RESEARCH ARTICLE

running head: cortex-dependent taste-odor association learning

Prior retronasal odor experience enhances taste-odor association learning via the insular cortex and posterior piriform cortex

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# ABSTRACT

## Olfactory experience drives many of the associations we develop for food and beverages. The influence of olfaction on eating is widely noted, however, the underpinnings of how exactly this influence creates the associations is understudied. In fact, previous work has demonstrated that olfactory stimuli can be processed in two different modes, retronasal (through the mouth) and orthonasal (through the nose). Though olfactory experience is conventionally thought of in terms of smelling things outside of the body, rats learn an odor-reward association preference learning task faster when the odor is retronasally experienced rather than orthonasally. Based on this difference and previous work concerning the impact of innocuous experience on taste aversion learning, we hypothesized that enriching the olfactory environment with novel retronasal olfactory experiences would similarly potentiate olfactory association learning. Our study tests this hypothesis, examining the impact of olfactory exposure (OE) on subsequent olfactory preference learning (preference for an odor paired with sucrose) in rats. Our results demonstrate that rats trained after OE develop a significantly stronger preference for the paired odor than unexposed rats. Given that retronasal preference learning and taste pre-exposure are both gustatory cortex (GC) dependent, we employ a complementary experiment to test whether the enhancement of this learning from prior OE is also dependent on GC, hypothesizing that optogenetically inhibiting GC function during learning will prevent the OE-induced potentiated learning we see in our first experiment, resulting in rats having a similar preference for the paired and unpaired odor regardless of prior OE. Finally, because of the role posterior piriform cortex (pPC) in multisensory integration and olfactory processing we repeat the experiment above while inhibiting pPC. Overall, our results indicate that prior innocuous olfactory experience improves preference learning and (like taste pre-exposure) that innocuous olfactory experience likely enhances learning *via* GC and pPC.

## **NEW & NOTEWORTHY**

In a novel investigation, innocuous olfactory experiences, stabilize individual differences in rat preference after odor association learning. Enriching olfactory environments with novel retronasal experiences significantly enhances the degree olfactory association learning. Furthermore, a pivotal involvement of gustatory cortex (GC) and posterior piriform cortex (pPC) are required for the development of taste-potentiated odor associations.

**Keywords:** Chemosensation, Multisensory, Lick Microstructure, Inter-Region, Flavor

# INTRODUCTION

Taste-odor associations are critical for organismal survival. Much work has been done on how taste-odor associations produce aversion to conditioned odors in taste-potentiated odor aversion (TPOA) tasks, however, parsing out how preferences develop over time remain somewhat elusive in terms of system neuroscience. Recent research has shown roles of gustatory cortex (GC) involvement in TPOA in an odor modality specific manner. TPOA is thought to act through within-compound association, requiring GC, such that the perception and recognition of novel tastes is required for the development of TPOA. GC has been determined as an area involved in the recall of not just the tastes experienced during associative learning but also the odors. Posterior piriform cortex an “association area” close to areas associated with hedonics, memory, and smell seems a likely area in which these taste-odor association may be created. To analyze whether this is the case we implemented a multi-cohort protocol testing rat taste-odor association learning is GC or pPC dependent. Additionally, we aimed to see how experience may override GC or pPC involvement in taste-odor associations.

*Olfactory preferences play a pivotal role in guiding feeding behaviors and are influenced by a complex interplay of sensory inputs and associative learning processes. Investigating how animals respond to different odors and their associations provides valuable insights into the mechanisms underlying olfactory perception and preference formation. Building upon previous research on olfactory conditioning and preference testing, the current study aims to elucidate the impact of prior odor experience on retronasal and orthonasal olfaction preferences using a brief access bottle task.*

*Previous studies have demonstrated that rats exhibit selective preferences for odors associated with palatable or aversive taste stimuli, reflecting the integration of olfactory and gustatory sensory cues during learning (Experiment 2; Multisensory Context). Moreover, the gustatory cortex (GC) has been implicated in the expression of retro-trained preferences, highlighting its role in mediating associative learning processes related to olfactory-taste associations (GCx Effect). Understanding the neural circuits involved in processing olfactory information and their modulation by prior experience is crucial for unraveling the complexities of olfactory preference formation.*

*In addition to the gustatory cortex, the piriform cortex (pPC) represents a key region involved in olfactory processing, contributing to the discrimination, and encoding of odor stimuli. However, the specific contributions of the pPC to olfactory preference learning and expression remain to be fully elucidated. By employing optogenetic manipulation techniques targeting the GC and/or pPC, the current study seeks to dissect the neural mechanisms underlying olfactory preference formation and the role of prior odor experience in shaping these preferences.*

*Given the importance of both orthonasal and retronasal olfaction in guiding feeding behaviors, investigating how prior odor experience influences preference for odors presented via these pathways is of particular interest. By employing a brief access bottle task paradigm, which allows for rapid assessment of olfactory preferences, this study aims to provide novel insights into the dynamics of olfactory preference formation and the neural substrates underlying these processes.*

*In summary, this study builds upon previous research on olfactory conditioning and preference testing by examining the influence of prior odor experience on retronasal and orthonasal olfaction preferences. By integrating behavioral assays with optogenetic manipulations targeting specific brain regions implicated in olfactory processing, this study aims to deepen our understanding of the neural mechanisms underlying olfactory preference formation and the role of prior experience in shaping these preferences.*

Experiment 1:

Rats avoided consuming stimuli containing an odor previously paired with an unpalatable concentration of citric acid.

Neophobia influenced avoidance of novel odorized stimuli.

Preference for stimuli paired with palatable sucrose over unpalatable citric acid was consistent.

Experiment 2:

Rats preferred stimuli containing a novel odor over stimuli paired with an unpalatable concentration of citric acid.

Preference for stimuli previously paired with palatable sucrose remained consistent.

GCx Inhibition Study:

Inhibition of the gustatory cortex (GCx) blocked expression of retro-trained preferences but not ortho-trained preferences.

Ortho preferences were unaffected by GCx, indicating GC involvement in expressing retro-trained preferences only.

Single pPC Neuron Response Analysis:

Single neurons in the piriform cortex (pPC) responded selectively to multiple odors.

Discriminability between odors increased post-conditioning, suggesting qualitative changes in odor-evoked responses.

Multisensory Context Influence on Flavor Preferences:

Extensive training on taste-smell mixtures resulted in the formation of associations between taste and odor components.

Preferences for congruent flavors in multisensory conditions were not significantly different from preferences in corresponding unisensory conditions.

Multisensory context increased the reliability of flavor preference decisions, independent of prior experience with specific taste-smell mixtures.

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### Subheading level 3

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#### Subheading level 4 (if needed)

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# MATERIALS AND METHODS

## Ethical approval

# Experimental procedures complied with the Brandeis University Institutional Animal Care and Use Committee guidelines.

## Animals

In total, 53 adult Long–Evans rats (www.criver. com), weighing between 250 and 400 g, served as subjects. All rats were individually housed and kept under a 12:12 h light/dark photocycle and received food and water ad libitum until the second day of habituation to the Davis MS-160 Lickometer apparatus (MedAssociates) in which they began water-restriction until the end of the experiment (10-15mL daily). Prior to experiments rats were acclimated to the room and handled for one week. Experiments were conducted during the light cycle. No animals were excluded from the analysis. Animals that exhibited weight loss below 80% of their baseline weight (measured before the start of water deprivation), complications during recovery from surgery or other signs of distress were excluded from further experimental procedures.

## Surgery

## Stereotaxic surgery was performed under isoflurane anesthesia. Adeno-associated virus (serotype 9) coding for ArchT (AAV-CAG-ArchT-GFP; www.genetherapy.unc.edu) was injected into GC bilaterally at three locations 4.8 mm, 4.6 mm, and 4.4 mm ventral from the surface of the brain (5 μl/hemisphere) using a Nanoject III (https://www.drummondsci.com/) at a rate of ~5 nl/s, three weeks before the experiment (rats in Experiment 2 only). AAV serotype 9 is known to spread well across the tissue and infect all cell types over this time course. GC was defined as the region of insular cortex where we and others have repeatedly found a high density of taste responses. This region corresponds with the region previously identified as receiving projections from the gustatory thalamus. Layer 3 of pPC was chosen as the target for pPC inhibition due to the large amount of odor responsive pyramidal cells and their long-range projections. Optic fibers (rats in Experiment 2 only) were implanted bilaterally in gustatory insular cortex (GC, 1.4 mm anterior to Bregma, 5 mm lateral to the midline, 4.7 mm ventral from the surface of the brain) or in posterior piriform cortex (PC, 1.4 mm posterior to Bregma, 5.5 mm lateral to the midline, 7.0 mm ventral from the surface of the brain). Skull screw and dental cement were used to keep fibers in place and dust caps were put on the ends of the optic fiber ferrules to prevent damage to the fibers. After a week of recovery, animals that returned to normal behavior and activity were started on the experimental protocol.

## Stimuli

Odor test stimuli were exemplars of odorants used in olfactory experiments (www.sigmaaldrich.com), >98% purity): methyl valerate, carvone, ethyl butyrate, cis-hexen-1-ol, and citral in aqueous solution (0.01% v/v in distilled water). Paired taste stimuli (www.fishersci.com) sucrose (200 mM) in distilled water. Odor pre-exposure was delivered in either the homecage drinking bottle or in cotton balls taped above the food tray. Training stimuli consisted of mixtures of a single odorant and a single tastant or an odor dissolved in distilled water.

## Stimulus presentation & recording procedures

## Rats (n = 53) were tested in a clean Davis MS-160 Lickometer apparatus at approximately the same time each day. Each rat was habituated to the testing chamber and trained to drink water across 5 days (a period during which they also acclimated to water restriction) and then put onto a training protocol consisting of a pre-preference test, training block, and post-preference test. Combined orthonasal and retronasal stimuli consisted of odors dissolved in water in bottles, retronasal only stimuli utilized a small fan to eliminate eminent odors from the bottle spout, and orthonasal only stimuli consisted of odors swabbed onto the outside of the bottle spouts at points where animals did not lick. Animals were given 60 seconds to commence licking when a bottle was presented and limited to 5 seconds from first lick onset to lick at the bottle spout. Odor solutions delivered pseudo-randomly over 60 trials were delivered for all conditions except for experiments which utilized lasers where it was reduced to 42 trials due to time constraints and this being the point where most animals stopped participating in trials.

**Table 1.** Experimental Groups

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Conditions** | | **Training Length** | **Training Sessions** | **N** |
| Orthonasal Stimuli | Naive | 6 Days | 6 | 5 |
| Experienced | 4 Days | 4 | 5 |
| Retronasal Stimuli | Naive | 6 Days | 6 | 5 |
| Experienced | 4 Days | 4 | 5 |
| Combined Ortho/Retro Stimuli | Naive | 3 Days/6 Days | 6/6 | 3/5 |
| Experienced | 6 Days | 6 | 5 |
| Laser Inhibition of GC during Retro | Naive | 6 Days | 6 | 5 |
| Experienced | 6 Days | 6 | 5 |
| Laser Inhibition of pPC during Retro | Naive | 6 Days | 6 | 5 |
| Experienced | 6 Days | 6 | 5 |

27/ 53 Animals Done

## Optical Inhibition

Transfected GC and pPC neuron populations were illuminated using 532nm light from a laser through a rotary patch cable splitter into two multimode optic fibers 200µm in diameter epoxied into stainless ferrule (www.thorlabs.com) with roughed sides calibrated to 40W power. Light strength was chosen based on previous experiments demonstrating inactivation of cells from at least 1mm from the tips of flat-tip optic fibers and confined to target areas. The recurrent circuitry and interneurons of GC and pPC creates a situation where manipulation of specific cell types produces disinhibition which causes activation of neurons as well as inactivation, however, the target of this optical inhibition is to disrupt normal responses from GC and pPC respectively by targeting a variety of cell types. This prevents complex network effects and neuronal kindling. Overall consumption rates of rats remained not significantly different from other conditions in the transfected animals during both testing and training.

## Quantification & Statistical Analysis

Quantification of viral expression was performed by Statistical analyses were performed in Python (Python Software Foundation). Mixed-design ANOVA, Wilcoxon-Rank Sum, and Effect Size (Cohen’s d) were implemented to test multiple groups. Pilot data from the combined group was used to calculate sample sizes using La Morte Power Calculations. The package pingouin was used for all significance tests. A p-value statistic of <0.05 was considered significant.

## Histology

Fibers were labelled with a drop of Fast Green or Vybrant® DiI cell-labelling solution (www.thermofisher.com), brushed onto the tip before implantation, facilitating postmortem histological reconstruction of the implant location. After experiments, rats were perfused trans-cardially with saline and 10% formalin, their brains extracted and placed in 30% sucrose for 3–5 days. Brains were then frozen; coronal sections were cut around the implant location using a Leica SM2010R sliding microtome, mounted on glass slides in Fluoromount-G medium (www.southernbiotech.com) and a cover slip was applied. Epifluorescence microscopy was used to visualize GFP and 1,1 -dioctadecyl-3,3,3 ,3 -tetramethylindocarbocyanine perchlorate (DiI). Figure 1 quantifies viral expression and confirms histological reconstruction of electrode placement in GC and pPC for all animals.

## Statistical analysis

## Data and Software Availability

# RESULTS

The observed difference in preference, as indicated by the difference in average lick counts per trial, between odors associated with sucrose and those unpaired with sucrose, reveals a significant increase in average licks per trial for both enriched and unenriched cohorts (p=0.03 and p=0.01). Strikingly, the enriched cohorts displayed a less significant difference in preference compared to the unenriched counterparts, contrary to our initial hypothesis. Furthermore, subjects subjected to an equivalent number of training sessions over a condensed period of 3 days, as opposed to the standard 6-day regimen, did not develop a discernible preference for either odor (p=0.38).

Upon scrutinizing the change in preference pre- and post-training, a discernible and significant increase in the change of average licks per trial was exclusively seen within the enriched group (p=0.01). The results suggest that the duration of the training regimen and environmental enrichment may modulate the expression of odor preference in a nuanced way, with there being no difference in the establishment of a preference but instead for how strongly that preference develops from pre- to post-training levels, thus, meriting further investigation into the underlying mechanisms governing these observed outcomes.

# DISCUSSION

Explain your interpretation of the data, especially compared with previously published material cited in the References. Significance and limitations may also be present.

Perspectives and Significance

This section is required at the end of all *AJP-Regulatory, Integrative and Comparative Physiology* DISCUSSION sections.

# APPENDIX

This section may be included in mathematical modeling or computational papers, e.g., to provide details of a solution strategy.

# GLOSSARY

This section is only included for equation-laden articles with many different symbols (such as mathematical modeling or computational papers). See [this article](https://doi.org/10.1152/advan.00171.2021) for an example.

Abbr. definition

# DATA AVAILABILITY

Provide a statement declaring where the source data can be found, including DOI / URL (accession number).

# SUPPLEMENTAL MATERIAL

List all cited Supplemental Figures, Tables, Audio, Videos, etc. and where they can be found, including DOI / URL (accession number).

# ACKNOWLEDGMENTS

This section is optional. This is where to thank people indirectly involved with the research (e.g., technical assistance, gifts of reagents, loans of equipment, suggestions during writing). Dedications of articles to persons living or deceased are not permitted. Do not include “promissory notes.” APS Journal policy is against inclusion of implicit or explicit promises that future work will be published.

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# DISCLOSURES

Authors are required at the time of submission to disclose any perceived or potential conflict of interest, financial or otherwise. See [**Author Conflict of Interest**](https://journals.physiology.org/author-info.ethical-policies). If the article is accepted for publication, information on the perceived or potential conflict of interest, or lack thereof, must be noted in the Disclosures section.

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# AUTHOR CONTRIBUTIONS

Identify which authors participated in the research: Conceived and designed research, performed experiments, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript. The information must be the same as in the online submission site.

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# FIGURE LEGENDS

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# TABLES

Footnotes should appear below table with all symbols and abbreviations defined. Example:

**Table 1.** p-values

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | **Combined Stimulus** | | ***P* Value** | **Ortho** | | ***P* Value** | **Retro** | | ***P* Value** |
| Post Training Results | | **Paired** | **Unpaired** |  | **Paired** | **Unpaired** |  | **Paired** | **Unpaired** |  |
| Average Licks per Trial | Enriched | 24.08(±6.62) | 19.61(±5.23) | 0.037\* |  |  |  |  |  |  |
| Unenriched | 27.16(±5.80) | 14.94(±8.01) | 0.013\* |  |  |  |  |  |  |
| Increase in Preference (Pre/Post) | Enriched | 1.1(±0.22) | 0.50(±0.11) | 0.019\* |  |  |  |  |  |  |
| Unenriched | 2.7(±2.98) | 0.59(±0.48) | 0.149 |
| Discrimination Index | Enriched |  |  |  |  |  |  |  |  |  |
| Unenriched |  |  |  |
| Average Bout Length Differences | Enriched |  |  |  |  |  |  |  |  |  |
| Unenriched |  |  |  |

Values are means ± SE; n = 7–9. ANG II, angiotensin II. Comparisons made vs. same-sex sham controls at 40 mmHg by two-tailed Student’s *t* test (males) or one-way ANOVA with Bonferroni post hoc correction (females).