective CS. Conditioned responding was similar to the last conditioning session with higher responding in the CS+ induction groups compared to the CS− induction groups (Supplementary Fig. 1 D-F). A Drug (Daun02, Vehicle) \times CS Elevation (CS+, CS−) ANOVA during the induction session confirmed an effect of CS ($F(1,38) = 61.50$, $p < 0.001$), but no effect of drug or interaction (F 's < 2.3 , p 's > 0.1). These results were expected since drug infusions occurred after the induction session and confirm similar responding between drug groups within their respective CS induction.

3.3. Reversal learning

3.3.1. Reversal Session 1

The group that received Daun02 following CS+ presentations during the induction session (CS+ Daun02), and thus CS+ neuronal ensemble inactivation, had lower conditioned responding to the same CS, now rCS−, during reversal session 1 (Fig. 2A). Analysis of conditioned responding with a Group \times CS Elevation repeated measures ANOVA showed a significant effect of CS ($F(1,39) = 273.57$, $p < 0.001$), but no effect of Group ($F = 1.80$, $p = 0.18$) or interaction ($F = 2.19$, $p = 0.13$). We analyzed responding to each rCS separately, because of an *a priori* prediction that they would be differently affected based on our design. The analyses on each rCS showed a Group effect on average responding to the rCS− ($F(2,39) = 4.08$, $p = 0.025$), with lower responding in the CS+ Daun02 group compared to the CS−Daun02 ($p < 0.01$) and Vehicle ($p = 0.05$) groups, and no difference between the CS−Daun02 and Vehicle groups ($p > 0.1$). No group differences were found in rCS+ responding ($F < 0.4$, $p > 0.5$). To evaluate the recall of the cue value

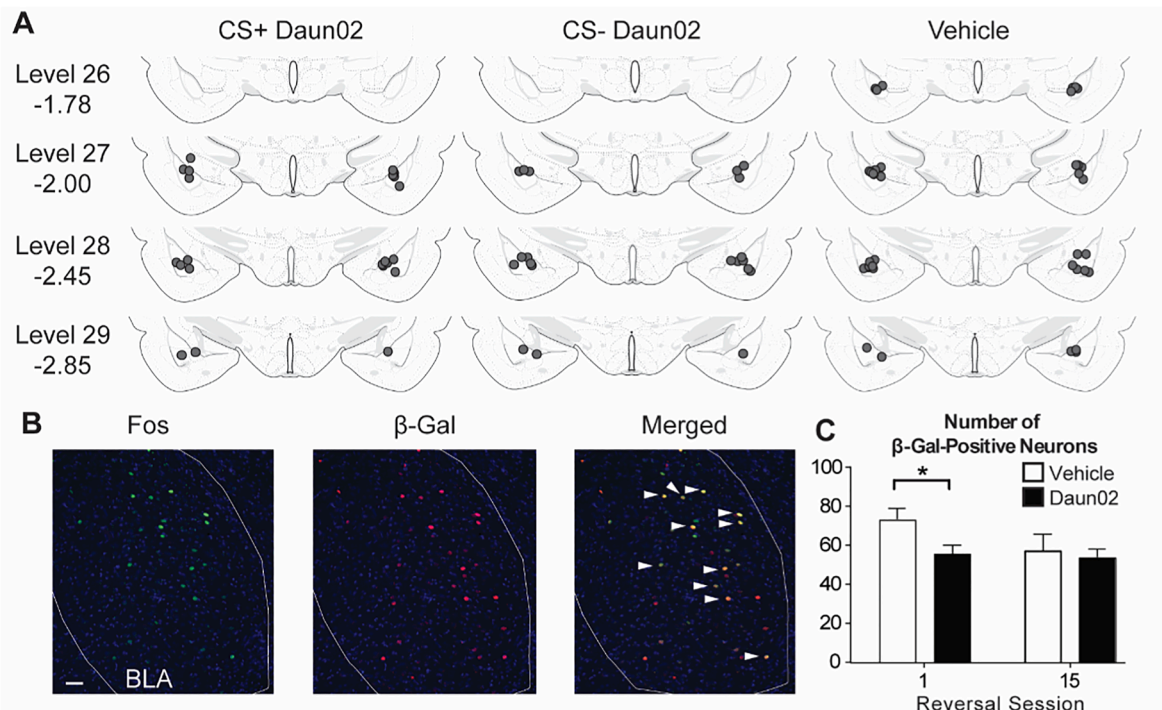


Fig. 1. Cannula placements and Fos and β -Gal induction. (A) All cannula placements were within levels 26–29 of the BLA (–1.78 to –2.85 mm from Bregma as indicated) and similar across drug groups. (B) Representative images showing Fos, β -Gal, and colocalization (white arrows). (C) There was a significant reduction in β -Gal-labeled neurons in the group that received Daun02 and were perfused after the first reversal session, but not the last reversal session. Scale bar = 25 μ m. * $p < 0.05$.

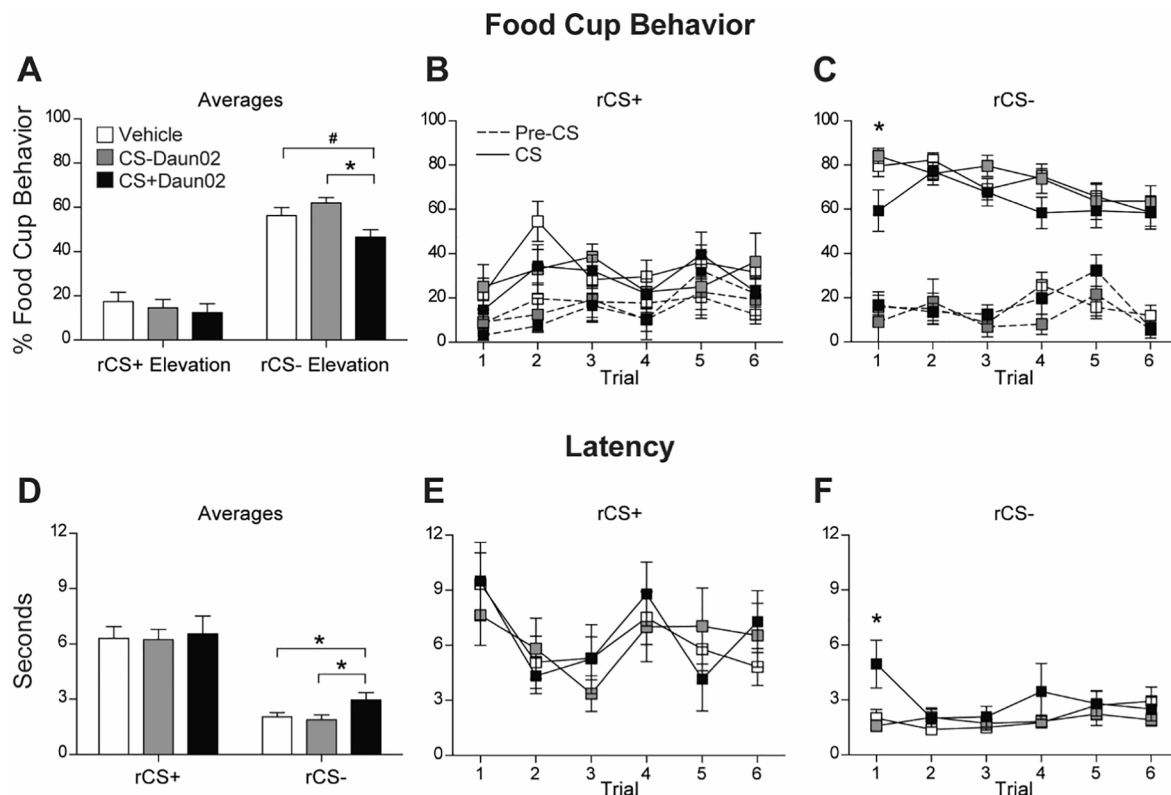


Fig. 2. Conditioned responding during Reversal Session 1. (A) Average food cup responding (mean \pm SEM) during rCS+ and rCS– during Reversal Session 1. Data shown as Elevation score (CS responding minus pre-CS [baseline] responding). (B,C) Food cup responding to each rCS+ trial (B) and rCS– trial (C) during the session. Solid line represents responding during the CS, and dashed line represents responding during the pre-CS (baseline) period. (D) Average latency to approach the food cup (mean \pm SEM) during the rCS+ and rCS– during the session. (E,F) Latency responding to each rCS+ trial (E) and rCS– trial (F) during the session. # $p = 0.05$; * $p < 0.05$.

following Daun02 neuronal ensemble inactivation, we analyzed each trial in the first reversal session with emphasis on the first trial. The CS+ Daun02 group showed lower conditioned responding the first time the CS+, now rCS-, was presented (Fig. 2C). There was a main effect of Group ($F(1,39) = 3.93, p = 0.028$) with the CS+ Daun02 group showing lower conditioned responding to the CS-Daun02 ($p = 0.015$) and Vehicle ($p = 0.023$) groups.

Additionally, we analyzed latency to approach the food cup after the onset of each cue and found rats approached the food cup faster during the rCS- compared to the rCS+, as expected since the rCS- was previously the CS+. A Group \times CS ANOVA showed an effect of CS on overall average latency to respond to the food cup ($F(1,39) = 125.36, p < 0.001$; Fig. 2D) indicating rats approached the food cup faster during the rCS- compared to the rCS+, but there was no Group effect or interaction (F 's $< 1, p$'s > 0.5). We analyzed responding to each rCS separately, because of an *a priori* prediction that they would be differently affected based on our design. The analyses on each rCS showed a Group effect on latency to the rCS- ($F(2,39) = 3.61, p = 0.037$), with slower latencies in the CS+ Daun02 group compared to the CS-Daun02 ($p = 0.021$) and Vehicle ($p = 0.026$) groups, and no difference between the CS-Daun02 and Vehicle groups ($p > 0.1$). No group differences were found in latency to the rCS+ ($F < 0.1, p < 0.5$).

Again, we analyzed the first trial during the first reversal session for latency to the rCS- and found a main effect of Group ($F(2,39) = 5.27, p < 0.01$) with the CS+ Daun02 group showing slower latencies to the rCS- compared to the CS-Daun02 and Vehicle groups (p 's < 0.01 ; Fig. 2F). We found no differences for other rCS- trials or rCS+ trials (p 's > 0.1).

3.3.2. Reversal learning across sessions

We analyzed responding in a group of rats that underwent 15 sessions of reversal learning to determine if inactivation of specific CS BLA neuronal ensembles interfered with updating the new values of the cues during reversal learning. The group that received Daun02 following CS+ induction showed a decrease in conditioned responding to the same CS, now the rCS-, throughout reversal learning (Fig. 3B; Supplementary Fig. 1 G-H). Analysis of responding to CSs with a Group \times CS Elevation \times Session repeated measures ANOVA showed CS Elevation \times Group interaction ($F(2,18) = 7.33, p < 0.01$), and expected main effects of Session ($F(3,54) = 5.23, p < 0.01$) and CS ($F(1,18) = 40.82, p < 0.001$) and a Session \times CS Elevation interaction ($F(3,54) = 81.38, p < 0.001$), but no other effects or interactions (F 's $< 2, p > 0.1$). Follow-up analyses confirmed a significant effect of Group on conditioned responding to the rCS- ($F(2,18) = 5.08, p = 0.018$) with the CS+ Daun02 group showing lower conditioned responding to the rCS-, which was previously their CS+, across reversal learning compared to the CS-Daun02 ($p = 0.010$) and Vehicle ($p = 0.014$) groups. The decrease was specifically during reversal session 1 as described above and session 15 ($F(2,18) = 4.71, p = 0.023$), where the CS+ Daun02 group had significantly lower responding compared to the Vehicle and CS-Daun02 groups (p 's < 0.015).

The analyses on latency responding showed the group that received Daun02 following CS- induction was slower to respond to the food cup after the same cue presentation, now rCS+, during reversal learning (Fig. 3C). A Group \times CS \times Session repeated measures ANOVA found expected main effects of Session ($F(3,54) = 14.51, p < 0.001$) and CS ($F(1,18) = 6.85, p < 0.02$), and a Session \times CS interaction ($F(3,54) = 40.79, p < 0.001$), but no Group effects or interaction (F 's $< 1.5, p$'s $>$

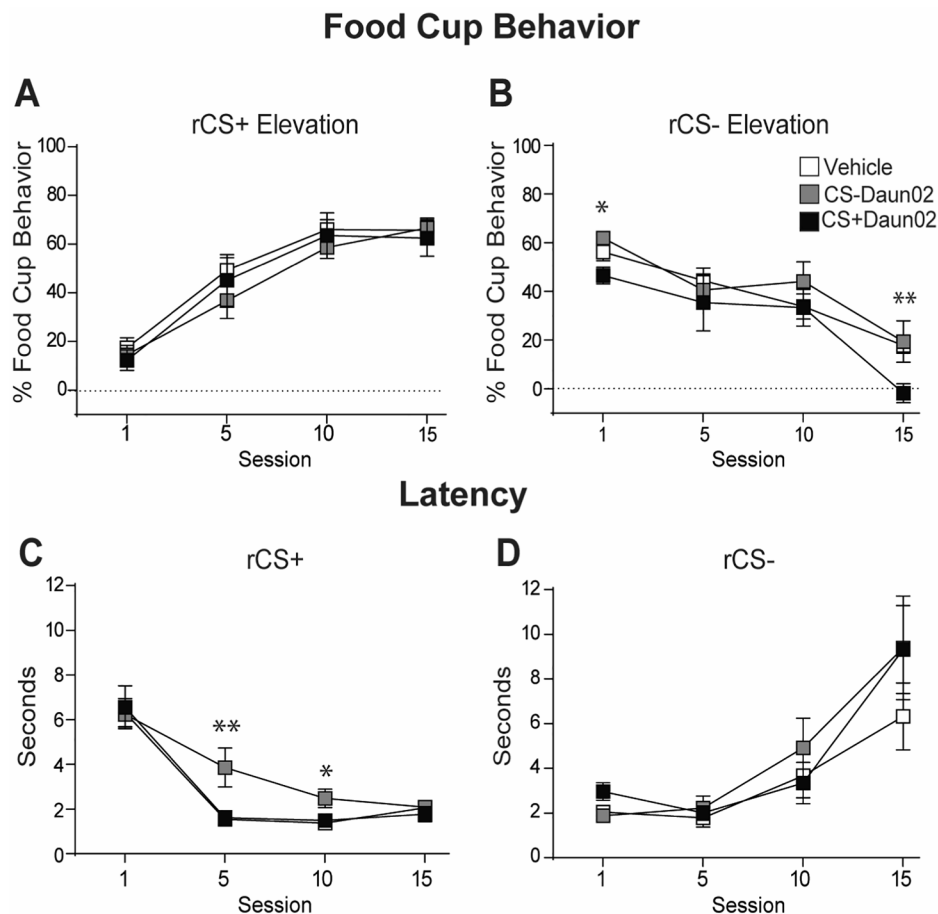


Fig. 3. Conditioned responding throughout reversal learning. (A,B) Average food cup responding (mean \pm SEM) during rCS+ (A) and rCS- (B) throughout reversal learning. Data shown as Elevation score (CS responding minus pre-CS [baseline] responding). (C,D) Average latency to approach the food cup (mean \pm SEM) during the rCS+ (C) and rCS- (D) throughout reversal learning. * $p < 0.05$; ** $p < 0.015$.

0.1). Simple effect analyses showed an effect of Group on responding to the rCS+ during session 5 ($F(2,18) = 7.39, p < 0.01$) and session 10 ($F(2,18) = 4.21, p < 0.05$). The CS-Daun02 group had significantly longer latencies to the rCS+ during session 5 compared to the CS+ Daun02 and Vehicle groups (p 's < 0.01) and session 10 compared to the CS+ Daun02 group ($p < 0.01$) and Vehicle group ($p = 0.037$). We found no differences in latency to respond to the rCS- (p 's > 0.1).

4. Discussion

The current study examined if potentially separate BLA neuronal ensembles that are activated during CS+ and CS- memory recall are necessary for value updating when the CS contingencies are reversed. This was accomplished by testing if inactivating the neuronal ensemble that responds to a particular learned CS altered the memory of that specific CS and not the other CS. Additionally, we examined if CS-specific neuronal ensembles are necessary to learn the new associations to the same CS when the outcome is changed during reversal learning. Using the Daun02 method, we specifically inactivated BLA neuronal ensembles that were activated by either a CS that was previously associated with food (CS+) or a CS not paired with food (CS-), and then evaluated conditioned responding when the outcomes of the cues were switched during reversal learning. We found that the group that received the Daun02 following the CS+ session (CS+ Daun02 group) showed a decrease in conditioned responding to the same CS, now the rCS-, during the first reversal session and specifically during the first presentation of that CS prior to any new information about the outcome. This decreased responding indicates that the original CS+ neuronal ensemble was necessary during recall of previously learned outcome of that cue and to incorporate this information during initial reversal learning. Second, we found that the group that received Daun02 following CS- induction (CS-Daun02 group) was slower to approach the food cup (i.e. longer latency) after the same cue presentation, now the rCS+, during reversal sessions 5 and 10. This slower latency suggests a decrease in motivation to attend to the cue and approach the food reward. Together, these results support our hypotheses that separate BLA neuronal ensembles mediate CS+ and CS- memory recall, and reactivation of each cue-specific neuronal ensemble is necessary for updating the value of that specific learned cue, in order to respond appropriately during reversal learning. Importantly, these results also indicate plasticity of BLA neuronal ensembles when cues' values are altered since all rats eventually showed the same levels of conditioned responding by the end of reversal learning.

The observed impairments in conditioned responding were specific to the CS to which the neuronal ensemble was inactivated and did not cause general impairments in behavioral responding. The CS+ Daun02 group was impaired on responding to rCS-, previously the CS+, and CS-Daun02 group was impaired on responding to rCS+, previously the CS-. This suggests that our preparation inactivated separate CS+ and CS- neuronal ensembles, which impaired subsequent, CS-specific reversal learning. This is in agreement with prior work that found specific effects of neuronal ensembles inactivation by Daun02 (Pfarr et al., 2015). Additionally, other studies have shown altered reward-seeking behaviors due to specific neuronal ensemble inactivation with this method (Caprioli et al., 2017; Cole, Keefer, Anderson, & Petrovich, 2020; Cruz et al., 2014; de Guglielmo et al., 2016; Fanous et al., 2012; Funk et al., 2016; George & Hope, 2017; Koya et al., 2009; Pfarr et al., 2015; Warren et al., 2016; Whitaker et al., 2017; Xue et al., 2017; Josselyn & Frankland, 2018; Josselyn & Tonegawa, 2020).

The results of the current study are in agreement with previous studies that showed separate BLA neuronal ensembles respond to distinct learned cues. Previous studies have shown that ~ 60% of BLA neurons respond to a distinct learned cue during appetitive learning, and then half of these neurons alter their responding when the outcomes are switched during reversal learning in rats (Schoenbaum et al., 1999; Zhang & Li, 2018) and primates (Paton, Belova, Morrison, & Salzman,

2006). Interestingly, in both studies the neurons responded selectively to specific cues prior to correct behavioral performance, indicating BLA neurons are tracking the outcome and the value of learned cues to guide behavior. Similarly, another study showed a subset of BLA neurons (~10%) respond to a well-learned reward predictive cue, but then distinctly alter their responses when food is no longer delivered during extinction ("reinforcement-omission" neurons; (Tye et al., 2010)), suggesting this subset of neurons may be tracking the outcome of the learned cues. This indicates that BLA neurons can alter their responses based on environmental changes, signifying neural plasticity.

The current results indicate that the BLA regulates the updating of the value of learned appetitive cues based on the current outcome. This was confirmed for both groups that received cue-specific neuronal ensemble inactivation by Daun02: the CS+ Daun02 group had lower conditioned responding to the same CS during reversal learning (rCS-), and the CS-Daun02 group was slower to approach the food cup after presentation of the same CS during reversal learning (rCS+). Indeed, previous studies have shown an intact BLA is needed to access the value of the learned cue in order to appropriately update it when the outcome is changed and alter behavioral responding (as reviewed in (Wassum & Izquierdo, 2015)). The BLA encodes the value of the cues during learning (Cole et al., 2013; Esber & Holland, 2014; Parkes & Balleine, 2013; Piette et al., 2012; Schoenbaum et al., 1999; Tye & Janak, 2007; Uwano, Nishijo, Ono, & Tamura, 1995) and is involved in appetitive cue discrimination (Ambroggi, Ishikawa, Fields, & Nicola, 2008; Ishikawa et al., 2008) and reversal learning (Churchwell, Morris, Heurtelou, & Kesner, 2009). However, several studies have shown the BLA may not be critical for initial acquisition of cue value learning (Balleine, Killcross, & Dickinson, 2003; Corbit & Balleine, 2005; Hatfield et al., 1996; Holland, Petrovich, & Gallagher, 2002; Parkinson, Robbins, & Everitt, 2000), but it is critical to encode and assess the representation of the learned associations to alter subsequent behavioral motivation and learning (Blundell et al., 2001; Corbit & Balleine, 2005; Coutureau, Marchand, & Di Scala, 2009; Everitt et al., 2003; Galarce, McDannald, & Holland, 2010; Hatfield et al., 1996; Holland et al., 2002; Holland & Petrovich, 2005; Johnson, Gallagher, & Holland, 2009; Ostlund & Balleine, 2008; Petrovich, 2013; Setlow et al., 2002; Tye & Janak, 2007; Wassum & Izquierdo, 2015; Hoang & Sharpe, 2021; Fisher et al., 2020). This suggests a specific role for the BLA in reward value representation when appetitive learning is altered, in agreement with the current findings.

5. Conclusions

The current study investigated the plasticity across a learning paradigm that requires value updating. We found that inactivation of the BLA neuronal ensemble responsive during a specific learned cue recall resulted in impaired conditioned responding to the same cue during reversal learning without interfering with responding to the other learned cue. These results show distinct neuronal ensembles within the BLA are activated during specific cue memory recall and are necessary to update the value of that cue during reversal learning.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nlm.2022.107663>.

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