Neurons in the Nonhuman Primate Amygdala and Dorsal Anterior Cingulate Cortex Signal Aversive Memory Formation under Sedation

Title Page

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Neurons in the primate amygdala and dorsal anterior cingulate cortex signal aversive memory formation under sedation and anesthesia

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Abstract:

Background: Anesthetics aim to depress consciousness and prevent memory of unpleasant experiences. Patients' explicit recall is rare, but it was suggested that even without explicit recall, implicit memory can form and impact long-term outcomes. The amygdala and dorsal anterior cingulate cortex (a part of the medial-prefrontal-cortex) participate in forging emotional and valence-driven learning, for both explicit and implicit memories. We hypothesized that this circuitry plays an active role in formation of aversive associations under sedation and anesthesia.

Methods: We recorded the activity of single neurons in the amygdala and the dorsal anterior cingulate cortex of sedated and anesthetized monkeys undergoing aversive tone-odor conditioning, and tested retention after recovery. We used ketamine - a non-competitive NMDA-receptor-antagonist; and midazolam - a GABA-co-agonist at a wide range of sedative and anesthetic states.

Results: Aversive memory formation occurred in 26/59 sessions under anesthetics with either drugs. Single-neuron responses in the amygdala and dACC were positively correlated between acquisition and retention (amygdala n =101, r= 0.51 p= $1e^{-9}$; dACC n =121, r=0.32 p= 0.0001 Pearson). Neural responses during acquisition under anesthetics were stronger in sessions exhibiting memory formation (amygdala n=101, p=0.02 dACC n=121 p=0.01). The magnitude of amygdala responses during acquisition was correlated with the magnitude of behavioral retention response (n =101, Pearson, r= 0.22 p= 0.026). Thus, amygdala and dACC responses during acquisition under anesthetics predicted retention. Interestingly, unconditioned responses under anesthetics did not differ in magnitude from saline controls.

Conclusions: Our results suggest that the amygdala-dACC circuit maintains its role in acquisition and maintenance of memories under anesthetic agents and that the stimulus valence is sufficient to drive memory formation under different sedation and anesthesia states. Future monitoring strategies of the amygdala and dACC may help predict and prevent adverse memory formation.

Introduction

Every year, approximately 250 million people undergo anesthesia for surgery [1] while increasing, and by some estimates larger, numbers receive different levels of sedation for a wide range of medical procedures outside the operation room [2,3]. Anesthetics aim to prevent pain, distress and memory of this unpleasant experience. However, in some cases patients report painful and distressing events that they experienced during medical procedures despite having been under sedation or anesthesia. The incidence of this phenomenon during general anesthesia is estimated at 0.1-0.2% of patients and as high as 1-2% in high-risk populations [4,5]. Such episodes may result in post-traumatic stress disorder [6] and other long-term physiological and psychological stress-related effects. Thus, with a limited mechanistic understanding and with limited tools to assess a patient's ongoing awareness and experience *during* the procedure, the evolution of anesthetic practice has been driven almost exclusively by a single variable: amnesia, more specifically the lack of explicit memory.

The absence of explicit memory however, does not ensure that the painful sensation was not experienced [7] and further, that implicit memory was not formed as a result [8]. Implicit memory manifests as an altered response to a previously encountered stimulus independent of conscious awareness. Although some aspects of implicit and explicit memory formation are shared, implicit memory recruits distinct neural mechanisms [9]. Although direct evidence to the extent of implicit memory formation under anesthesia is limited [10,11], previous studies [12,13] suggest a preserved capacity to learn and recall information presented under anesthetics. These studies point to functioning amygdala circuits as the possible substrate [14,15].

The amygdala processes emotionally salient stimuli [16,17] and reciprocal connections with the dorsal anterior cingulate cortex (dACC) a part of the medial prefrontal cortex (mPFC), modulates associations and regulates their expression [18,19]. A properly functioning amygdala-mPFC circuit optimizes the attribution of valence to stimuli and their association, whereas a dysregulated amygdala-mPFC circuit may underlie anxiety disorders and PTSD [20]. Anesthesia may be less effective in preventing implicit memory compared with explicit and contextual memory (for which it has been historically tested), harboring unexpected maladaptive learning leading to psychologic damage [15,21].

We therefore hypothesized that associations and implicit memory formation may occur, at least partially, under sedation and anesthesia; and that neural activity in the amygdala and the mPFC contributes to the acquisition of such memories.

To test this, we recorded single-cell spiking activity from sedated and anesthetized non-human primates while undergoing classical aversive conditioning, a well-established paradigm of associative learning and implicit memory formation [22,23]. We chose two widely used anesthetic agents manipulating two distinct mechanisms, excitatory and inhibitory: ketamine, an NMDA receptor antagonist and midazolam, a GABA co-agonist. We explored a wide range of doses and anesthetic states from mild sedation to a general anesthetic plain. We used a tone-odor conditioning paradigm that relies on respiratory responses as the unconditioned response (UR) and the conditioned response (CR) [24], and therefore does not require consciousness during acquisition.

Materials and methods:

Study design

We aimed to study the effect of anesthetics on stimulus valence, acquisition and memory *in-vivo* and to identify correlates in the mPFC-amygdala circuit using a non-human primate model and clinically relevant doses of anesthetics.

Our hypothesis posited aversive valence and implicit memory formation are maintained under anesthetics and that amygdala-mPFC activity is sufficiently resilient to anesthetics during acquisition to allow later retention.

To this end, two behaving non-human primates engaged in a classical tone-odor conditioning task. This is a useful translational model allowing for invasive neural recording under anesthetics. The paradigm traces respiratory responses and does not require conscious volition making it suitable as an implicit measure of learning and memory in both anesthetized and awake conditions. We simultaneously recorded the responses of single neurons in the amygdala and dACC and compared the behavioral responses to preceding and simultaneous neural responses.

We defined two *a priori* possible outcomes for both the behavioral and neural results: (i) Learning and memory (i.e. acquisition and retention); A statistically significant difference ($p<0.05$) between habituation and retention OR a statistically significant difference between habituation and acquisition and no statistically significant difference between acquisition and retention. (ii) No response; behavioral and neural results not fulfilling the

previous criteria hence NO statistically significant difference between habituation and acquisition and no statistically significant difference between habituation and retention.

Animals

We implanted male *macaca fascicularis* (4–7 kg) with a recording chamber (30 x 30 mm) above the baso-lateral amygdalae and dorsal anterior cingulate cortices under deep anesthesia and aseptic conditions. All surgical and experimental procedures were approved and conducted in accordance with the regulations of the Weizmann Institute Animal Care and Use Committee (IACUC), following NIH regulations and with AAALAC accreditation. Food, water, and enrichments (e.g., fruits and play instruments) were available ad libitum during the whole period, except for the six hours prior to a recording session due to the required anesthesia.

MRI-based electrode positioning

To ensure accurate recordings from the target anatomical structures, we performed MRI scans before, during, and after the recording period using a 3-tesla MRI scanner: (MAGNETOM Trio, Siemens) with a CP knee coil (Siemens). T1 weighted and 3D gradient-echo (MPRAGE) pulse sequence with TR of 2500 ms, TI of 1100 ms, TE of 3.36 ms, 8° flip angle, and 2 averages. We extracted images in the sagittal plane, with a 192x192 matrix and 0.83mm or 0.63mm resolution. We used the first scan before surgery to align and refine anatomical maps for each individual animal (relative location of the amygdala, dACC and anatomical markers such as the inter-aural line and the anterior commissure). This scan guided the positioning of the chamber on the skull at surgery. After surgery, we performed another scan with two electrodes directed toward the amygdala, and another two

at the dACC. Three observers reviewed the scan separately to inspect the images and calculate the amygdala anterior–posterior and lateral-medial borders relative to each of the electrode penetrations and the location at the dACC. We calculated the depth of the two structures from the Dura surface based on the MRI at all penetration points. Clear anatomical markers and visual similarity to identify these structures based on MRI images from primate atlas[25] were used.

Recordings

Respiration: The conditioned response previously described in this paradigm consists of an augmented respiratory response. Hence, we recorded a continuous airway pressure trace throughout the paradigm. We extracted and quantified the volume of each inspiration, peak pressure and rise time.

Detailed descriptions of the odor delivery system (olfactometer) have been previously reported [26]. In brief, three hoses attach to a silicon made nasal mask placed over the monkey's nose. The first hose delivers air into the mask at a constant flow. When stimuli is commanded, silent vacuum solenoids divert away the clean air and allow odorized air into the mask. Importantly, we delivered the odorized air at the same pressure and flow as the clean air, and commanded it from outside the room to guarantee that the monkey receives no cues regarding odor delivery. The aversive odor is discharged for 1sec. The second hose evacuates air from the mask at an equal flow to that delivered into the mask, and quickly removes odors right after their release while maintaining stable pressure within the mask. The third hose is connected to two pressure sensors with different sensitivity range (1⁄4" and 1" H_2O pressure range, AllSensors), that allow measurement of respiratory

behavior with minimal time lag. To load air with odor, filtered air is flowed through a Teflon made odor canister. We used real-time detection of spontaneous inhalation onsets to trigger tones and odors.

Single neuron recordings: The recording chamber allowed simultaneous recording from the amygdala and dACC of both hemispheres. During each recording session, we lowered 4-8 microelectrodes, up to two at each structure (0.6–1.2 M glass/narylene-coated tungsten microelectrode, Alpha Omega or FSH). The monkey's head was fixated and we lowered the electrodes into the brain inside a metal guide (Gauge 25xxtw, Cadence) using a head-tower and electrode positioning system (Alpha Omega). The guide penetrated and crossed the Dura and stopped ~0.5mm in the cortex. We then moved the electrodes independently further into the amygdala and dACC respectively (mapping sessions in each animal were performed moving slowly and identifying electro-physiological markers of firing properties tracking the known anatomical pathway into the target structures). Electrode signals were pre-amplified, 0.3 Hz-6 KHz band pass filtered and sampled at 25 KHz (Alpha Lab Pro, Alpha Omega). We allowed 15 min for the tissue and signal to stabilize before starting the behavioral protocol. At the end of the recording period, we performed off-line spike sorting for all sessions to improve neuron isolation (offline sorter, Plexon Inc).

We synchronized and recorded the behavioral paradigm and all variables using Matlab software and Alpha-Omega analog and digital recorders.

Depth of sedation and anesthesia measurement: We used the Ramsay score [27] to assess the depth of anesthesia during the study sessions: The scale ranges from 1-5 and corresponds to the baseline state of the subject and the level of response to discrete stimuli:

1. Agitated or restless or both, 2. Alert and tranquil, 3. Brisk response to a light glabellar tap or loud auditory stimulus, 4. Sluggish response to a light glabellar tap or loud auditory stimulus, 5. No response to a light glabellar tap or loud auditory stimulus.

Following the injection of an anesthetic, we allowed 5 minutes for induction and clinical effect to take place. An investigator (human sedation and anesthesia experts, N.S. & E.K) then briefly entered the recording chamber and scored the clinical sedation \ anesthesia depth [28].

Behavior

Under sedation or anesthesia at a discrete drug dose, the monkeys engaged a classical conditioning task, a learning paradigm of tone-odor conditioning. The conditioned response (CR) described in this paradigm is increased breath effort in response to the auditory conditioned stimulus (CS), which is locked to a breath onset in preparation for the aversive odor discharge, the unconditioned stimulus (US) timed to the following breath onset. The unconditioned response is decreased breath volume in response to the noxious odor. We performed the experiment and recorded variables while the monkeys sat in a customized chair, in a dark, acoustically isolated room. We placed a mask over the monkey's nose for respiratory measurements and odor delivery.

Experimental sessions consisted of habituation to tones, injection of anesthetics, and the acquisition of tone-odor associations followed by testing retention of learned associations.

During habituation, an auditory conditioned stimuli (CS) was generated randomly at the range of 500-5000Hz. CS differed from CS of the previous session by 500Hz and not

repeated in additional sessions during the following 2 weeks. Tones were pure sinus waves of 1000 ms duration with 5ms onset and offset ramps, generated with a standard computer and delivered through a speaker (Adam 5 studio monitor, ADAM Audio) located 40 cm behind and to the center of the animal at 75dB. During habituation, the daily CS was presented 10 times (inter-trial interval (ITI) approximately 60s).

Following habituation, we delivered anesthetics by intra-muscular injection. We used ketamine, an NMDA receptor antagonist, and midazolam, a benzodiazepine GABA co-agonist. We used four different doses of each drug (1, 2, 4 and 8 mg/kg, and 0.1, 0.2, 0.4 and 0.8 mg/kg for ketamine and midazolam respectively) as well as a normal saline control condition. The dose range aimed to include light sedation to deep anesthesia according to estimates from human and primate studies [29-31]. In addition to existing data we also evaluated the drug dose/response effects on 3 monkeys according to the Ramsay sedation scale[27].

During the experiment period, we randomized the order of testing sessions for drug and dose to avoid the potential bias of a consistent increase or decrease of doses or desensitization to a given agent throughout the study period.

After injection, we allowed 5 minutes for the gradual rise of drug blood and CNS levels to effective concentrations and clinical effect to take place.

Acquisition followed by presenting 12 CS/US pairs randomly intermingled with five unpaired CS presentations (ITI approx. 60s). For the aversive odor, we chose a 1:20 solution of propionic acid (Sigma-Aldrich) diluted in mineral oil, a highly aversive agent

to monkeys and humans that triggers both olfactory and trigeminal receptors. This specific solution has been previously tested in this behavioral paradigm [24].

We tested retention 45 minutes from the time of injection, after recovery, with 10 presentations of CS (ITI approx. 60s). This interval was chosen after pre-experiment trials revealed it was the maximal time required for return to baseline behavior (A Ramsay score [27] of 1-2) and is in-line with similar reports [31].

Statistical analysis

We measured breath augmentation by quantifying peak inspiratory pressures, time to peak pressures (the inverse being inspiratory velocity) and inspiratory volumes during the first 40% of the respiratory cycle (the average inspiratory phase). These measures are commonly used to assess breath dynamics and responses [26,32]. As anesthetics may significantly modulate respiration, we corrected the respiratory variables to avoid direct drug effects on our results. To correct for effects on the absolute inspiratory pressures, the pressure traces were z-scored using the pre-stimuli, inter-trial interval as a reference baseline. To correct for possible direct drug effects on the respiratory rate, time points along the respiratory cycle were normalized to cycle length and reported as a fraction of the respiratory cycle. We evaluated differences in response to stimuli across habituation, acquisition and retention according to peak inspiratory pressures and inspiratory velocity [32] using multivariate analysis of variance (MANOVA).

Respiratory rate changes in response to anesthetics:

As a measure of anesthetic effect on respiratory rate, we sampled the pre-stimuli respiratory rates during the acquisition phase and compared them to the pre-stimuli respiratory rates during the habituation phase prior to anesthetics using the paired t-test.

Neuronal Responses:

For each trial, we calculated the response to stimuli as the absolute difference in pre and post stimuli firing rates divided by their sum.

$$TR = | (FR_{Post} - FR_{Pre}) / (FR_{Post} + FR_{Pre}) |$$

TR trial response, FR firing rate Pre/Post refers to stimulus

We chose a time window of 500ms pre and post stimuli. This is in line with previously described epoch of neural responses to fearful stimuli [33]. When sorting there is an inherent tradeoff between sensitivity (in our case utilizing all "true" neurons) and specificity (in our case using only "true" neurons). We explicitly favored specificity. Previous works used a cutoff of 1Hz average firing rates [34]. Given that anesthetics, especially midazolam, may (and did see figure 1.C) lower baseline firing rates we chose a cutoff of 0.5Hz. We included only neurons that maintained a median firing rate of >0.5Hz throughout the paradigm, during habituation, acquisition and retention.

In a similar fashion to the behavioral analysis, differences in response to stimuli across habituation, acquisition and retention were evaluated using analysis of variance (ANOVA). We calculated the neuron strength of acquisition and retention as the mean response during the respective phases across trials.

Firing rate changes in response to anesthetics:

As anesthetic effect on neuronal firing rate, we sampled the pre-stimuli firing rates during the acquisition phase and compared them to the pre-stimuli firing rates during the habituation phase prior to anesthetics using the paired t-test.

We calculated the behavioral and neuronal strengths of acquisition and retention as their mean responses during the respective phases across trials. We used the Pearson correlation to test their association.

We evaluated differences between results in multiple subgroups (e.g. doses, drugs etc.) using ANOVA. When comparing two discrete groups (e.g. outcome A Vs. outcome B), we used the two-sample t-test.

We considered p. values<0.05 significant. Where relevant we corrected for multiple comparisons. We excluded from analysis trial outliers (not whole neurons or sessions) deviating more than 3SD from the mean.

Results

We injected two *Macaca fascicularis* monkeys (Monkey $S_{n\ sessions} = 26$, Monkey $L_{n\ sessions} = 42$) with low (1-2 mg/kg ketamine or 0.1-0.2 mg/kg midazolam) or high (4-8 mg/kg ketamine or 0.4-0.8 mg/kg midazolam) doses of anesthetics. Following injections, monkeys underwent tone-odor (CS-US) aversive conditioning. We used the respiratory pattern to detect successful conditioning (CR) as shown in previous studies [24,26].

Modified Ramsay sedation scale [27] was measured 5 minutes after injection and showed a dose dependent effect of drug on behavioral parameters (low vs. high dose 2.9±0.13, 4.4±0.11 mean ± SEM, one way ANOVA, df = 4, F= 23.4, p = 9.9725e-09; figure 1.A). Midazolam and ketamine produced similar effects on the depth of anesthesia as measured by Modified Ramsay sedation scale (mean+/-SEM, 3.7+/-0.18, 3.5+/-0.18 respectively, post-hoc test, p = 0.95; figure 1.A). As expected, respiratory rates under anesthetics decreased (figure 1.B), absolute firing rate under midazolam slightly decreased and absolute firing rates under ketamine slightly increased (figure 1.C).

We recorded a total of 68 sessions, 119 amygdala and 132 dACC neurons met inclusion criteria (see methods),

Associative learning and implicit memory formation under anesthetics

The respiratory pattern measured by pressure sensors was used to identify conditioning: a change following the CS is indicative of successful conditioning (namely, a CR). Each daily session began with habituation to a novel CS tone followed by drug injection. We allowed 5 minutes after injection, for the drug effect to reach a stable sedative \ anesthetic

plain and assessed the depth of sedation using a modified Ramsay score (see methods). Anesthetic induction was followed by an acquisition phase where CS-US pairs were presented 12 times. The animals were then allowed to naturally recover from the drug, and once fully alert, but not before 45 minutes have passed, we presented the CS 10 times to test retention of the learned association (figure 1.D).

We found that significant CRs during retention existed in 44% of all anesthesia sessions, suggesting memory formation under both types of anesthetics and in all doses (n=59, MANOVA, see examples and cumulative incidence figure 2.A, C). Interestingly, this result was not significantly different from following injection of normal saline (44.4%, Chi^2 p = 0.98).

Anesthetics may impair motor responses and hence acquisition of conditioned responses is usually examined just after recovery [23]. In the current case, the use of a tone-odor paradigm allowed us to overcome this challenge by measuring breathing responses and so we turned to examine the behavioral dynamics of learning under anesthetics. We observed a gradual development of the conditioned response throughout acquisition, and then a gradual decrease during retention likely due to the unpaired CS presentations (namely, an extinction-like process) [35]. Interestingly, sessions that did not culminate in memory formation also displayed a gradual (albeit smaller) acquisition response. The behavioral response to the CS during acquisition under anesthetics was not statistically different between sessions with, or without subsequent retention (two sample t-test p.= 0.25) suggesting that behavioral responses under anesthetics may not accurately predict later retention and memory formation (figure 2.B).

In sessions with subsequent retention (n=26), the magnitude of the CR during acquisition was positively correlated with the magnitude of the CR during retention [24] (Pearson correlation, r = 0.62, p = 0.0008). This was not the case in sessions when retention was not evident (n=33, r = -0.06, p = 0.74, figure 2.D) or between habituation and retention (n=26, r=0.28 p=0.15) suggesting an effect of learning. The correlation between the strength of acquisition under anesthetics and the strength of the subsequent retention provides additional support for the link between the two, and further suggests that memory strength/magnitude is also maintained [35] (figure 2.E).

In sum, we found behavioral evidence of learning and aversive memory formation under anesthetics with both types of drugs from minimal sedation to general anesthesia. Although ketamine and midazolam modulate different systems (excitatory vs. inhibitory), we observed similar levels of retention after recovery with both drugs.

Neural activity in the amygdala and dACC signal acquisition

To investigate the neural dynamics of memory formation under anesthetics, we recorded the activity of single neurons in the amygdala and dACC simultaneously (n=101 & 121, respectively).

Responses of single neurons were measured as the absolute difference in firing rates between pre- and post- stimuli and divided by their sum (response index). We found that responses during acquisition were higher in sessions with behavioral CRs, namely, sessions exhibiting memory formation under anesthetics (sessions with CRs vs. sessions with no CRs, t-test, n=101, p=0.02 and n=121 p=0.01 for the amygdala and dACC, respectively). Interestingly, dACC neurons, but not amygdala neurons, exhibited this difference also

during retention (t-test, p=0.003, p=0.09 for the dACC and amygdala, respectively; figure 3A).

To explore learning and memory at the single cell level we divided the responses into two possible outcomes - similar to the behavioral data; no change in responses to the CS or a development of significant response during acquisition that extended into retention (figure 3B). Similar to behavior, both these outcomes were present under anesthetics with both drugs, in all doses and across the sedation-anesthesia continuum (ANOVA over habituation-acquisition-retention). Overall, 21% of amygdala neurons and 21% of dACC neurons showed significant changes in firing rates during acquisition under anesthetics and the following retention (Binomial test, $p_{amygdala} = 2.6*10^{-6}$ and $p_{dACC} = 6.4*10^{-3}$, see example and cumulative incidence figure 3B, C).

Neural activity during acquisition under anesthetics predicts later retention

To further test whether acquisition responses extend to the retention following recovery from anesthesia, we tested the correlation between neural responses in acquisition and retention. Single-neuron response magnitudes in the amygdala and dACC were positively correlated between acquisition and retention (amygdala n =101, Pearson, r= 0.51 $p= 1e^{-9}$; dACC n =121, r=0.32 p= 0.0001 Pearson, figure 3D). This correlation does not seem to stem from a baseline response to the CS, since the correlation between habituation and retention responses to the CS was much weaker (amygdala r=0.16 p=0.1 dACC r=0.19 p=0.03) and increased significantly in the amygdala during conditioning (Fisher's transformation amygdala p = 0.005, dACC p = 0.28) suggesting an effect of learning.

The magnitude of neural responses during acquisition was correlated with the magnitude of behavioral retention responses, but only in the amygdala (amygdala n =101, Pearson, r= 0.22 p= 0.026; dACC n =121, r= -0.13 p= 0.15, figure 3E). This result provides important evidence for the link between amygdala activity during acquisition under anesthetics and the memory as tested during awake retention.

Because amygdala and dACC synchrony in response to the CS has been implicated in aversive memory formation in awake conditions [24], we turned to look at the signal-correlation between all possible pairs of neurons within the amygdala-dACC circuit under anesthetics (excluding pairs recorded on the same electrode). Indeed, during acquisition, we found correlated activity in dACC-dACC pairs (n= 142, r= 0.3 p= 2.1e-04, Pearson), amygdala-amygdala pairs (n=81, r= 0.25 p= 0.023, Pearson) and amygdala-dACC pairs (n=221, r= 0.26 p= 6.83e-05, Pearson, figure 3F).

In sum, we found that a significant portion of single neurons in both regions exhibit progressive changes in response to the CS and corresponding behavioral evidence of memory formation. Amygdala and dACC responses were elevated during acquisition when memory was successfully formed under anesthetics and synchrony between neuron pairs within the amygdala-dACC circuit was maintained.

Aversive valence supports memory formation under anesthetics

Anesthetics may affect learning and memory by interfering with the acquisition process itself and/or by attenuating the valence of the aversive stimulus. To distinguish between these two options, we tested if the anesthetics modulated the response to the US (aversive odor) and whether this modulation affected memory formation.

The stereotypical unconditioned response (UR) to the aversive odor in our paradigm is a reduction of inhale volume once the odor is encountered (see cumulative incidence and examples figure 4A, B). Importantly, this response did not differ between the different anesthetic conditions (agents and doses; ANOVA p. = 0.14, figure 4D top left), and yet we found that inhale volumes in response to the aversive odor were 9.5% lower in sessions with no retention when compared to sessions with successful retention (t-test p=0.01 figure 4D top right). Nevertheless, this was not the case in high dose (deeper sedation) sessions, (namely, no difference in UR between session with to without retention), suggesting that under deep sedation/anesthesia, the behavioral response fails to predict subsequent memory. This can result from differences in perception and/or different levels of arousal.

Notably, we did not observe any habituation of the UR under anesthetics, and in contrast, an escalating trend towards the end of acquisition in sessions with retention (*paired t-test*, p=0.08). Moreover, responses at the end of acquisition were more robust in sessions with successful retention (t-test, $p=8.08e^{-04}$). This dynamics may stem from US facilitation [36] that in turn leads to stronger association and more reliable memory formation.

We evaluated single neuron responses to the aversive odor (US) (see examples figure 4C). Neural responses to the US did not differ in magnitude between anesthetics and saline controls (wake vs. anesthesia, p=0.12 and p=0.23 for the amygdala and dACC, respectively). Yet neural responses to the US under midazolam were significantly higher than under ketamine (t-test, p=9.04e-18 and p=1.1e-11[1] for the amygdala and dACC, respectively). As expected, neural responses to the US were more robust in sessions culminating in successful retention than those that did not (t-test, $p=5.2e^{-5}$ and p=0.002 for the amygdala and dACC respectively, fig. 4C).

To conclude, we found evidence in both the behavioral response and the neural response that the aversive valence is preserved under anesthetics in both agents, and its magnitude contributes to memory formation.

Discussion:

In this study, we demonstrated implicit aversive memory formation occurring under sedation and anesthesia in non-human primates using two commonly used anesthetics that leverage two distinct mechanisms, GABA and NMDA transmission. We also noticed a maintained representation of aversive valence despite anesthetics administration, suggesting that anesthetics directly affect memory formation.

We recorded changes in neural responses in the amygdala and dACC that varied by the different behavioral outcomes and correlated with aversive stimulus association and successful implicit memory formation. The large proportion of neurons showing these changes supports the assumption this is indeed a manifestation of memory formation.

NMDA and GABA communication have an essential and extensive role in memory formation including in the dACC and amygdala. Targeting these two structures and these two mechanisms offered a natural hypothesis.

A large body of rodent studies has suggested that anesthetics negatively affect acquisition and retention of learned associations [23]. Yet a number of studies did suggest simple associative learning to be possible under anesthetics [37,38] . Indeed, in our study, although retention was successful in approximately 44% of sessions and occurred at all anesthetic depths it was not uniform. This allowed us to compare different aspects of successful versus aborted implicit memory formation under anesthetics.

Interestingly, we found the trajectories of learning and memory under anesthetics to be similar to those observed in awake animals. We found incremental acquisition slopes under

anesthetics followed by decrement responses of extinction once awake and found the behavioral response during acquisition under anesthetics correlated the strength of retention following it. This suggests learning and memory under anesthetics follow similar rules to pharmacologically naïve conditions and that the function of structures and circuits serving these processes remains conserved despite the presence of anesthetics.

The amygdala is considered sufficient for encoding simple aversive associations [39]. It gathers and associates diverse sensory inputs [16].This suggests that regardless of global neural deficits induced by anesthetics and more specifically hippocampal deficits [15], amygdala function under anesthesia may suffice for associative memory formation. Previous studies have shown that shielding the amygdala from anesthetics by local injection of antagonists enables acquisition under anesthesia [40].

The mPFC and more specifically the dACC forms a tight circuit with the amygdala and is thought to appraise and regulate its acquired inputs and associations [20]. Unlike the amygdala, only a few studies focused on mPFC function under anesthesia [41]. These were inconclusive and demonstrated cases where the activity decreased [42] whereas in other cases it was maintained [41].

In our study, the magnitude of amygdala and dACC responses to the CS during acquisition correlated with their response to the CS during retention testing following recovery. Furthermore, amygdala acquisition responses under anesthetics correlated in magnitude with behavior following recovery. Elevated and incremental amygdala and dACC responses to the CS during acquisition under anesthetics correlated with the behavioral trajectory and heralded successful memory retention following recovery. This suggests that

maintained amygdala and dACC function is both possible and necessary for acquisition under anesthetics and that monitoring their activity under anesthetics may serve to predict future memory. Noteworthy, unlike amygdala neurons, which only responded preferentially during acquisition, in retention positive sessions, dACC neurons showed elevated responses to the CS during retention testing after recovery as well which may hint at an extended role for the dACC in consolidation and retrieval.

When assessing simultaneous activity in pairs of neurons, a significant correlation in inter-amygdala, inter-dACC and amygdala-dACC activity emerged. This is in line with previous findings of the role of amygdala-dACC synchrony in aversive learning [24] and suggests a functioning dACC-Amygdala circuit under anesthetics.

When anesthetics affect memory formation, a seminal question is whether the effect stems from a change in the emotional state, a diminished integration of aversive stimuli or from a diminished integration and association of the environment presented. These options are not mutually exclusive. Although it is well accepted that primary representations of stimuli persist under anesthesia [43] the level of stimuli integration remains an open question [44]. Eloquently stated, stimuli are often assumed to be "received but not perceived" [45]. Our results suggest that under the continuum of sedation to anesthesia, stimuli not only reach primary cortices but also go further upstream and are integrated by secondary association cortices and nuclei.

An accurate attribution of salience by amygdala neurons to the aversive stimulus is required to drive learning and memory and for the transfer of salience to the conditioned stimulus [46]. Studies that assess the direct effect of anesthesia on aversive valence are relatively few

and often contradicting [47,48]. We chose an olfactory stimulus, a sensory modality well suited for dissecting both valence and intensity [49]. The aversive nature of the chosen odor is based on previous studies [17,24,26] in awake animals. Although we did not compare aversive and rewarding (e.g. appetitive) stimuli, our results suggest this known aversive stimulus maintains its salience under anesthetics.

Our study has several limitations. Our sample size did not allow for a quantitative comparison between anesthetic conditions. Despite this, we show implicit learning under all anesthetic conditions, a continuum from awake animals through increasing levels of sedation to full anesthesia. We chose ketamine and midazolam for their different mechanisms and their ubiquitous clinical use in a variety of settings yet our results may not apply to other agents.

Our results suggest that implicit memory formation under anesthetics is likely in clinical setting. Intact aversive valence precedes implicit memory formation, as reflected in behavioral responses and more robustly in neural responses to the US suggesting it is sufficient to drive memory formation. The patterns we observed are similar to those found in conditioning studies in wake animals suggesting implicit aversive memory may be resilient to anesthetics. A major strength of our study lies in pairing behavior and invasive electrophysiological recordings of a non-human primate brain under increasing levels of sedation and anesthesia by commonly used anesthetics, mechanisms and doses [50]. This improves the translatability and generalizability of our results and may help to bridge the gap between the methodological and ethical limitations of human studies and the limitations of rodent studies caveated by evolutionary distance.

Conclusion:

Our study suggests that under sedation and anesthesia implicit aversive memory formation, as well as the integration of stimuli persist. Acquisition and retention of aversive information seems to follow similar rules and engage the same structures and mechanisms as those described in awake animals. We show patterns in the amygdala-dACC circuit that predict this and may serve future monitoring strategies of anesthetized patients.

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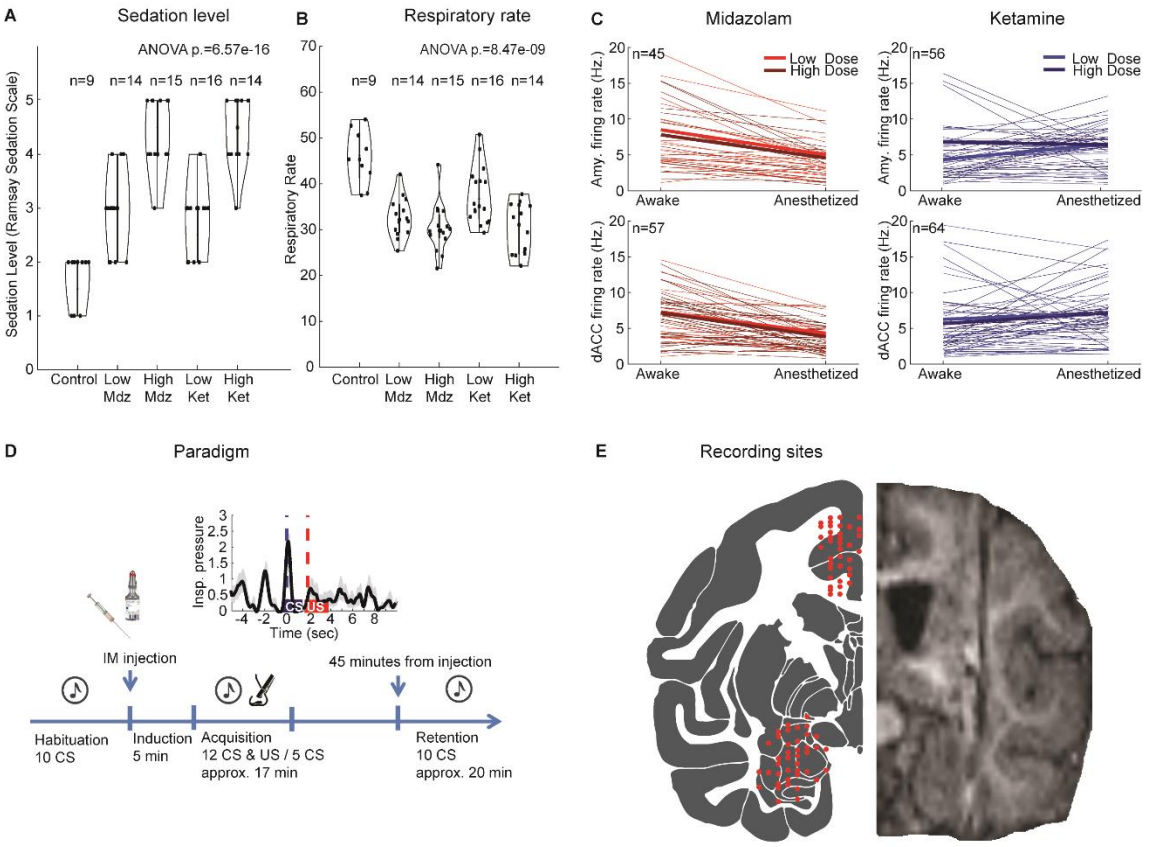


Figure 1. Experimental paradigm and baseline physiology under sedation and anesthesia.

A. The Ramsay sedation scale[27] was evaluated by two senior human anesthesiologists (N.S. & E.K; Scale: 1. Agitated or restless or both, 2. Oriented and tranquil, 3. Brisk response to a light glabellar tap or loud auditory stimulus, 4. Sluggish response to a light glabellar tap or loud auditory stimulus, 5. No response to a light glabellar tap or loud auditory stimulus). Circles indicate individual sessions; bars present median +/-IQR. Similar sedative effects were observed for midazolam and ketamine in low and high doses respectively.

B. Respiratory rates per-session (dots) with medians +/-IQR for both agents and doses. No major differences were observed across anesthetic conditions.

C. Firing rate changes from awake to under anesthetics, shown for all neurons in both regions, both agents, and the two doses (thick line marks the mean in each panel). As previously reported, Midazolam induced a reduction in most neurons, whereas ketamine induced an increase.

D. Paradigm flow: sessions consisted of habituation to tones, induction of sedation/anesthesia, acquisition of tone-odor associations, a pause for recovery and then testing for retention of associations. During habituation, an auditory conditioned stimulus (CS) was presented 10 times. Sedation and anesthesia were induced by intra-muscular (IM) injection and allowing 5 minutes for induction. Acquisition followed by presenting 12 CS-US pairs, randomly interleaved with 5 unpaired CS presentations (namely, partial reinforcement). Retention of 10 CS presentations was tested 45 minutes from the time of injection following recovery. The Inset shows a typical session displaying conditioned and unconditioned responses. The conditioned response (CR) is an increase in inhale volume following the tone CS, delivered locked to breath onset. The aversive-odor (US, red) delivery is locked to the next breath onset. The unconditioned response (UR) is a decrease in volume.

E. Left, A coronal representation of recording sites superimposed on an anatomical plate of M. Fascicularis [25], reconstructed from MRI with positioning electrodes and grid-alignment (example, Right).

(Mdz. Midazolam, Ket. Ketamine, Insp. Inspirium)

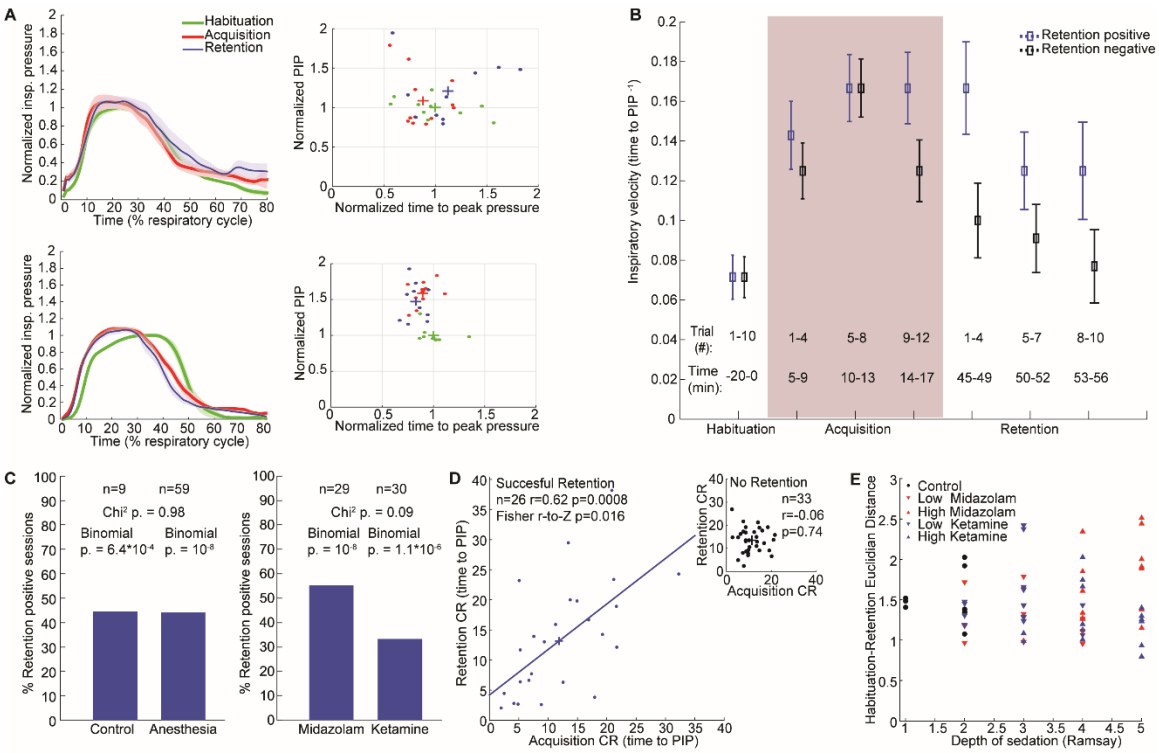


Figure 2. Implicit memory formation under anesthetics.

A. Representative examples of conditioned responses (CR) during habituation, acquisition and retention; Left column: mean+/- SEM over trials; Right column: showing peak pressure and time-to-peak for all trials. Top row: a session where acquisition or retention did not occur. Bottom row: a session showing evidence for acquisition and retention.

B. Conditioned responses (CR) under anesthetics during acquisition and retention, sorted according to the session classification, namely separately for sessions where there was vs. when there was no evidence for retention. The CR increases with acquisition and decrease following unpaired CS presentation during retention (suggesting extinction). The conditioned response is higher in retention positive sessions (blue), and importantly, already during the end of the acquisition i.e. under sedation/anesthesia.

C. Proportion of sessions showing successful retention post-anesthetics.

D. CR during acquisition plotted against the CR during retention, shown per session (averaged over trials). A positive correlation is seen in sessions when retention was evident (blue main plot, Pearson), but was absent when in sessions where no retention was evident (black, inset).

E. The difference between the acquisition CR and the retention CR plotted against the depth of sedation and anesthesia.

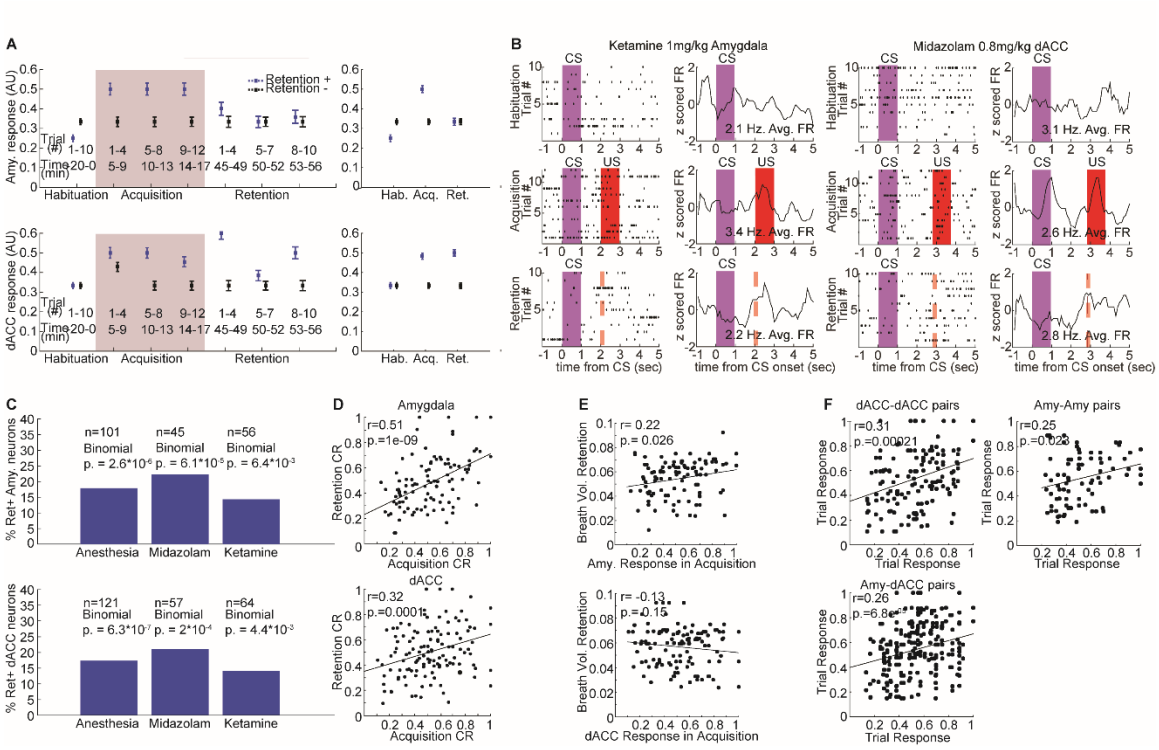


Figure 3. Neural correlates for memory formation under anesthetics.

A. Neural responses during acquisition and retention presented separately for session with retention and sessions with no evidence for retention. Main plots show time from drug administration. Right insets show results pooled across stage (median+/-SEM). Amygdala (top) and dACC (bottom) neurons responded more during acquisition under anesthetics in sessions with retention. Moreover, in retention positive sessions, dACC responses remain elevated during awake retention testing.

B. Example of amygdala neuron under ketamine (1mg/kg, left) and a dACC neuron under midazolam (0.8mg/kg, right), showing evidence of acquisition and retention. Notice the response to the expected (but nonexistent) US during retention.

C. Proportion of neurons with significant responses to the CS during post-recovery retention trials, for Amygdala (top) and dACC (bottom).

D. Single neuron responses (averaged over trials) in acquisition against retention (Pearson), for Amygdala (top) and dACC (bottom).

E. Single unit responses during acquisition plotted against the behavioral response (CR) during retention, for amygdala neurons (top) and dACC neurons (bottom). Amygdala response magnitude to the CS during conditioning under anesthetics correlates the magnitude of post-recovery retention behavioral responses.

F. Pairwise activity (signal correlations) for intra-dACC, intra-amygdala and amygdala-dACC pairs during acquisition under anesthetics (Pearson).

(CS conditioned stimulus, US unconditioned stimulus, Ret. Retention, Vol. volume, Amy amygdala, FR firing rate)

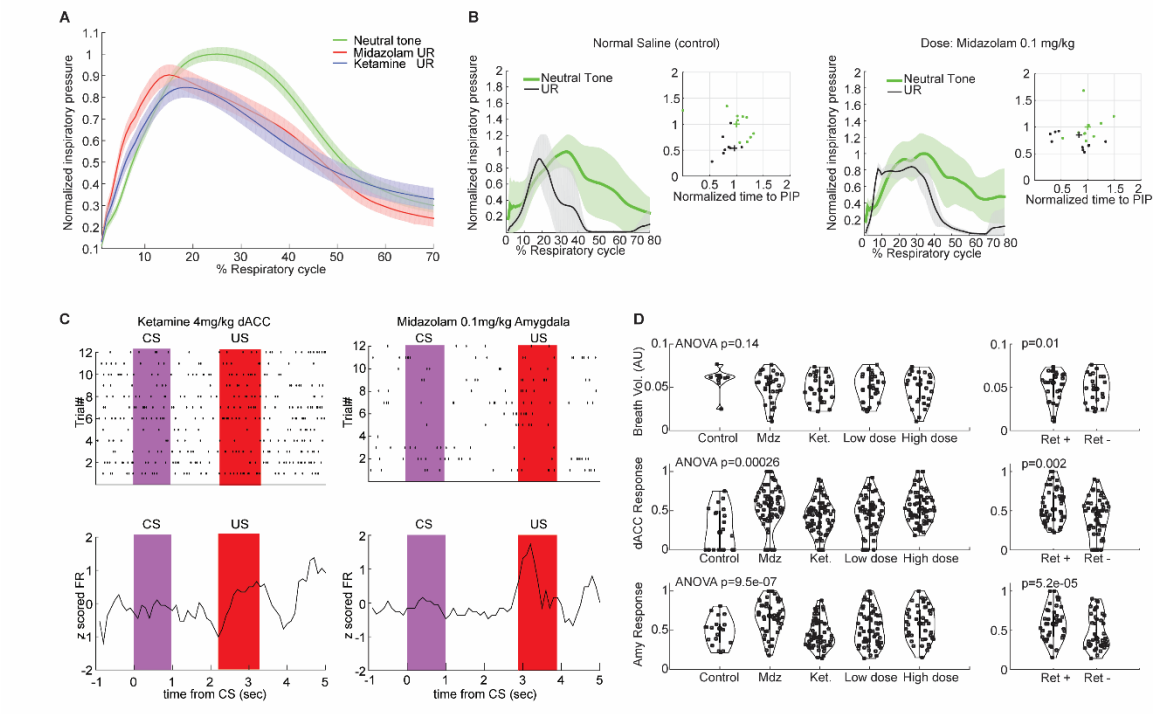


Figure 4. Behavioral and neural representation of valence under sedation and anesthesia.

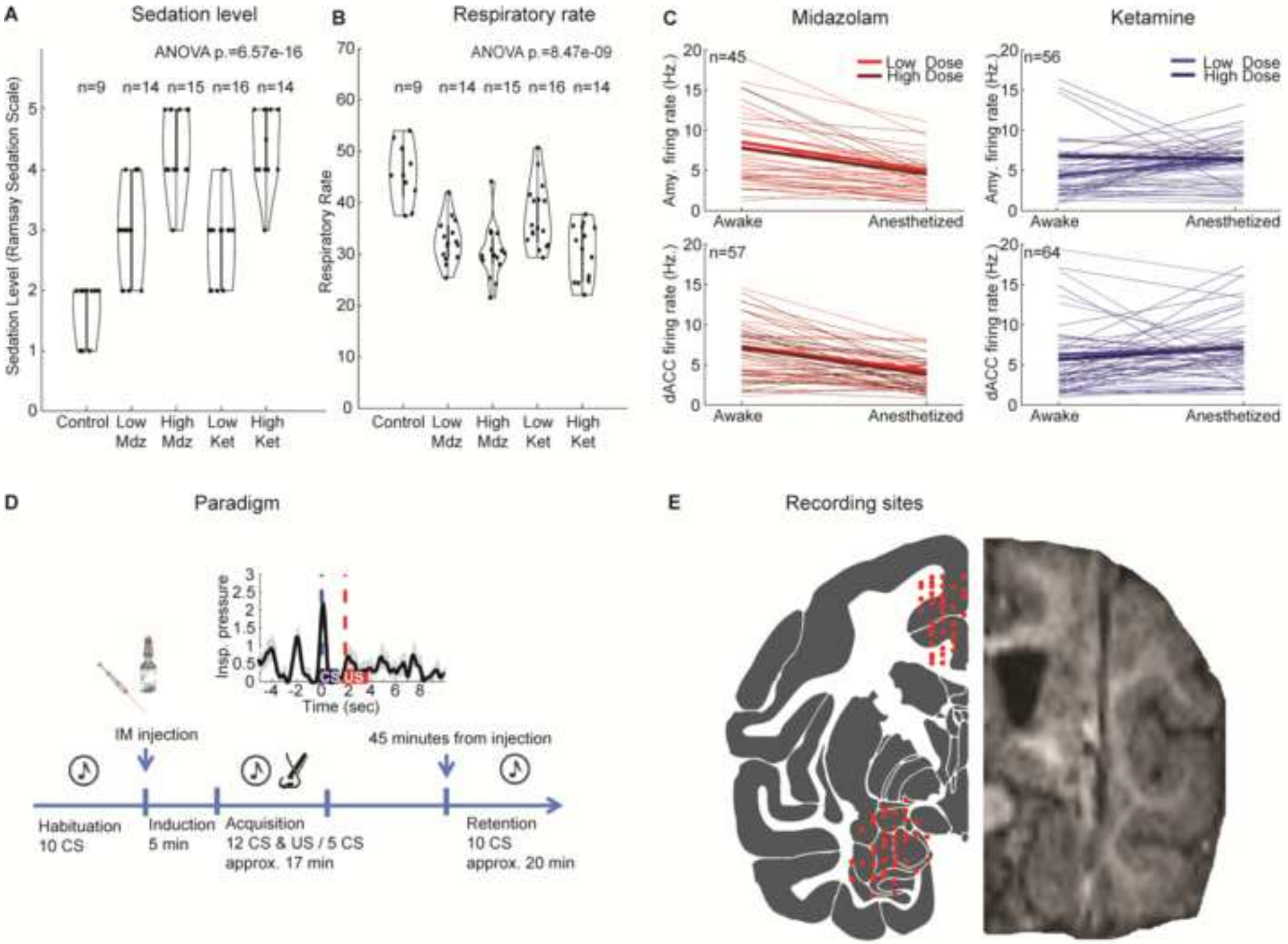
A. Unconditioned respiratory responses (UR) pooled across sessions under anesthetics (Mean+/- SEM).

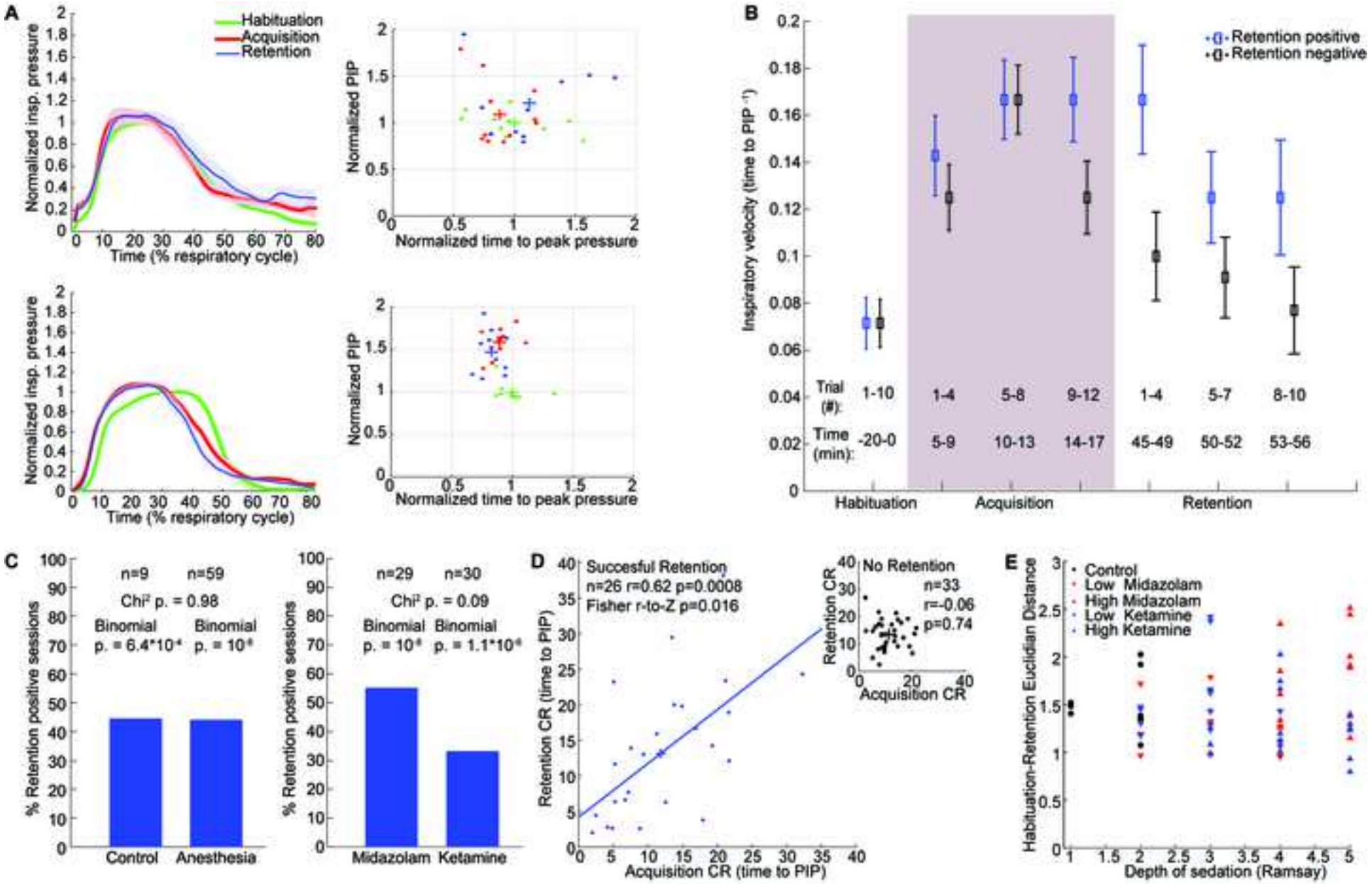
B. Individual session examples of unconditioned responses (UR) elicited by the aversive odor (black, mean+/- SEM overs session trials), compared to a neutral tone (green, mean+/- SEM). The aversive odor induce early termination of the inhale with decreased duration and volume.

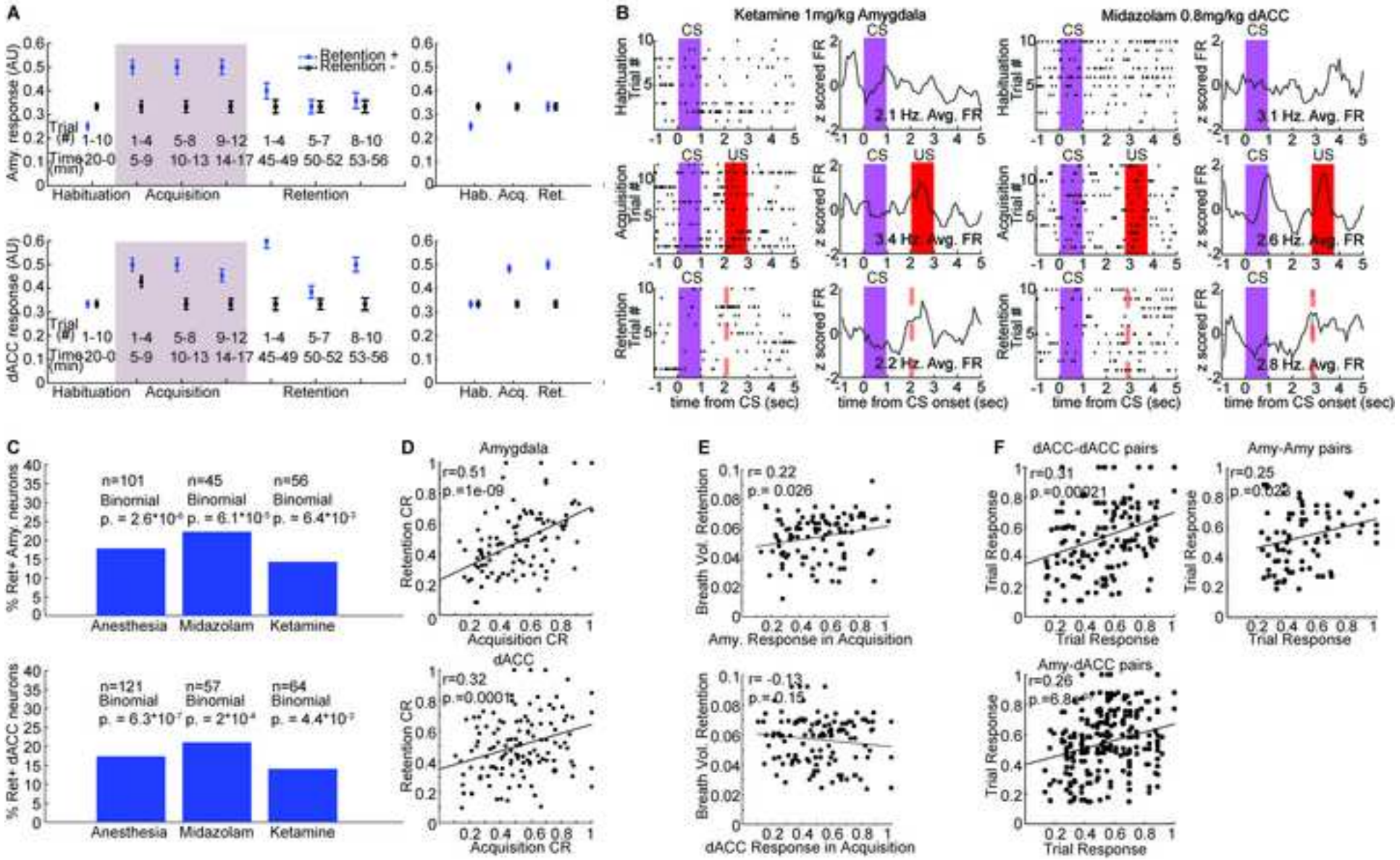
C. Individual examples of dACC and amygdala neurons under ketamine and midazolam showing an increased firing rate in response to the unconditioned stimulus, namely the aversive odor.

D. Unconditioned responses by agent and dose, for the behavioral response (top), dACC neural responses (middle), and amygdala neural responses (bottom).

(CS conditioned stimulus, US unconditioned stimulus, Ret. Retention, Vol. volume, Amy amygdala, FR firing rate, PIP peak inspiratory pressure, Mdz. Midazolam, Ket. ketamine)







Figure(s)

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