Primary auditory cortex is necessary for the acquisition and expression of categorical behavior

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Summary

The primary auditory cortex (ACtx) is critically involved in the association of sensory information with specific behavioral outcomes. Such sensory-guided behaviors are necessarily brain-wide endeavors, requiring a plethora of distinct brain areas, including those that are involved in aspects of decision making, motor planning, motor initiation, and reward prediction. ACtx comprises a number of distinct excitatory cell-types that allow for the brain-wide propagation of behaviorally-relevant sensory information. Exactly how ACtx involvement changes as a function of learning, as well as the functional role of distinct excitatory cell-types is unclear. Here, we addressed these questions by designing a two-choice auditory task in which water-restricted, head-fixed mice were trained to categorize the temporal rate of a sinusoidal amplitude modulated (sAM) noise burst and used transient cell-type specific optogenetics to probe ACtx necessity across the duration of learning. Our data demonstrate that ACtx is necessary for the ability to categorize the rate of sAM noise, and this necessity grows across learning. ACtx silencing substantially altered the behavioral strategies used to solve the task by introducing a fluctuating choice bias and increasing dependence on prior decisions. Furthermore, ACtx silencing did not impact the animal's motor report, suggesting that ACtx is necessary for the conversion of sensation to action. Targeted inhibition of extratelencephalic projections on just 20% of trials had a minimal effect on task performance, but significantly degraded learning. Taken together, our data suggest that distinct cortical cell-types synergistically control auditory-guided behavior and that extratelencephalic neurons play a critical role in learning and plasticity.

Introduction

Effectively engaging with the world around us requires intricate perceptual processing that enables us to interpret the sensory environment and react accordingly within the given context. The learning process of associating sensory information with specific behavioral outcomes is essential for the rapid selection of optimal motor actions and often depends on the categorization of stimuli based on their expected outcome [1, 2]. Categorization is a computational process that involves a complex transformation of sensory information into discrete perceptual categories [3–5]. Although much work has implicated multiple sensory cortices as being involved in different aspects of categorization (and sensory-guided behavior in general) [6–9], the role of sensory cortex in both the acquisition and expression of categorical behavior is unclear.

In hearing, while many studies have reported that primary auditory cortex (ACtx) inactivation can impair or completely abolish auditory-guided behavior [10–15], several studies have shown that ACtx inactivation can lead to little or no effect [16–18]. Although there exists significant heterogeneity in task conditions, inactivation method, and animal model [19], most evidence points towards ACtx necessity depending critically on both stimulus choice and complexity of task parameters [20]. More complex acoustic objects, especially those that require temporal integration, are more likely to necessitate ACtx involvement. Such involvement is less likely to be critical for simple feature discrimination. Exactly how such ACtx involvement changes as a function of learning represents a significant gap in knowledge.

Learning to respond accurately to incoming sound stimuli is necessarily a brain-wide endeavour, requiring a plethora of distinct brain areas, including those that are involved in aspects of decision making, motor planning, motor initiation, and reward prediction [21–24]. Indeed, ACtx is comprised of distinct excitatory cell-types that facilitate the flow of sensory information to many downstream intra- and extratelencephalic targets in parietal and frontal cortices, as well as in the midbrain, thalamus, and striatum [25–30]. Although several studies have suggested distinct behavioral roles for excitatory projections (across multiple sensory modalities) [28, 31–38], few studies to date have assayed their specific contributions to the acquisition and expression of a sensory-guided behavior using a standardized task.

Here, we designed a head-fixed categorization task (adapted from [8]) and used transient cell-type specific optogenetics to probe ACtx necessity across the duration of learning. Our data demonstrate that ACtx is necessary for the ability to categorize the rate of amplitude modulated noise, and this necessity grows across learning. We demonstrate that ACtx silencing substantially alters the behavioral strategies the animal uses to solve the task, by introducing a fluctuating choice bias, and increasing dependence on prior decisions. Furthermore, we show that ACtx silencing does not impact the motor report of the decision, suggesting that ACtx is necessary for the conversion of sensation to action. Finally, we show that targeted inhibition of extratelencephalic projections

on just 20% of trials has a minimal affect on task performance, but significantly degrades learning. Taken together, our data suggest that distinct cortical cell-types synergistically control auditory-guided behavior and that extratelencephalic neurons play a critical role in learning and plasticity.

Results

ACtx is necessary for accurate categorization of sAM noise

To investigate the involvement of ACtx in categorical decision making, we designed a two-choice auditory categorization task in which water-restricted, head-fixed mice were trained to categorize the rate of a sinusoidal amplitude modulated (sAM) noise burst (**Figure 1A**). Mice were tasked with categorizing sAM noise as either "slow" or "fast" by directionally licking left or right spouts to earn a small water reward. The stimuli consisted of sAM rates spanning 2 - 32 Hz in 0.5 octave increments, presented at 70 dB SPL, including an 8 Hz stimulus at the category boundary that was rewarded probabilistically (50% left, 50% right). Mice were initially introduced to an easy version of the task in which they had to categorize only the two slowest (2, 2.4 Hz) and two fastest sounds (22.8, 32 Hz), allowing them to learn the association between category and spout. To facilitate the inhibition of pan-excitatory neural activity we selectively expressed a Soma-targeted *Guillardia theta* anion-conducting channelrhodopsin (stGtACR2) in CAMKII-expressing neurons within ACtx [39]. The stGtACR2 was activated via blue LED light (1.1 sec, 2 mW) delivered through an optic fiber, initiated 100 ms prior to sound onset and lasted for the duration of the sound (**Figure 1B**). Optogenetic inhibition was introduced in a pseudo-random 20% of trials once mice demonstrated performance above chance levels in the easy task (>60% correct over 200 easy trials). Mice were considered proficient at the easy task when they achieved 85% accuracy over 200 trials (**Figure 1C**). Subsequently, all additional sAM rates (4 - 16 Hz) were introduced and mice were trained to reach an expert level, defined as accuracy above 75% correct across "hard" sounds (4, 5.6, 11.2, 16 Hz). Mice readily learned this task. Mice achieved the threshold for optogenetics within 2.95 $\pm$ 0.25 sessions (924.8 $\pm$ 64.5 trials), achieved the proficient threshold in 8 $\pm$ 0.51 sessions (2966.6 $\pm$ 164.0 trials) and achieved the expert threshold in 12.75 $\pm$ 0.70 sessions (4932.4 $\pm$ 293.1 trials). On average, mice completed 408.4 $\pm$ 10.8 trials per session.

Optogenetic inhibition of the ACtx significantly impaired the performance of expert mice, reducing their accuracy from $83.9 \pm 0.7\%$ to $61.0 \pm 3.9\%$ (**Figure 2A**, N=4, paired t-test, p=0.012). To control for the impact of light, a separate cohort of mice, implanted with optic fibers but lacking a viral injection, were also tested. This group did not show a significant difference in accuracy between LED-off and LED-on trials (N=4, paired t-test, p=0.611), indicating that the observed decrease in performance is specifically attributable to optogenetic inhibition of the ACtx. To investigate the effect of ACtx inhibition across different stimuli, we fit psychometric curves [40–42] to the data. These curves characterize the probability of categorizing a sound as "fast" as a function

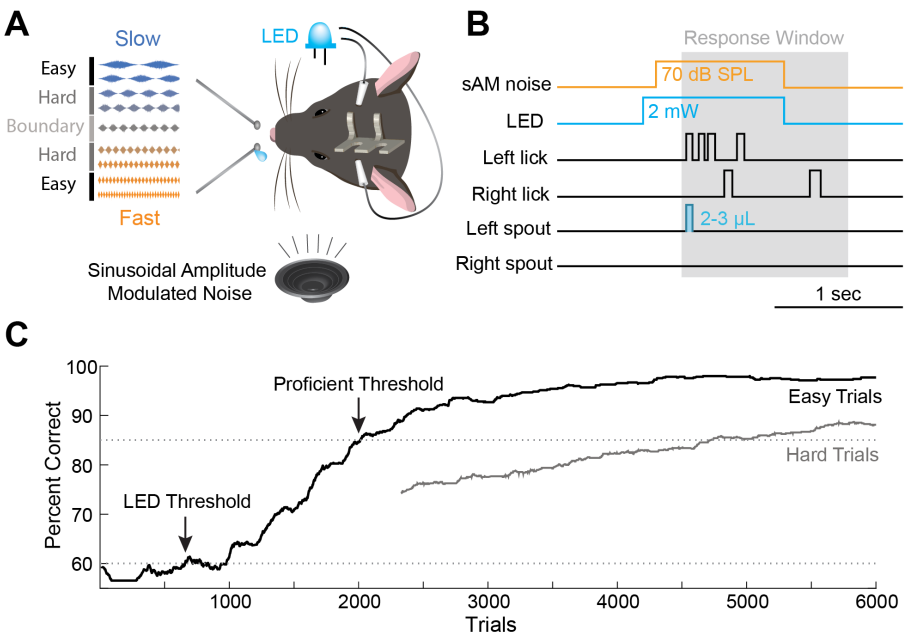

Figure 1: sAM Categorization Task. **A**: Schematic representation of the stimulus set and behavioral setup. Head-fixed mice are trained to categorize sAM noise as "slow" or "fast", based on the rate of sinusoidal modulation. Blue light is delivered on 20% of trials via optic ferrules cemented over the ACtx. **B**: Schematic illustration of the trial structure, outlining the sequence of events within a single behavioral trial, from the presentation of sAM noise to the mouse's behavioral response, and details the timing of the optogenetic inhibition relative to the sound onset. **C**: Exemplar task performance across multiple trials for a single mouse. Performance is visualized as a rolling accuracy calculated over a 200 trial window. Thresholds for LED optogenetic introduction and task proficiency are denoted by horizontal dotted lines at 60% and 85%, respectively.

of sAM rate (**Figure 2D-E**). Optogenetic inhibition of the ACtx led to a shallower psychometric curve with choice probability approaching 50% across all stimuli. This flattening of the psychometric curve also manifested as a significant increase in the lapse rate (**Figure 2B,** N=4, paired t-test, control: p=0.072, pan-excitatory: p=0.008) as well as a decrease in the psychometric slope (**Figure 2C,** N=4, paired t-test, control: p=0.295, pan-excitatory: p=0.021), differences that were not present in the control group.

Although the average psychometric curve across the population flattened towards chance levels, the psychometric curves exhibited variability across different mice and different sessions. We frequently observed that inhibition drove mice to categorize all sounds uniformly as either "slow" or "fast", without a consistent bias towards a specific spout or sound association (**Figure 3A**). To further investigate this phenomenon, we utilized an asymmetry index to quantify directional bias by computing the difference between left and right choices, normalized by the sum of all choices. This measure yields a value whose distance from 0 indicates either left-preferring (>0) or right-preferring (<0). Considering that half of all stimuli were associated with each spout, we expected expert mice to exhibit a bias close to 0. This expectation was upheld in control mice and in LED-

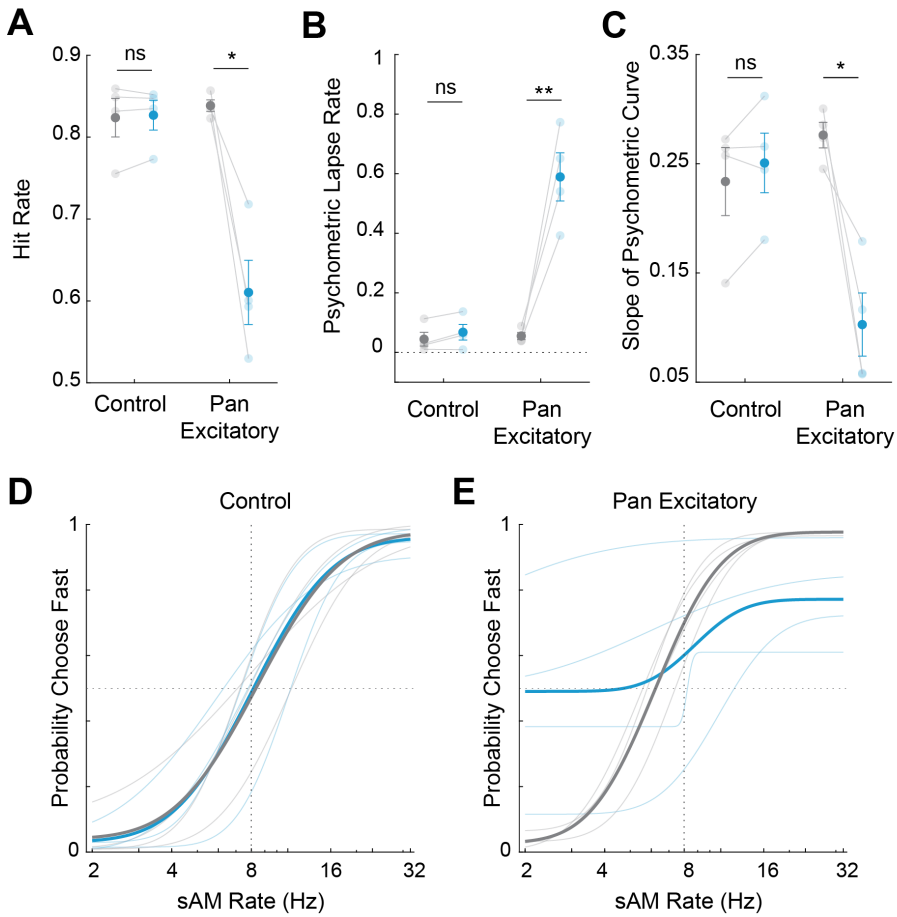

Figure 2: ACtx is necessary for categorization in expert mice. **A**: Hit rate (percent correct) for control and pan-excitatory groups, with (blue) and without LED (grey). **B**: Psychometric slope for control and pan-excitatory groups, with and without LED. **C**: Lapse rate for control and pan-excitatory groups, with and without LED. **D**: Psychometric curves from control animals for LED-off (grey) and LED-on (blue) conditions. Lighter shades represent individual animals, and bold represents the mean. **E**: Psychometric curves with pan-excitatory suppression (same color conventions as **D**.

off trials of pan-excitatory mice, where the bias was found to be near 0 (**Figure 3B-C**, control: -0.032 ± 0.061, pan-excitatory: -0.119 ± 0.066). However, inhibiting the ACtx led to a significant increase in absolute bias in expert mice (**Figure 3C,** N=4, unpaired t-test, p=0.002), consistent with our observation that individual mice often chose one spout in the absence of ACtx activity. To further examine the consistency of these biases over sessions, we analyzed the bias as a function of the number of complete trials. This visualization revealed strong biases in every mouse that flipped sides across learning (**Figure 3D**).

In visual decision-making, task history has a greater impact on choice when the task is more challenging such that mice are more likely to repeat their previous choice when faced with perceptual uncertainty [43]. We examined this in our task by quantifying the proportion of trials in

which mice repeated their previous choice as a function of stimuli distance to category boundary. Consistent with previous findings, we found that LED-off trials in both groups exhibited the highest proportion of repeated choices close to the category boundary that decreased with distance (**Figure 3E**, N=4, two-way ANOVA, main effect for distance, control: $p < 0.0001$, pan-excitatory: p=0.041). While there was no effect of LED on control animals (main effect for LED: p=0.165, interaction between LED and distance: p=0.725), pan-excitatory inhibition led to a significant change in repeated choices as a function of distance (main effect for LED: p=0.048, interaction between LED and distance: p=0.002). Notably, with "hard" sounds, 1 - 2 octaves from the category boundary, inhibition led to significantly more repeated choices compared to LED-off controls (Sidak's multiple comparisons test, 1 octave: p=0.002, 1.5 octaves: p=0.009, 2 octaves: p=0.013), comparable to the proportion observed at and near the category boundary. This suggests that mice rely primarily on auditory information when the stimulus is easy, and resort to the use of prior information when the stimulus is ambiguous or ACtx is silenced.

Inhibiting ACtx affects categorization but not perception

These findings collectively suggest that the ACtx is necessary for the accurate transformation of bottom-up auditory information into perceptual categories. In the absence of ACtx, mice disregard perceptual features when categorizing sounds, instead demonstrating a tendency to lick one spout repetitively. This failure to categorize sounds does not appear to stem from an inability to perceive the sAM rates. Given the unpredictable onset of sound, one would predict that mice would not exhibit reliable licking behavior if they could not perceive the stimulus. However, optogenetic inhibition did not result in a significant change in the rate of no-go trials, during which mice fail to respond within the response window (**Figure 4A,** N=4, paired t-test, control: p=0.766, pan-excitatory: p=0.099). Moreover, reaction times for LED-on trials in pan-excitatory mice aligned closely with the distribution of both LED-off trials and control reaction times (**Figure 4B-C**). We observed a notable separation of reaction times to stimuli based on the sAM rates, likely due to more or less acoustic evidence being necessary to identify "slow" or "fast" rates, respectively. Indeed, slower rates were associated with slower reaction times on average in control and LED-off trials (**Figure 4C, top/middle**). This separation of reaction times was still evident during trials with pan-excitatory inhibition (**Figure 4C, bottom**), suggesting that, during inhibition, mice perceive the differences in sAM rates but fail to accurately categorize them into perceptual groups.

Learning amplifies ACtx contributions to categorization

If ACtx is necessary for the transformation of auditory stimuli into discrete perceptual categories then it suggests that the impact of ACtx inhibition should correlate with the extent to which a mouse has learned to categorize sounds. We therefore analyzed hit rate as a function of trials for both control (**Figure 5A**) and pan-excitatory (**Figure 5B**) mice to examine the effect of ACtx inhi-

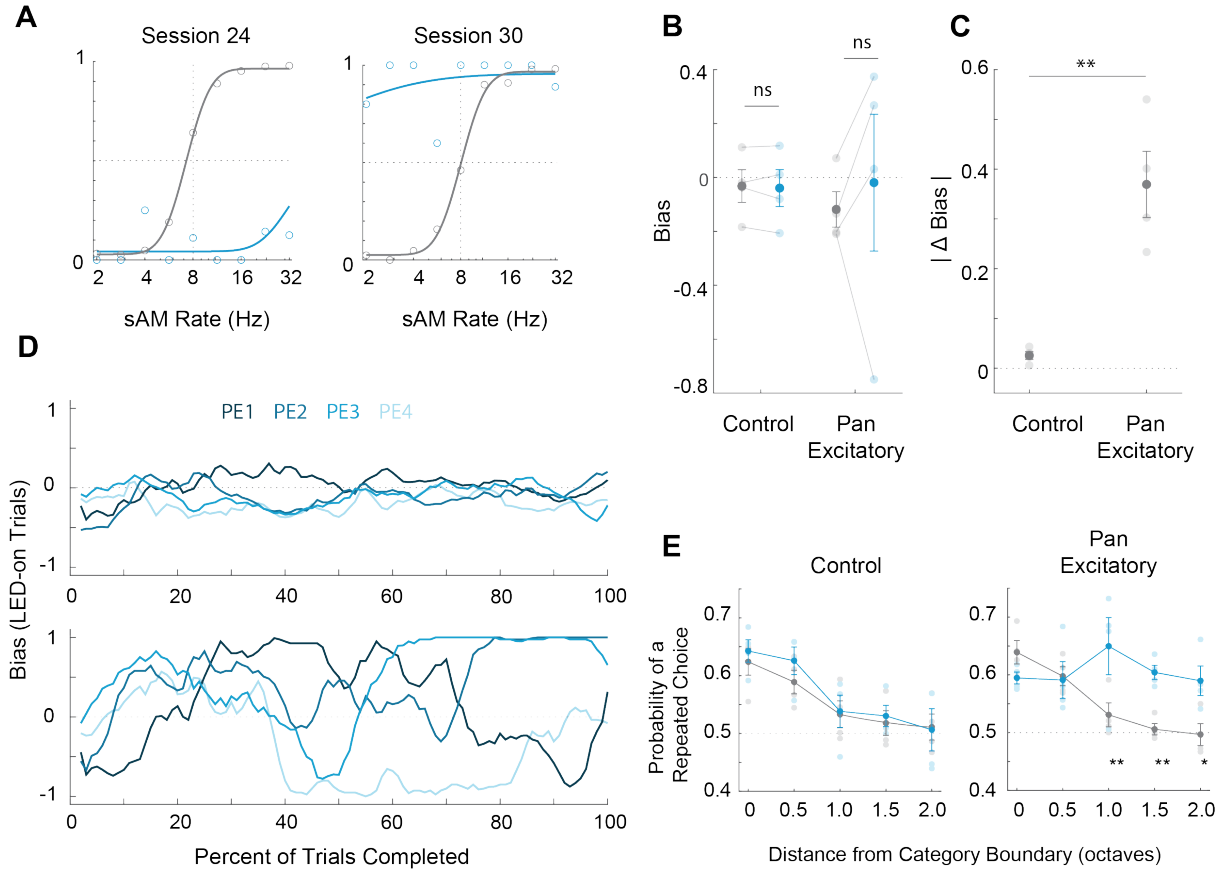

Figure 3: ACtx inhibition leads to a fluctuating choice bias. **A**: Exemplar psychometric curves from two sessions from the same mouse showing a switch in bias from "slow" to "fast". **B**: Difference in bias between LED-on and LED-off conditions. **C**: Difference in absolute bias between LED-on and LED-off conditions. **D**: Bias as a function of training for pan-excitatory animals in LED-off (top) and LED-on (bottom) trials. **E**: Proportion of repeated choices as a function of distance from category boundary (octaves).

bition over learning. As expected, control mice performance improved over time with no significant differences between performance on LED-on and LED-off trials (**Figure 5A**, mixed-effect model, fixed effect for time: $p \leq 0.0001$, fixed effect for LED: p=0.707). In contrast, with pan-excitatory inhibition, the performance on LED-on trials differed significantly from LED-off trials (**Figure 5B**, mixed-effect model, fixed effect for time, $p \leq 0.0001$, fixed effect for LED: p=0.004). Although the initial difference in performance with ACtx inhibition was relatively minimal, (**Figure 5C**, Δ Hit Rate: LED-off - LED-on, -12.2% ± 9.6) the effect had tripled by the end of training (-36.5% ± 3.5), suggesting a learning-dependent influence of inhibition.

To directly probe the relationship between the amount of training and the effect of ACtx inhibition, we opted to bin trials across mice based on task performance. This method enabled us to account for variations in learning rates across mice. Trials were grouped into four bins based on distinct performance thresholds: optogenetic introduction (performance >60% on easy trials),

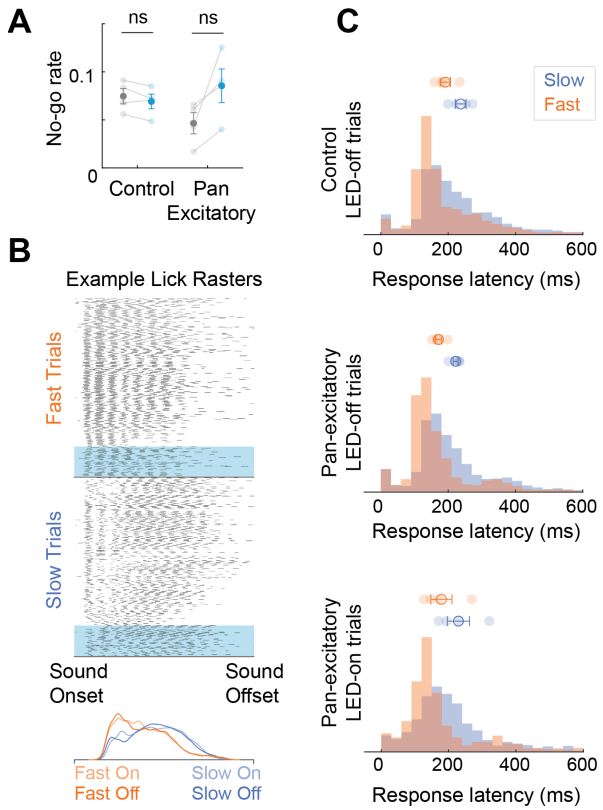

Figure 4: Inhibiting ACtx affects categorization but not perception. **A**: No-go rate for control and pan-excitatory groups, with and without LED. **B**: Exemplar lick rasters from one session showing the difference in response latency as a function of sAM rate, with "fast" stimuli (top) associated with shorter latency responses compared to "slow" stimuli (middle) in both LED-on and LED-off conditions. Probability densities for all groups are shown (bottom). **C**: Response latency distributions for control LED-off trials (top), pan-excitatory LED-off trials (middle) and pan-excitatory LED-on trials (bottom) for both fast (orange) and slow (blue) stimuli, with averages plotted above.

proficient (performance >85% on easy trials) and expert (performance >75% on hard trials). This analysis revealed that the contribution of ACtx to sAM categorization was dependent on performance. Early in training, when optogenetic trials were first introduced, ACtx inhibition had a negligible impact on performance (**Figure 5D**, repeated-measures ANOVA, Sidak multiple comparisons test, opto to proficient: p=0.119). However, as mice improved their performance on control trials, ACtx inhibition started to significantly affect behavior (**Figure 5D**, repeated-measures ANOVA, Sidak multiple comparisons test, proficient to expert: p=0.0194, expert: p=0.003). Consistent with the influence on performance, we also found that LED-induced bias due to inhibition changed as a function of training (**Figure 5E**). Notably, when optogenetic trials were first introduced, pan-excitatory inhibition had no effect on bias (one-sample t-test: p=0.068). Yet, at proficient and expert performance levels, there was a significant increase in absolute bias during LED-on trials compared to LED-off trials (one-sample t-test, proficient to expert: p=0.002, expert: p=0.011).

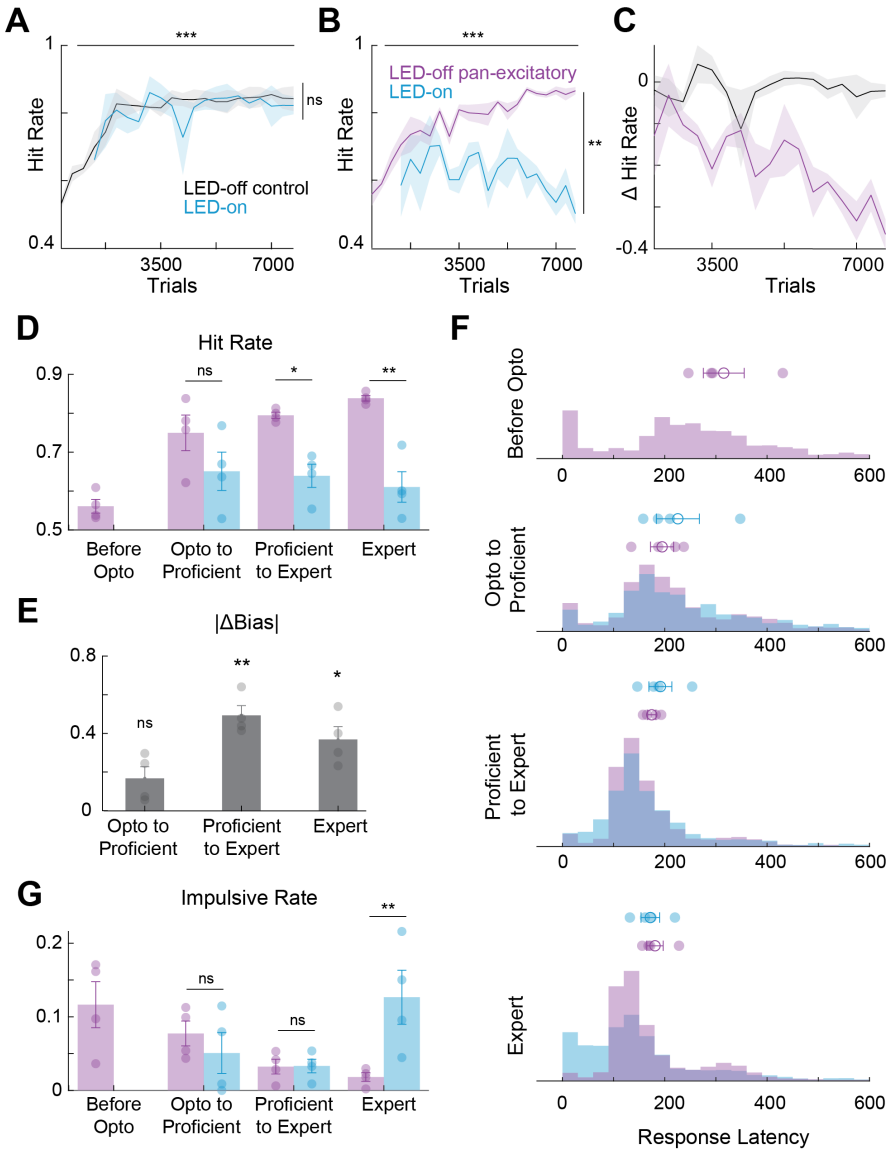

Figure 5: Learning amplifies ACtx contributions to categorization. **A**: Hit rate for LED-off (black) and LED-on (blue) trials in control mice. **B**: Hit rate for LED-off (purple) and LED-on (blue) trials in pan-excitatory mice. **C**: Difference between LED-on and LED-off performance as a function of trials. **D**: Hit rate on LED-off (purple) and LED-on (blue) trials during four training periods defined by behavioral thresholds. **E**: Change in bias with LED for each behavioral training period **F**: Response latencies for LED-off and LED-on trials across learning. **G**: Rate of impulsive licks as a function of training period.

We also examined how the response times evolve as a function of learning by plotting the distribution of response latencies across performance bins for pan-excitatory animals. In the absence of inhibition, response latencies became shorter over the course of training (**Figure 5F**, one-way ANOVA, main effect for response latency: p=0.0049). Inhibition of ACtx led to no significant

change in average response latencies (**Figure 5F,** paired t-test, opto to proficient: p=0.54, proficient to expert: p=0.50, expert: p=0.72) . However, once mice became expert, the LED-on distribution shifted such that more response times occurred early after sound onset. Given that the first amplitude modulation of the fastest stimulus occurs 31.25 ms after sound onset, we assumed that any reaction prior to this could not be a result of categorizing the sAM rate. These might instead represent impulsive responses or failure to inhibit anticipatory off-target licking. Early in training (before optogenetic inhibition), impulsive licks were frequent, accounting for 11.7% of all trials (**Figure 5G**). As performance improved, the frequency of impulsive licks decreased in LED-off trials (**Figure 5G**, two-way ANOVA, main effect for time: p=0.048). However, the opposite trend occurred in LED-on trials, leading to a significant difference in impulsivity in expert animals (two-way ANOVA, Sidak multiple comparisons test, interaction between time and LED: p=0.007, expert LED-on versus LED-off: p=0.0053), suggesting that increased impulsivity is related to decreased performance and increased choice bias (**Figure 5D-E**).

Inhibition of ET neuron impairs speed and efficacy of categorical learning

The ascending auditory system is critical for the perception and processing of auditory information. Many downstream targets of the ACtx, including the striatum, are critically involved in the conversion of sensation to action [32, 33]. Given that our prior optogenetic manipulations targeted the ACtx without specificity, we chose to replicate our experiments focusing on the three major excitatory cell-classes to understand how the flow of information out of the ACtx through specific projections contributes to the acquisition and expression of categorical behavior.

We selectively expressed stGtACR in populations of either layer 5 intratelencephalic (IT), extratelencephalic (ET), or corticothalamic (CT) neurons using intersectional viral techniques (see Methods). These mice then underwent the same behavioral training and optogenetic protocols as our previous pan-excitatory and control groups. Unlike pan-excitatory inhibition, cell-type specific inhibition did not significantly impair performance in expert mice (**Figure 6A-B**, paired t-test, ET: p=0.76, CT: p=0.25, IT: p=0.43). Although we did not observe a significant change in performance during inhibition of distinct projections in expert mice, we hypothesized that inhibition might impair task learning.

To examine the learning rate, we analyzed performance as a function of trials for each group compared to control mice (**Figure 6C-D**). Performance on "easy" stimuli was plotted as a function of trials and the resulting curves were fit with an error function (see Methods). This revealed that ET animals' performance plateaued at lower hit rate compared to controls (**Figure 6C,** ET=0.89, control=0.94) and required more trials to reach half-maximum performance (ET=2131.5, control=1806.2). In contrast, CT and IT animals plateaued at, or above, control levels (CT=0.96, IT=0.94) and required comparable number of trials to reach half maximum (CT=1745.5, IT=1864.2), suggesting the effect was specific to ET. Next, performance on "hard" stimuli was similarly plotted

and the resultant curves were fit with a line. Similar to the learning trajectory of "easy" stimuli, ET mice exhibited slower learning compared to controls as evidenced by the slope of the linear fit (**Figure 6B,** ET: 0.48% per 1000 trials, control: 1.9% per 1000 trials). IT mice exhibited a similar learning rate to control (1.8% per 1000 trials), whereas CT mice learning rates were larger (3.7% per 1000 trials).

To further quantify the effect of inhibition on learning efficacy, we calculated the maximum performance of each experimental group as measured by performance across the first eight expert sessions. Consistent with our earlier observation, we found that the performance of ET mice plateaued at a lower hit rate compared to control, IT, and CT mice (**Figure 6E**, one-way ANOVA, Tukey's multiple comparisons test, control versus ET: p=0.0314, IT vs ET: p=0.0348, CT vs ET: p=0.0003). Expert ET mice reached an average performance of 79.6% across all stimuli, whereas control mice reached 82.4% correct, IT mice reached 82.3% correct, and CT mice reached 83.8% correct . A significant impairment in learning was not present with inhibition of IT or CT neurons (**Figure 6E**, one-way ANOVA, Tukey's multiple comparisons test, control versus CT: p=0.4712, control versus IT: p>0.999), suggesting that this effect is specific to ET neurons. We quantified the differences in learning rate by analyzing the number of trials to reach our optogenetic, proficient, and expert performance criteria and found that ET mice required more trials to reach expert thresholds (**Figure 6D,** One-way ANOVA, p=0.0396, control versus ET: p=0.0177). ET mice also took more trials to reach the threshold for introducing optogenetics (**Figure 6F**, control versus ET: p=0.0158). To control for these differences in initial learning, we also calculated how many trials it took to move between our performance thresholds (i.e. opto to proficient, proficient to expert). We found a significant increase in the number of trials between the proficient and expert thresholds (**Figure 6G**, one-way ANOVA, p=0.0462, control versus ET: p= 0.0322). Together, this suggests that inhibition of ET neurons on just 20% of trials leads to a significant reduction in learning rate.

Discussion

The role of ACtx in facilitating the acquisition and expression of auditory-guided behaviors is widely reported [10–15, 20]. While previous studies have predominantly focused on the specific stimuli and behavioral paradigms that underscore whether ACtx is necessary for task expression, our understanding as to how this necessity is sculpted across learning remains incomplete. In the current study, we used an auditory choice task alongside transient optogenetics to explicitly probe how ACtx necessity changes across learning. Our results show that ACtx necessity increases throughout learning, suggesting that ACtx is crucial for both initial task acquisition, as sound categories and associations are being formed, and for the gradual refinement of performance as learning progresses. Crucially, ACtx never became dispensable for task expression, reaffirming the necessity of ACtx for complex sound feature discrimination.

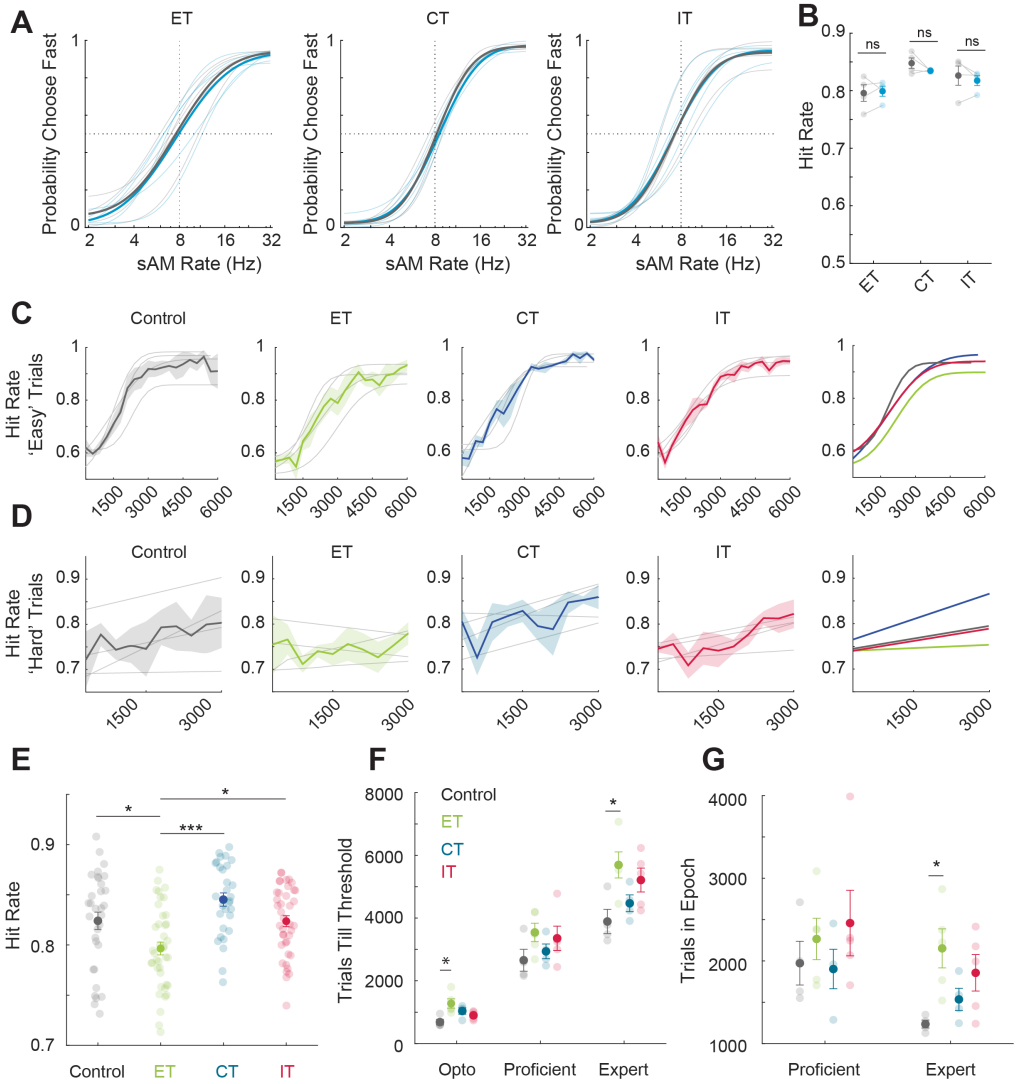

Figure 6: Inhibition of ET neurons impairs speed and efficacy of categorical learning. **A**: Psychometric curves from ET (left), CT (middle), and IT (right) mice for LED-off (grey) and LED-on (blue) conditions. Lighter shades represent individual animals, and bold represents the mean.**B**: Hit rate for ET, CT, and IT groups, with (blue) and without LED (grey). **C**: Hit rate on "easy" trials as a function of trial for control, ET, CT, and IT groups. Rightmost panel shows the fit to the average hit rate for each group. **D**: Hit rate on "hard" trials as a function of trials. Rightmost panel shows the linear fit to the average hit rate for each group. **E**: Hit rate for first eight sessions after reaching expert threshold. **F**: Number of trials for each group to reach optogenetic, proficient, and expert thresholds. **G**: Number of trials between optogenetic and proficient thresholds and proficient and expert thresholds.

Transient ACtx silencing substantially altered the behavioral strategies the animal uses to solve the task, by introducing a fluctuating choice bias, and increasing dependence on prior decisions. During difficult decision making, mice typically resort to using prior information to guide their

current decisions [43]. Here, transient ACtx silencing led to a dependence on prior information, even for easy decisions. Such a dependence on prior information is known to be associated with parietal networks, where targeted silencing has been shown to improve performance on choice tasks [44], suggesting that feedback circuits to the ACtx represent an important avenue for future study.

Response latency analysis suggests that late in learning, ACtx silencing alters the behavioral report of perception. At first glance, this latency observation suggests that the transformation of sensory information to motor action is being disturbed. However, when mice do not categorize sounds with high accuracy early in learning, they still reliably lick in response to sounds with similar latencies to control mice, suggesting that it is the categorization of the sound, not the motor report, that is disrupted in the absence of ACtx activity. As the ability to categorize improves, the perceptual report changes perhaps as a mechanism to maximize reward given perceptual uncertainty. Critically, our study demonstrates that mice categorize sounds based on differences in their putative meaning, rather than acoustic variations, mirroring findings in non-human primates [45–49].

Although much work in recent years has started to assign functional roles to distinct excitatory sub-types [28, 31–38], the necessity of these cell-types to behavior remains unknown. Although we observed no significant reduction in task expression when individual cell-types were silenced, our data is consistent with a growing literature suggesting that ET neurons play a critical role in learning and plasticity across multiple sensory modalities [31, 35, 50, 51] and suggests that, even if a cell-type is dispensable, that perturbation during learning can have lasting effects. An additional possibility that cannot be ruled out however is that the categorization task is not challenging enough for the descending projections to play a critical role [52].

Ultimately, everything is connected. Cortical contributions to auditory categorization will likely be sculpted by myriad top-down inputs from frontal and parietal regions, by local inhibitory subnetworks, and further modulated by incoming neuromodulatory signals, providing many distinct avenues for future work.

Materials and Methods

Mice

All procedures were approved by the University of Pittsburgh Animal Care and Use Committee and followed the guidelines established by the National Institute of Health for the care and use of laboratory animals. This study is based on data from 20 mice (aged 6-8 weeks, both male and female). All mice were maintained under a 12 hr/12 hr periodic light cycle. 12 C57/Bl6 mice (#000664, Jackson Labs) were used for control groups, as well as viral access to excitatory cells

and ET-type cells, 4 Tlx3-Cre mice (B6.FVB(Cg)-Tg(Tlx3-Cre)PL56Gsat/Mmucd, MMRRC) were used to provide genetic access to IT-type cells, and 4 Ntsr1-Cre mice (B6.FVB(Cg)-Tg(Ntsr1-Cre)-GN220Gsat/Mmucd, MMRRC) were used to provide genetic access to CT-type cells.

Surgical Procedures

Virus-mediated gene delivery

Mice were anesthetized using isoflurane (4%) and placed in a stereotaxic plane (Kopf model 1900). A surgical plane of anesthesia was maintained throughout the procedure using continuous infusion of isoflurane (1-2%) in oxygen. Mice lay atop a homeothermic blanket system (Fine Science Tools) that maintained core body temperature at approximately 36.5°C. The surgical area was shaved and cleaned with iodine and ethanol before being numbed with lidocaine (5 mg/ml). For viral delivery to both ACtx, incisions were made to the left and ride side of the scalp to expose the skull around the caudal end of the temporal ridge. The temporalis muscle was then retracted and two burr holes on either hemisphere (approximately 0.3 mm each) were made along each temporal ridge, spanning a region of 1.5-2.5 mm rostral to the lambdoid suture. A motorized stereotaxic injection system (Nanoject III, Drummond Scientific) was used to inject 300 nl of either a non-specific GtACR2 (pAAV1-CamKIIa-stGtACR2-FusionRed, Addgene #105669), or a Cre-dependent GtACR2 (pAAV1-hSyn1-SIO-stGtACR2-FusionRed, Addgene #105677) into each burr hole approximately 0.5 mm below the pial surface with an injection rate of 10-20 nl/min. Following the injections, the surgical areas were sutured shut, antibiotic ointment was applied to the wound margin, and an analgesic was administered (Carprofen, 5 mg/kg). Mice had ad libitum access to a carprofen-laced medigel (Clear H^2O) for three days post surgery.

Implantation of headplate and optic fibers

Mice were brought to a surgical plane of anesthesia, using the same protocol for anesthesia and body temperature described above. The dorsal surface of the skull was exposed and the periosteum was removed. The skull was first prepared with 70% ethanol and etchant (C&B Metabond) before attaching a custom titanium head plate (eMachineShop) to the skull overlying bregma with opaque dental cement (C&B Metabond). A small craniotomy (0.5 mm x 0.5 mm, medal-lateral x rostral-caudal) was made bilaterally and a ferrule stub (Thorlabs) was implanted over each ACtx surface. Once dry, the cement surrounding the fiber implant was pained black with nail polish to prevent light from escaping.

Acoustic and optogenetic stimulation

Acoustic stimulation

Stimuli were generated with a 24-bit digital-to-analog converted (National Instruments model PXI-4461) using custom scripts programmed in MATLAB (Mathworks) and LabVIEW (National Instruments). Stimuli were presented via a free-field speaker (PUI Audio) facing the left (contralateral)

ear. Stimuli were calibrated using a wide-band ultrasonic acoustic sensor (SPM0204UD5, Knowles Acoustics).

Light delivery

Blue light (488 nm) was generated by either an LED (470 nm, M470F3, Thorlabs) or a diode laser (473 nm, LuxX, Omicron) and delivered bilaterally to the brain via implanted multimode optic fibers coupled to a bifurcating optical patch cable with ceramic mating sleeves. LED/laser power through the optic fiber assembly was calibrated prior to implantation with a photodetector (Thorlabs).

Mouse Behavior

Behavioral training

Approximately a week before training, mice were water restricted to 4% of their baseline weight of water per day. Mice were first acclimated to handling and head-fixation. After three days of acclimation, mice were introduced to a single spout Pavlovian task in which water reward was dispensed following a 1-second sAM noise stimulus. Once a mouse consistently licked the spout in response to the stimulus, the task was changed to require the mouse to lick in a 1.5 sec response window, 200 ms after sound onset, to trigger the water reward. After the mouse successfully licked for 10 trials in a row in response to sound, a second spout was introduced. Water was dispensed following "fast" sounds (22.8 and 32 Hz) from one spout and "slow" sounds (2 and 2.4 Hz) from the other. To control for potential side biases, the speed/spout association was varied across mice.

Once a mouse was consistently licking both spouts in response to sound, the task was changed to require a lick to the appropriate spout within the response window to trigger the reward. Licks outside of the response window were not counted as a response. An incorrect lick triggered a 5 sec timeout before the start of the next trial. Trials with no lick in the response window were counted as "no go" trials with no consequence. Behavioral sessions continued as long as the mouse was engaging in the behavior and sessions were ended when a mouse exhibited persistent "no go" behavior (i.e. >10 in a row) suggesting satiation. Once mice had a session with >60% correct over 200 trials (LED threshold), optogenetic inhibition was randomly introduced on 20% of trials for all following sessions. Once mice reached the threshold of 85% correct across 200 LED-off trials (proficient threshold), the remaining sAM noises were introduced (2 - 32 Hz in 0.5 octave steps). A stimulus at the category boundary was included which was not associated with either "fast" or "slow" categories but instead was probabilistically rewarded (50% left/50% right). Mice (N=5) were excluded for failure to appropriately learn the task, defined as > 8 sessions to reach the LED threshold or > 12 sessions to reach the proficient threshold.

De-biasing

To prevent the occurrence of spout bias, in which a mouse repeatedly licks one spout regardless of stimulus, we performed a heuristic de-biasing procedure whenever a mouse licked a spout >6 times in a row in response to all "easy" stimuli. This de-biasing consisted of repeated presentations of only sounds associated with the non-preferred spout until the mouse correctly responded 3 times in a row. These de-biasing sessions occurred frequently early in learning and tapered off as the animals became proficient at categorization. Optogenetic trials were not included in de-biasing and were not counted towards the criteria for triggering de-biasing. At the beginning of each session, 20-30 trials of "easy" stimuli were presented to ensure unbiased behavior.

Statistical Analysis

All statistical analysis was performed with MATLAB (Mathworks) and Prism (GraphPad). Data are reported as mean *pm* SEM unless otherwise stated.

Author Contributions

RFK and RSW conceptualized all experiments. RFK collected and analyzed all data. CNC and MPA conducted all surgical procedures. LIB, JC, RD, HBK, HJM, and JMW assisted with behavioral training. RFK and RSW prepared figures and wrote the manuscript.

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Declaration of Competing Interests

The authors declare no competing interests.

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