Development of Innate Odor Encoding in the Mouse Brain

A Thesis

submitted to

Indian Institute of Science Education and Research, Pune, in partial fulfillment of the requirements for the BS-MS Dual Degree Programme

by

Subhransu Shekhar Pattanayak

Indian Institute of Science Education and Research, Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

Date: April 2025

Under the guidance of

Supervisor: Dr. Ron Yu,

Case Western Reserve University

From June 2024 to April 2025

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled ‘Development of Innate Odor Encoding in the Mouse Brain’ towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Subhransu Shekhar Pattanayak at the Indian Institute of Science Education and Research under the supervision of Dr. C. Ron Yu Professor, Department of Neuroscience, Case Western Reserve University during the academic year 2024-2025.

Dr. C. Ron Yu

Committee:

Dr. C. Ron Yu 

Dr. Nixon M. Abraham

This thesis is dedicated to my family and my guru, Mr. Sudipta Bhanu Hota

Declaration

I hereby declare that the matter embodied in the report entitled Development of Innate Odor Encoding in the Mouse Brain are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Nixon. M. Abraham and the same has not been submitted elsewhere for any other degree.

Subhransu Shekhar Pattanayak

Subhransu Shekhar Pattanayak

Date: 20/05/2025

Table of Contents

List of Tables

List of Figures

Abstract

Innate behaviors are crucial to the survival of animals in their natural environment. It enables them to avoid and escape predation risk, mate and reproduce, and find food sources. Innate odor identity and valence establishment is an elusive and highly regulated developmental process. How does the brain develop to pre-assign certain odors with specific valence innately? The first step towards addressing such bigger questions is understanding the process of neuronal circuit maturation in various parts of the brain. The current project addresses when and where the circuits potentially involved in processing innate odor identity and valence are established during the critical period. The project uses the TRAP2; R26Rtdt line, and innate odor exposures to mimic natural encounters with such odors to identify the neuronal population involved in innate odor processing during the critical period. It uses neuronal activity as a handle to explore circuit maturation. By labeling cells involved in processing innate odors at various time points during the critical period and comparing them to mature circuits processing the same odor reveal postnatal day 12 to day 16 is the time window during the critical period when the innate odor processing circuits are established. Further analysis shall also reveal the circuit maturation in all anatomical areas involved in innate odor processing.

Acknowledgments

I would like to express my gratitude to my supervisor, Dr. Ron Yu, for his mentorship and continuous guidance throughout my thesis work. His insightful advice and crucial contributions were of great help to me throughout my project. I am also grateful to my supervisor, Dr. Nixon. M. Abraham for his insightful suggestions and encouragement. I would also like to thank my mentor, Dr. Qiu Qiang, who not only taught me the necessary techniques but also guided and encouraged me throughout the project. I am grateful to Dr. Rahul Garg, Dr. Julin Wang, Dr. Ai Fang, and Ian Torres for their constant support and insightful suggestions throughout the project. I would also like to thank all the other lab members of the Yu Lab for making me feel at home: Devin, Max, Happiness, Thelema, Shubhangi, Vikas, Jae, Ian Freed, and Damie. I am also grateful to the core facility at Stowers Institute for Medical Research for providing all the pieces of equipment necessary for the project, and special thanks to the Animal facility and LASF for providing and maintaining all the animals and biosafety. I also thank IISER Pune for allowing me to work at Stowers Institute For Medical Research. Finally, I am extremely thankful to my parents and my brother, who supported and motivated me throughout the year. I wish to thank my friends, especially Anuj, Archana, and Lucy, for all the lighthearted discussions, motivation, and fond memories that they have given me.

Contributions

Contributor name	Contributor role
C. Ron Yu	Conceptualization Ideas
C. Ron Yu, Qiang Qiu	Methodology
	Software
-	Validation
Subhransu Pattanayak, Qiang Qiu	Formal analysis
Subhransu Pattanayak, Qiang Qiu	Investigation
	Resources
Subhransu Pattanayak, Qiang Qiu	Data Curation
Subhransu Pattanayak	Writing - original draft preparation
Subhransu Pattanayak, C. Ron Yu	Writing, review, and editing
	Visualization
C. Ron Yu	Supervision
C. Ron Yu, Qiang Qiu	Project administration
C. Ron Yu	Funding acquisition

This contributor syntax is based on the Journal of Cell Science CRediT Taxonomy[1].

[1] https://journals.biologists.com/jcs/pages/author-contributions

Chapter 1 Introduction

Identity and valence are two crucial concepts in understanding animal behavior. Identity is how the brain differentiates unique objects. Valence is the value, i.e, positive, which can induce an approach with the motivation for reward, or negative, which causes aversion to avoid punishment or danger. Valence imposition on an object's identity mostly occurs in higher cortical regions of the brain, like the amygdala, habenula, striatum, and prefrontal cortex (Gadziola MA, et al., 2015). Animals learn to assign valence to different objects through associative learning. Animals can be conditioned to associate a neutral stimulus with a positive or negative valence. Such associations are learned and are plastic and can, therefore, be modified throughout the animal's life span. However, valence associations with certain dynamics of sensory inputs are innate to animals. Behaviors like freezing, escaping, and grooming are innate behaviors observed in rodents in response to innately recognized cues like visual and auditory looming cues and predator odors. Such innate associations are crucial to survival in natural environments. Animals exhibit two qualities of innate responses towards the corresponding innate cues: (i) appetitive/ attractive and (ii) aversive/ fear response. Such responses can be studied in a controlled environment using behavioral assays and readouts. Using such approaches, the current project explores one of the aspects of the development of innate behavior in the context of innately recognized olfactory cues in mice.

1.1. RODENT OLFACTORY SYSTEM

The mouse olfactory system consists of the olfactory epithelium (OE), the olfactory bulb (OB), and the olfactory cortex (OC). The organization of the olfactory system is depicted in Figure 1. The olfactory epithelium is lined with olfactory sensory neurons (Buck and Axel, 1991). Each sensory neuron expresses only one type of receptor, to which only a specific set of molecules bind; a single odorant molecule may bind to multiple receptor types (Jiang et al., 2015; Malnic et al., 1999). The Olfactory sensory neurons (OSNs) of specific receptor types have convergent projections onto one or two glomeruli in the olfactory bulb (see Figure 1). An odor can consist of only a single odorant or may contain multiple types of odorant molecules. Depending on the composition of the odor, a specific set of glomeruli are activated in the olfactory bulb, thereby encoding the odor identity in the form of a glomerular topographic map. Thus, a pattern of glomerular activity as a whole encodes the odor identity and is used to recollect the memory associated with the odor. The OSNs synapse onto the glomerulus's mitral/tufted (M/T) cells. The M/T cells directly or indirectly project to downstream cortical and subcortical brain regions.

1.2. INNATE ODORS BEHAVIORS AND CIRCUITS

Odorants like 2-phenylethylamine (PEA), acetophenone, and peanut butter (PB) are innately recognized. These odors have innately assigned valence. PEA is innately aversive to mice, whereas PB is innately attractive to mice. In the case of non-innate or novel odors, such valence is assigned through conditioning and associative memory acquired through experience. For learned odor associations, the associated memory engram and the odor scene may reactivate the valence circuit connected to the memory engram in past odor experiences (Mori and Sakano, 2021). In contrast to learned odors, it has been hypothesized that the innate odor information is directly relayed to the valence regions in the amygdala. The valence of such sensory inputs is thus genetically assigned by a predetermined connection to appropriate valence centers in the brain. However, these circuits remain subject to postnatal changes and retain their plasticity during the critical period. Changes in the spontaneous activity of neurons during the early postnatal period are known to influence innate odor preference (C Ron Y, et al., 2021). The amygdala plays a crucial role in processing and assigning innate valence to sensory inputs. Sensory input for all modalities except for olfactory inputs are relayed to the amygdala via two major pathways: (i) the shorter thalamo-amygdala circuit (Phelps EA, LeDoux JE, 2005) and (ii) the longer thalamo-cortico-amygdala circuit (McDonald AJ. 1998). All olfactory input signals are relayed directly to the amygdala without a detour to the thalamus. These relays are of two types: (i) the direct Mitral Cell (MC) pathway and (ii) the indirect polysynaptic Tufted Cell (TC) Pathway (Price JL. 2003; Alheid GF, 1995). Most olfactory signals are relayed to the

Cortical Amygdala (CoA) and the Medial Amygdala (MeA). There is also an apparent anatomical segregation along the dorsoventral axis of the OB depending on the quality of odor detected and the downstream projections to the olfactory cortex(OC). MC pathway from the dorsal OB to the posteromedial CoA mediates innate aversive responses.

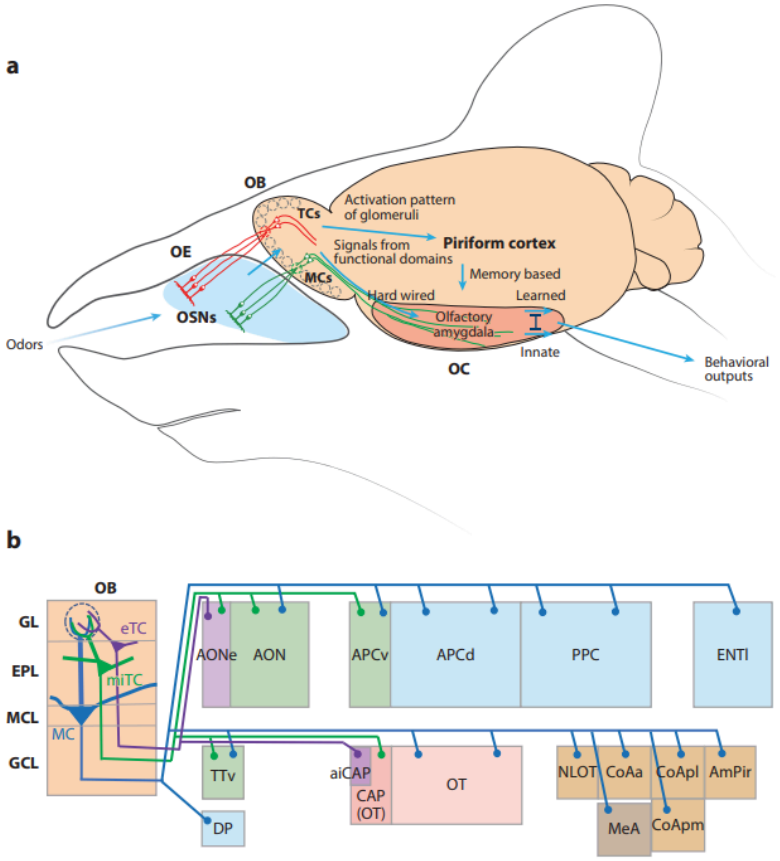

Figure 1: Mouse Olfactory System *Mori K, Sakano H. Olfactory Circuitry and Behavioral Decisions. Annu Rev Physiology. a. Organization of the olfactory system. Odors bind to OSNs lining the Olfactory Epithelium, and the activated OSNs of a specific receptor type convergently project to a single glomeruli in the OB b. Projection cells of the OB and their downstream targets. Mitral cell projections (in blue) are direct and project to the olfactory cortex.*

Mitral cells from the posteroventral OB that project to the anterior MeA mediate attractive social responses. Mitral Cells are the earliest-born neurons in the OB. Given their direct projection onto valence centers in the amygdala, their development and targeting play a crucial role in developing innate odor responses. When and how the connections between the Mitral cells and the valence centers in the amygdala are established remains to be studied. The genetic landscape that instructs the formation of the connections from and within the OB to the valence centers in the amygdala remains elusive. Past studies (Inokuchi et al., 2017) show that Nrp2+ (neuropilin2) MCs in the ventral OB preferentially target anterior MeA; this targeting is lost in conditional knockouts of Nrp2 in MCs. Nrp2 is primarily a ligand for Sema3F, and thus, Sema3F is necessary to segregate the MC targeting the MeA. It has also been shown that the dorsal domain is necessary for processing and relaying innate fear responses to TMT in mice and that the innate aversive response circuit differs from the innate fear response circuit (Kobayakawa, et al, 2007).

However, at the sensory level, innate fear and aversion are mostly processed in the dorsal domain of the OB. At the cortical level, the pathway seems to directly connect projection neurons to the valence centers in the amygdala. Photoactivation of a specific subpopulation in the cortical amygdala is sufficient to elicit an innate aversive response in mice (C.Root et al., 2014; C.Root et al., 2024). The

development of the circuit in the amygdala remains elusive. It has also been shown that the innate odors are encoded via a generalized population code (Qiu Q. et al., 2021) that challenges the labeled line connection to elicit innate odor behaviors. This raises further questions, such as how the representation of the innate odor differs from non-innate odor in the piriform cortex and how the piriform cortex cells for innate odors are connected to valence centers in the amygdala. During development, the olfactory map formation is instructed by a genetic program (Imai et al., 2006; Nakashima et al., 2013). The segregation of the glomerular structures in the OB is dependent on the OSN activity. The intrinsic activity of OSNs influences OR-specific glomerular segregation (Serizawa et al., 2006; Nakashima et al., 2019). During the critical period, the newly formed glomerular map is further influenced by odor-evoked OSN activity, enabling the imposition of attractive quality even on an innately aversive odor, caused by glomerular enlargement of exposed odor leading to odor-memory imprinting and attractive quality imposition on innately aversive odors. Hence the signalling systems underlying postnatal development at the glomerular and cortical level must be explored.

1.3. IMMEDIATE EARLY GENES AND CIRCUIT MATURATION

Immediate Early Genes (IEGs) are a class of activity-regulated genes (ARGs), genes that are produced in response to cellular stimuli. IEGs are a class of ARGs whose transcriptional activity increases rapidly within an hour of neuronal activity like neurotransmitter release. Arc, Erg1, and cFos are some examples of IEGs. cFos gene is a protoncogene, and its expression can be used as a marker for neuronal activity (Bullitt E. et al., 1990). One of the primary aspects of neuronal circuits is the complicated synaptic connections among its neurons. While the circuit develops, the synaptic connections and their plasticity are driven by neuronal activity at the cellular level. A matured circuit will contain a balanced mixture of excitatory and inhibitory neurons (Stiles et al., 2010; Goodman, 1993; Kolodkin, 2011; Winnubst, 2012; Tau, 2010; Seng, 2022). Therefore, using neuronal activity as a marker serves as a good starting point for exploring circuit maturation. As described earlier to explore signaling pathways underlying circuit development it is required to pin down the time during the critical period when the olfactory circuits mature.

Chapter 2 **Materials and Methods**

2.1 ANIMALS USED

Mice of TRAP2; R26RtdT strain were used. All the animals were maintained in the Lab Animal Services Facility of Stowers Institute at 12:12 h reversed light cycle and provided with food and water *ad libitum*. Experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee (IACUC) at Stowers Institute and in compliance with the NIH Guide for Care and Use of Animals. TRAP (Luo et. al., 2013) stands for Targeted Recombination in Active Population; refer to Figure 3. The R26RtdT genetic line has a LoxP-flanked STOP cassette upstream of tdTomato in the Rosa26 locus. There is a robust expression of tdTomato (red fluorescence) upon Cre-mediated excision of the LoxP-flanked STOP cassette. Figure 3 illustrates how the transgenic line works. The inducible CreER is expressed downstream of the IEG cFos, and in the presence of 4-hydroxytamoxifen (4OHT), a downstream metabolite of tamoxifen, is sequestered into the nucleus and excises the STOP cassette flanked by the LoxP sites. Thus permanently labeling only the active cells with tdTomato. In the absence of 4OHT the CreER remains in the cytoplasm, thus no excision of the STOP cassette and no tdTomato expression in any cell. The excision in TRAP cells is irreversible, leading to the permanent labeling of cells. Significant excision occurs in cells only as long as the 4OHT is present in the cells.

2.2. EXPERIMENTAL WORKFLOW

Separate groups of mice were exposed to PEA immediately following a 4OHT injection during Postnatal Day (P) - 4, 8, 12, 16, 21, and 28 in their home cage. The mice were then reexposed to PEA in their home cage, during the adult stage, around P60-70 and followed by immediate intracardiac perfusion and brain dissection. The brains were then sectioned, and the slices were stained for DAPI, tdTomato, and IEG cFos. The images were collected using the slide scanner, and further processing was done using the software VS-Desktop, ABBA, QuPath, and some custom-written scripts in MATLAB and Python.

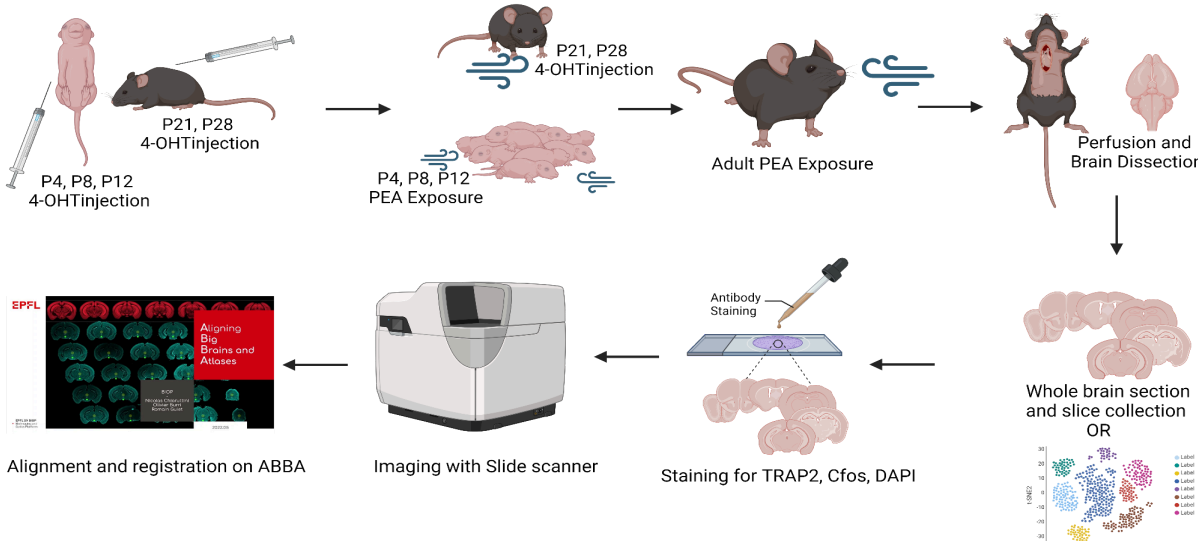

Figure 2: Experimental Workflow

Separate groups of mice are injected with 4-OHT on P4, 8, 12, 16, 21, and 28 and immediately exposed to PEA in their home cage. The same mice are then re-exposed to PEA during the adult stage, followed by whole-brain sectioning and staining.

2.3. ODOR EXPOSURE

The behavioral assay involved exposing the mice to the innately aversive odor PEA (1:1000 concentration) immediately following a 4OHT injection (50 mg/kg) by placing the odor in a cotton pad in their home cage. Mice of age P21 and P28 were exposed to odor post-weaning. The same group of mice was then exposed to PEA during P60- P70 in their home cage. The one-time exposure following the 4OHT injection during the early postnatal period permanently stained the cells active immediately after exposure.

2.4. HABITUATION

Prior to the first early postnatal exposure, mice were habituated for 5-7 days with a mock injection and a cotton pad with no odor during the same time of the day as the actual exposure. Mock injections for P4 and P8 were not performed, as the mice are too young to sustain injections before P4 and P8 without incurring long-term physiological damage.

Prior to adult exposure, mice were single-housed and habituated with a cotton pad without odor and mock injection at the same time of the day as the actual exposure for 5-7 days.

2.5. WHOLE BRAIN SECTIONING AND FOS IMMUNOSTAINING

After 1hr of adult exposure, mice were anesthetized using Urethane and then intracardially perfused with PBS (phosphate-buffered saline) followed by 4% PFA (paraformaldehyde). After which, the brain was dissected, ensuring minimal damage to the tissue. All samples were postfixed overnight at 4℃ in 4% PFA. Postfixed samples were then washed thrice for 15 mins each in PBS. The samples were then mounted in 4% agarose in a mold and cooled for 20 mins. The mounted samples were then sectioned using the Lecia Vibratome 1000s. The following section describes the protocol for vibratome section collection and immunostaining.

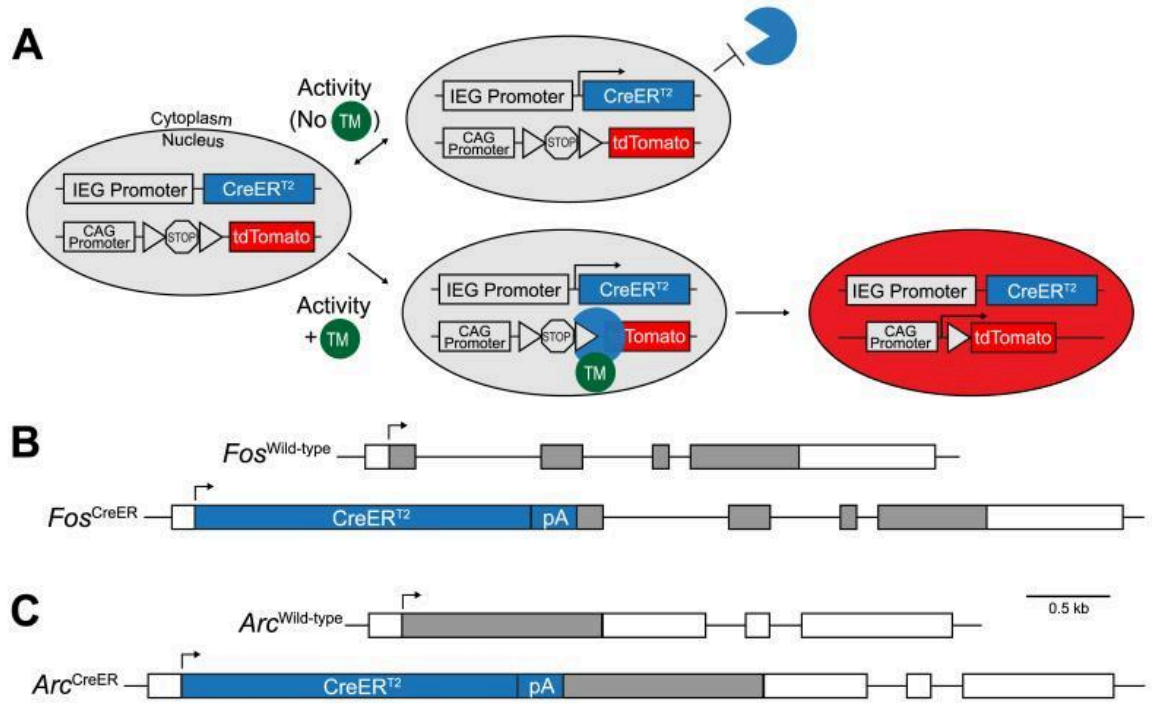

Figure 3: TRAP2:R26RTdt mouse line Genetic Construct

Strategy for Targeted Recombination in Active Population. Luo et. al 2013. A. In the presence of Tamoxifen, IEG-driven CreERT2 is expressed and sequestered into the nucleus in active cells, whereas no nucleus sequestering occurs in the absence of Tamoxifen. Once sequestered, the active cells in the presence of tamoxifen express nuclear tdTomato. B,C. The TRAP2;R26R

2.5.1. Protocol for free-floating whole brain FOS immunostaining:

1. Initial 60um vibratome sections of the OB of a brain mounted in 4% agarose gel were collected on charged slides and kept in a coupling jar filled with citrate buffer(PH-6).
   Free-floating slices (60µm vibratome sections) of the whole brain were collected in 12 serial wells in citrate buffer PH-6.
   I. The slices collected then underwent heat-induced antigen retrieval. This was performed by putting the coupling jars filled with citrate buffer in the microwave and heating until bubbles were seen. The same heating was repeated three times, with a gap of four minutes between each heating session.
   II. The well plates were kept on a slide warmer set to 9 on the heating scale for 10 minutes.
2. After heat-induced antigen retrieval, the citrate buffer was removed, and the slides and wells were washed once with PBST (0.1% Triton in PBS) for 10 minutes.
3. After the wash, the primary antigen was prepared with the following dilution and reagents: *NOTE - The quantities mentioned are for 60µm whole brain vibratome sections collected for two brains, with each brain collected into 12 wells in two different 24-well plates. Thus, there are a total of 12+12 wells and 2+2 OB section slides.*

Required Amount	Dilution antibody	DMSO	PBST
~5.7ml	1:1000 (5.7µl)	10% (520µl)	Req-DMSO = PBST (~5.2ml)

Table 1: *Table describing antibody solution preparation protocol for all antibodies used*

4. The following primary antibodies were used:

	Rabbit Anti-cFos	Goat TdTomato	DMSO
Dilution	1:1000	1:1000	10%
Final Quantity	5.7µl	5.7µl	520µl

Table 2: *Table describing Primary antibody solution composition*

5. 200ul was used for each well, and 200ul for each slide mounted with OBs.
6. Two days of primary incubation were performed. Slides were incubated at room temperature, and well plates were incubated at 4 degrees for 2 days on a belly dancer shaker.
7. After the primary incubation, samples were washed with PBST twice for 5 minutes each.
8. After the washing, the following secondary antibodies were used with the same preparation as in step 4. Secondary incubation was performed for 12 hours.
   I. Slides' secondary incubation was at room temperature.
   II. Well plates were covered with aluminum foil and were incubated at 4 degrees on a belly dancer shaker for 12 hours.

	Donkey AntiRabbit 647	Donkey Antigoat 546	DAPI	DMSO
Dilution	1:1000	1:1000	1:1000	10%
Final Quantity	5.7µl	5.7µl	5.7µl	520µl

Table 3: *Table describing secondary antibody solution composition*

9. Following secondary antibody incubation, the slices were washed once with PBST for 10 minutes and then were serially mounted on charged slides.

2.5.2. Serial whole brain vibratome section mounting:

1. As mentioned, each brain's 60µm whole brain sections were collected in 12 wells serially in a 24-well plate.
2. Following the secondary antibody incubation, sections were checked under the microscope for fluorescence signals.
3. Then the mounting was performed by picking one section from each well, starting from the 1st proceeding serially to the 12th, and then repeating the cycle again, until all slices were mounted. A schematic is presented below: Start from 1A to 6B and then begin again.
4. Meanwhile, warm the water-based mounting media, and mount the slides with coverslips without letting the slices dry too much.

Key resources table

REAGENT OR RESOURCE	COMPOSITION	SOURCE
Buffers and Fixatives		
Phosphate Buffer Saline (PBS) pH - 7.4, 1X	per liter: 8g Sodium Chloride, 0.2g potassium chloride, 1.44g sodium phosphate, 0.44g potassium phosphate	Stowers Institute for Medical Research, Media Prep
PBST	0.1% v/v of Triton in 1X PBS, pH	Yu Lab
Paraformaldehyde (PFA)	4% Paraformaldehyde	Yu Lab
Antibodies		
Rabbit Anti-cFos		Ab190289
Goat tdTomato		Origene
Donkey Anti-Rabbit 647		Invitrogen
Donkey Anti-Goat 546		Invitrogen
DAPI		Life Technologies Corp
Odorants		
Phenylethylamine (PEA)		Sigma Aldrich
Other reagents		
Dimethyl Sulfoxide (DMSO)		Sigma Aldrich
Urethane	2g/10ml 1X PBS	Sigma Aldrich
Agarose		Sigma Aldrich
4-Hydroxytamoxifen	2mg/ml, 50mg/kg per animal	Sigma Aldrich
Mineral Oil		Sigma Aldrich
Software and Algorithms		
ImageJ (Fiji)		
Custom Python Scripts		wormLove Github repo
ABBA and QuPath		ABBAv0.8.0, QuPathv0.4.3
VS-Desktop		

2.6. DATA ACQUISITION AND ANALYSIS

2.6.1. Image Acquisition and Processing

Images of all sample slides were acquired using the Olympus Slide Scanner at Stowers Institute for Medical Research. The images acquired were in Virtual Slide Images and were converted to TIFF in the Olympus VS-Desktop. Images were further processed with the macro by Richard Alexander on ImageJ2.0.

2.6.2. Image Analysis

The TIFF images obtained were first corrected for left-right alignment by visual inspection. After this, the images were arranged anterior to posterior by referring to the mouse brain atlas. The arranged images were then registered and aligned on ABBA (Aligning Big Brain Atlas) using Elastrix to warp the images and align them with the registered brain annotations. Further manual warping was performed to align the images more precisely with the registered annotations. (Refer to Figure 4) After alignment, the registered images were exported to QuPath. Using the ABBA plugin on QuPath, the annotations for the images registered on ABBA were imported onto the image. Finally, custom-written scripts on Groovy cell detection were performed on the image. The cell detection measurements were then exported, and the post hoc analysis was performed using custom-written scripts in Python and Matlab.

2.6.3. Post Hoc Analysis

All cell detection measurements for all annotations across all sections of one brain sample were exported as TSV files to designated folders. A custom-written Python script was used to read, merge, and organize cell detection measurements such that cell detection numbers across all sections of all unique annotations are combined and stored. This data was used to generate meaningful quantifications. All graphs and figures were generated using GraphPad Prism and InkSpace.

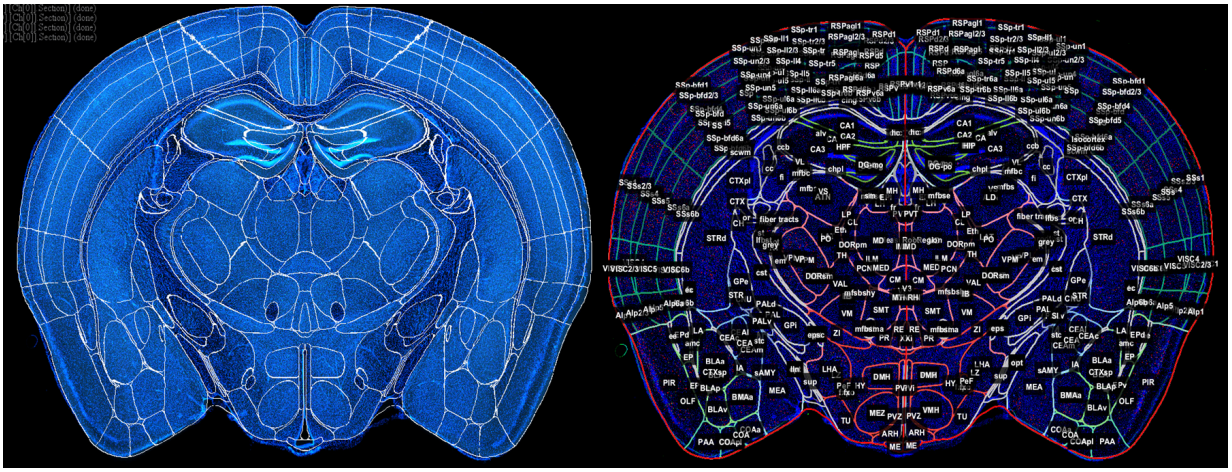

Figure 4: ABBA alignment/registration and QuPath annotations and cell detections
Left panel: Post ABBA alignment using image warping software BigWarp, two kinds of registration of the images to the Allen Brain Atlas annotations of the brain regions on ABBA were performed: (i) Elastrix Registration Affine and (ii) Elastrix Registration Spline, both involving different image warping methods. Right Panel: ABBA-registered and aligned brain sections displayed on QuPath using the ABBA extension. QuPath displays the cell detection channels' images for all sections superimposed on the ABBA annotations.

Chapter 3 Results

3.1. Whole-brain images indicate P12-16 as the maturation age for tdT-labeled Cells:

The TRAP2; R26RTdt genetic line permanently labels the neurons active immediately after the first early postnatal PEA exposure. Hence, examination of the tdTomato signal (Red) across all the brain slices of the whole brain reveals a spatiotemporal map of the circuit maturation. The piriform cortex shows (Figure 5-6) an increase in the Red signal till P12 (Figure 5), after which, P16-P21 there is no significant difference in the Red signal (Figure 6). The mice brains were fixed one hour after the adult exposure to PEA and stained for IEG cFos (Green). The Green signal indicates the activated neurons in the mature circuit in response to adult PEA exposure. There is a significant difference in the cFos between the adult control and PEA groups.

Similar observations were made for the nuclei of the amygdala. Figures 7 and 8 show the tdTomato signal for coronal sections around Bregma: -1.23. The coronal sections show both the Piriform cortex and the observable regions of amygdala nuclei (square dashed bracket - Cortical Amygdala, circular dashed bracket - Piriform Cortex) tdTomato signal significantly increasing till P12 (Figure 7). Although an increase is observed, it is not as significant after P12 (Figure 8). The Green signal is consistent across all sections for animals exposed to PEA as adults. There is a significant difference in this signal between the adult PEA exposed and the adult control group for regions in both the piriform cortex and amygdalar nuclei. Although all cFos activity in all the neurons need not necessarily be a result of the response to PEA, the cFos activity map may be used as a handle to study the circuits involved.

Due to methodological heterogeneity (differences in the angles of the sectioning plane, the starting plane of sectioning, and variability in brain sizes), the representative images are approximately from the same distance from bregma-0. They have been chosen by visual inspection, hence only providing an approximate but qualitative measure of the circuit development. Also, relatively brighter Red signals for the P4, P8 samples are nerve fibers and not cell bodies, thus posing a difficulty for accurate cell segmentation and quantification.

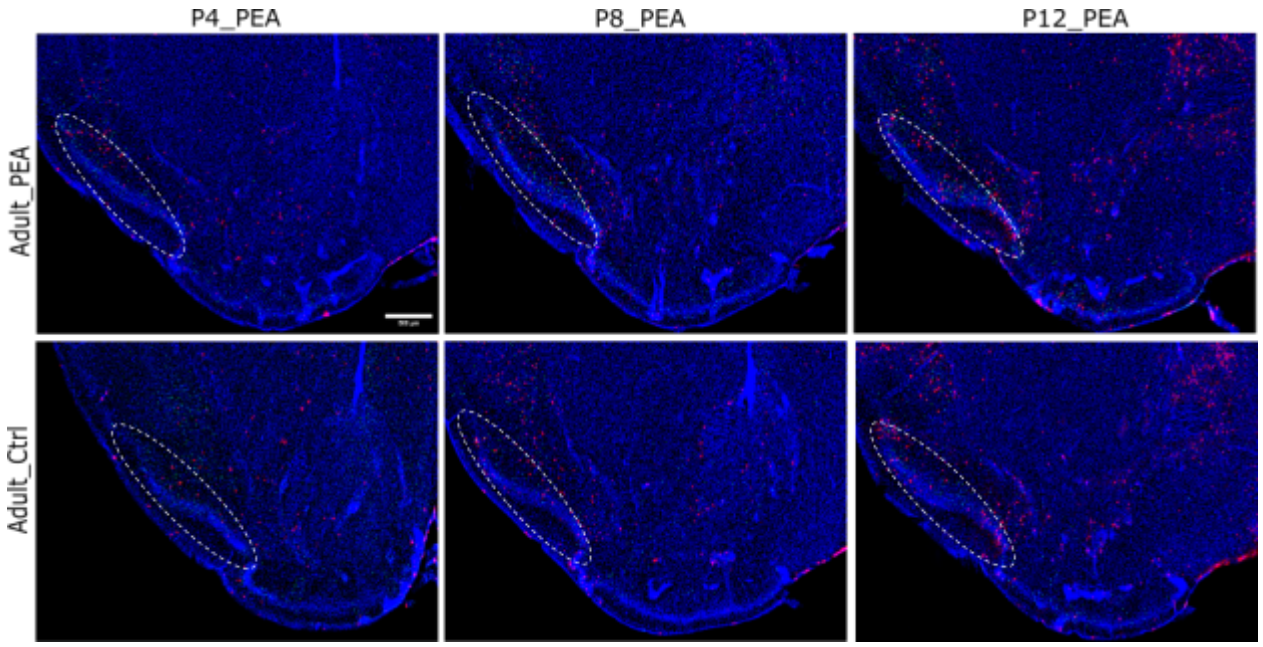

Figure 5: Representative Coronal section images of circuit maturation of active neurons in response to postnatal (P4, 8, 12) PEA exposure: *Red: TRAP2; R26Rtdt, Green: cFos, Blue: DAPI. Scale Bar, 500-µm, Dashed circle, piriform cortex, Bregma-1.10. Top Panel - Coronal section of the mouse brain, Bregma, 1.10, exposed to PEA at P4 (Left), P8 (Middle), and P12(Right) and re-exposed to PEA in the adult stage(Adult_PEA, P50-60). Bottom Panel - Coronal section of the mouse brain, Bregma, 1.10, exposed to PEA at P4 (left), P8 (Middle), and P12 (Right), and no exposure during the adult stage (Adult_Ctrl, P50-60). A significant increase in the Red signal is observed from P4 to P12 across all samples in and around the dashed*

circle. Significant difference in the Green (cFos) signal was observed between the Adult_PEA and Adult_ctrl groups across all samples.

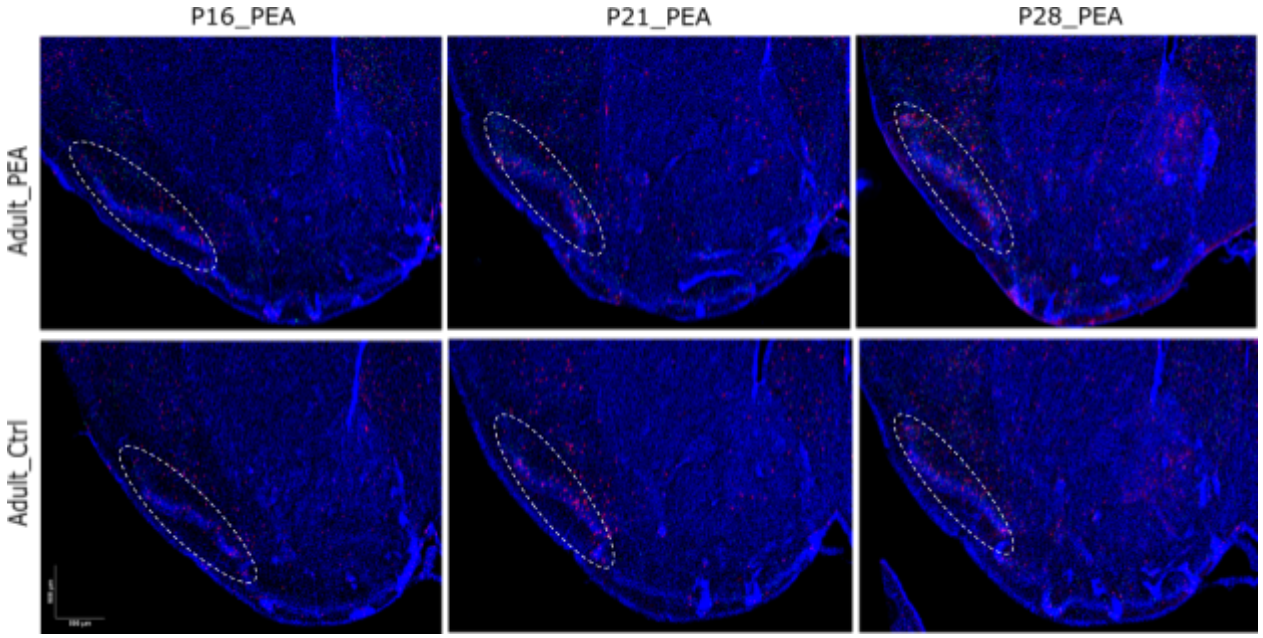

Figure 6: Representative Coronal section images of circuit maturation of active neurons in response to postnatal (P16, 21, 28) PEA exposure: *Red: TRAP2; R26Rtdt, Green: cFos, Blue: DAPI. Scale Bar, 500-µm, Dashed circle, piriform cortex, Bregma-1.10. Top Panel - Coronal section of the mouse brain, Bregma, 1.10, exposed to PEA at P16 (Left), P21 (Middle), and P28 (Right) and re-exposed to PEA in the adult stage (Adult_PEA, P50-60). Bottom Panel - Coronal section of the mouse brain, Bregma, 1.10, exposed to PEA at P16 (left), P21 (Middle), and P28 (Right), and no exposure during the adult stage (Adult_Ctrl, P50-60). A less significant increase in the Red signal than P4-P12 is observed from P16 to P28 across all samples in and around the dashed circle. A Significant difference in the Green (cFos) signal was observed between the Adult_PEA and Adult_Ctrl groups across all samples.*

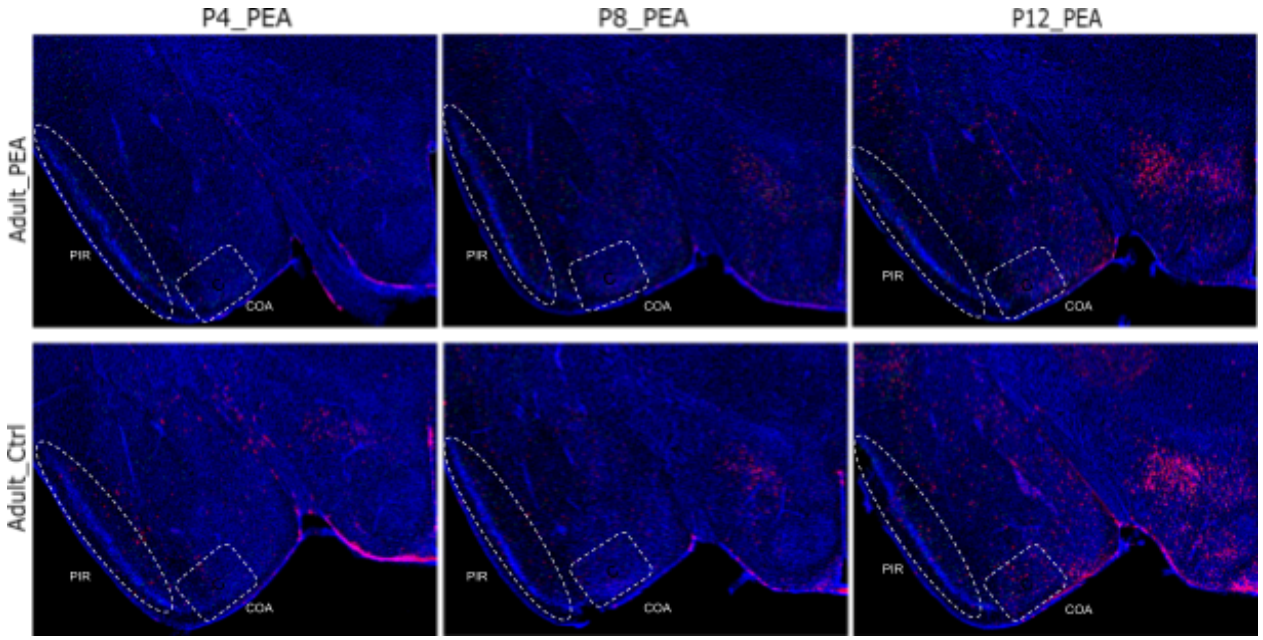

Figure 7: Representative Coronal section images of circuit maturation of active neurons in response to postnatal (P4, 8, 12) PEA exposure: *Red: TRAP2; R26Rtdt, Green: cFos, Blue: DAPI. Scale Bar, 500-µm, Dashed circle, piriform cortex, Bregma-1.10. Top Panel - Coronal section of the mouse brain, Bregma, -1.31, exposed to PEA at P4(Left), P8(Middle), and P12(Right) and re-exposed to PEA in the adult stage (Adult_PEA, P50-60). Bottom Panel - Coronal section of the mouse brain, Bregma,-1.31, exposed to PEA at P4(left), P8(Middle) and P12(Right), and no exposure during the adult stage (Adult_Ctrl, P50-60). A significant increase in the Red signal is observed from P4 to P12 across all samples in and around the Piriform cortex (dashed circle) and Cortical Amygdala (dashed square). Significant difference in the Green(cFos) signal was observed between the Adult_PEA and Adult_ctrl groups across all samples.*

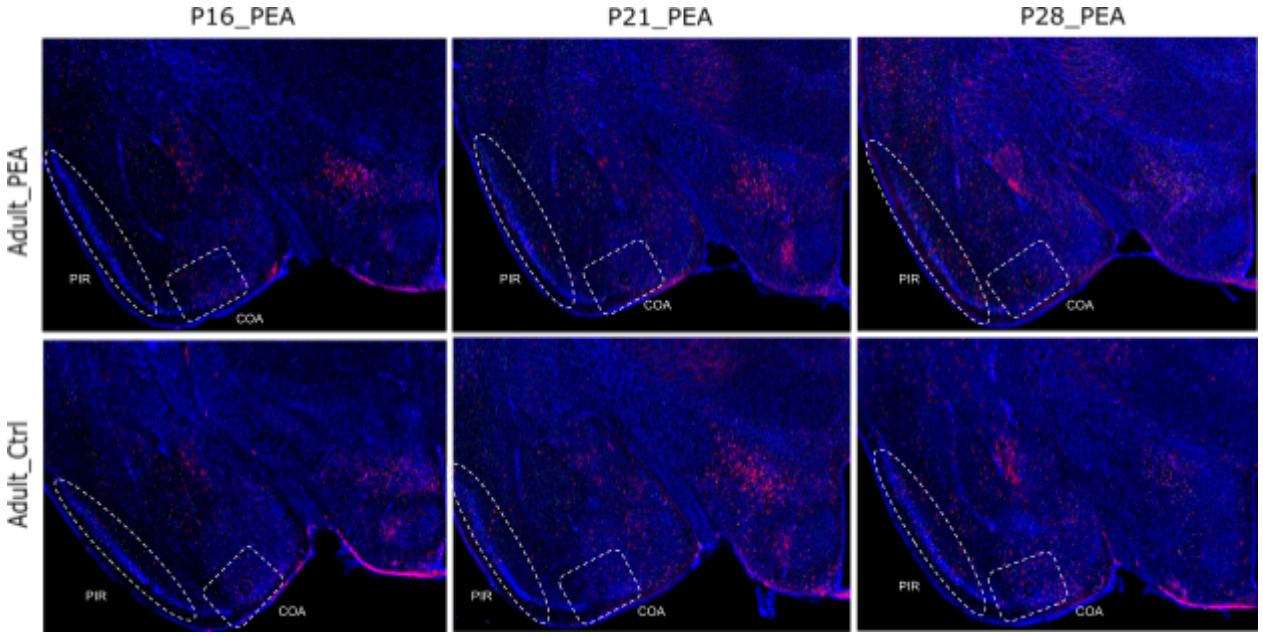

Figure 8: Representative Coronal section images of circuit maturation of active neurons in response to postnatal (P16, 21, 28) PEA exposure: *Red: TRAP2;R26Rtdt, Green: cFos, Blue: DAPI. Scale Bar, 500-µm, Dashed circle, piriform cortex, Bregma-1.10. Top Panel - Coronal section of the mouse brain, Bregma, -1.31, exposed to PEA at P4(Left), P8(Middle), and P12(Right) and re-exposed to PEA in the adult stage(Adult_PEA, P50-60). Bottom Panel - Coronal section of the mouse brain, Bregma,-1.31, exposed to PEA at P4(left), P8(Middle), and P12(Right), and no exposure during the adult stage (Adult_Ctrl, P50-60). A significant increase in the Red signal is observed from P4 to P12 across all samples in and around the dashed circle. Significant difference in the Green(cFos) signal was observed between the Adult_PEA and Adult_ctrl groups across all samples.*

3.2. cFos and TRAP2; R26RtdT double-positive cells' map indicates the maturation age between P12-16:

The IEG cFos positive cells in response to adult PEA exposure represent the active neuronal population of the mature circuit, whereas the tdTomato positive cells in response to early postnatal exposure are a representation of the active neuronal population of the developing circuit. An overlapping population indicates cells that formed and remained a part of the circuit as it developed and matured. Although 100% is never expected, an increasing overlap percentage approaching an asymptote will indicate the age at which the circuit becomes mature and stable. Figures 9 and 10 show representative images of the overlapping cells in coronal brain sections of mice exposed to PEA during the early postnatal and adult stages. Different groups of mice were exposed to PEA in their home cage at different time points postnatally. The time points include P4, 8, 12, 16, 21, and 28.

The coronal sections for representation and quantification were chosen by visual inspection. They have been selected primarily based on their gross distance from the bregma and the number and diversity of anatomical areas visible in the section. The sections in Figure 9 contain the hypothalamus, basomedial, basolateral, Medial, cortical amygdala nuclei, and Piriform cortex in the ventral portion. Major dorsal regions include the hippocampal complex (not shown in the image above) (CA1, CA2, CA3, and dentate gyrus). Thus a set of similar sections captures key areas of the brain known to be involved in processing innate odor. The top panel shows images of separate groups of mice exposed to PEA on P4, P8, and P12, processed for cell segmentation using custom-written codes in the lab. All detections in yellow (double-positive) indicate cFos (Green) and tdTomato (Red) double-positive cells (yellow). The filters and thresholding methods used for cell segmentation are standardized and adjusted based on individual brain section images. There is a significant increase in the double positive cell numbers from P4 to P8 and from P8 to P12.

.

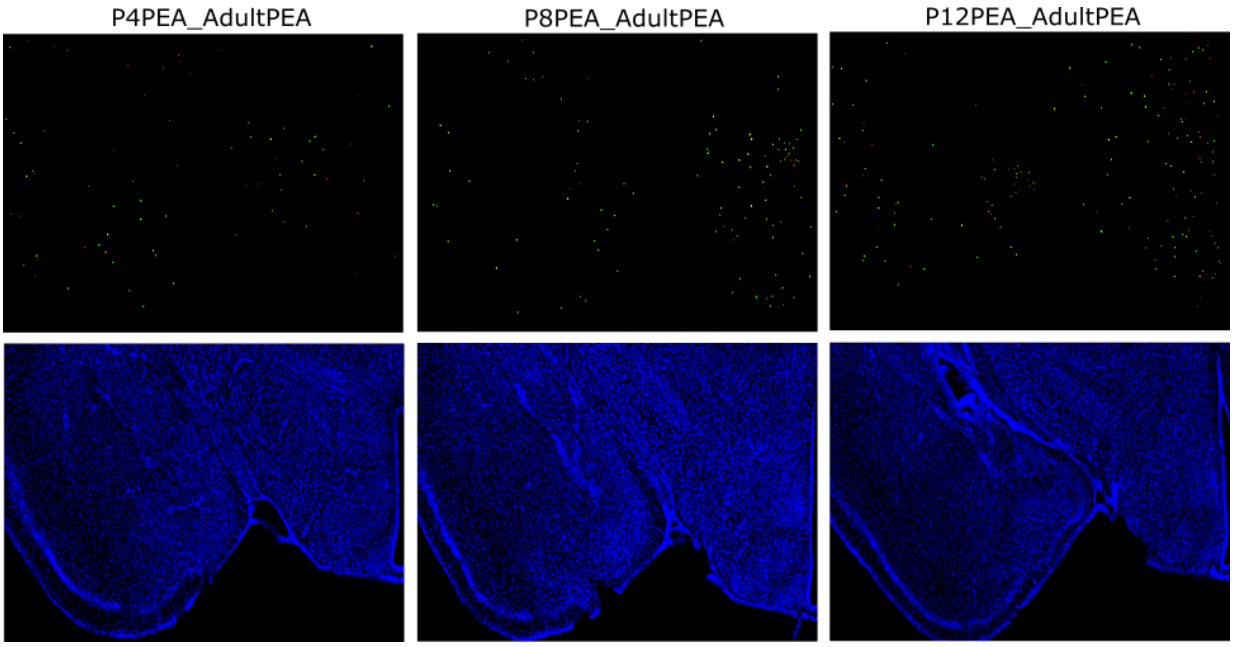

Figure 9: Representative Coronal section images of cFos and tdTomato double positive cells, and DAPI staining of P4, 8, 12 PEA exposed animals: *Red: TRAP2; R26Rtdt, Green: cFos, Blue: DAPI. Scale Bar, 500-µm, Bregma-1.10. Top Panel - Images of TRAP2; R26Rtdt and cFos double-positive cells in a coronal section of the mouse brain, Bregma, -1.31, exposed to PEA at P4 (Left), P8 (Middle) and P12(Right) and re-exposed to PEA in the adult stage (Adult_PEA, P50-60). Bottom Panel - Images of DAPI staining of samples in the top panel for comparison of anatomical areas showing double-positive cells.*

Figure 11 shows the number of cFos and tdTomato double-positive cells across the entire section for the sections displayed in Figures 9 and 10. 3 Selected sections from 2 animals of each age were used to quantify the number of double-positive cells. There is a significant increase in the overlap from P12 to P16, indicating that the circuits potentially mature between postnatal days 12 and 16

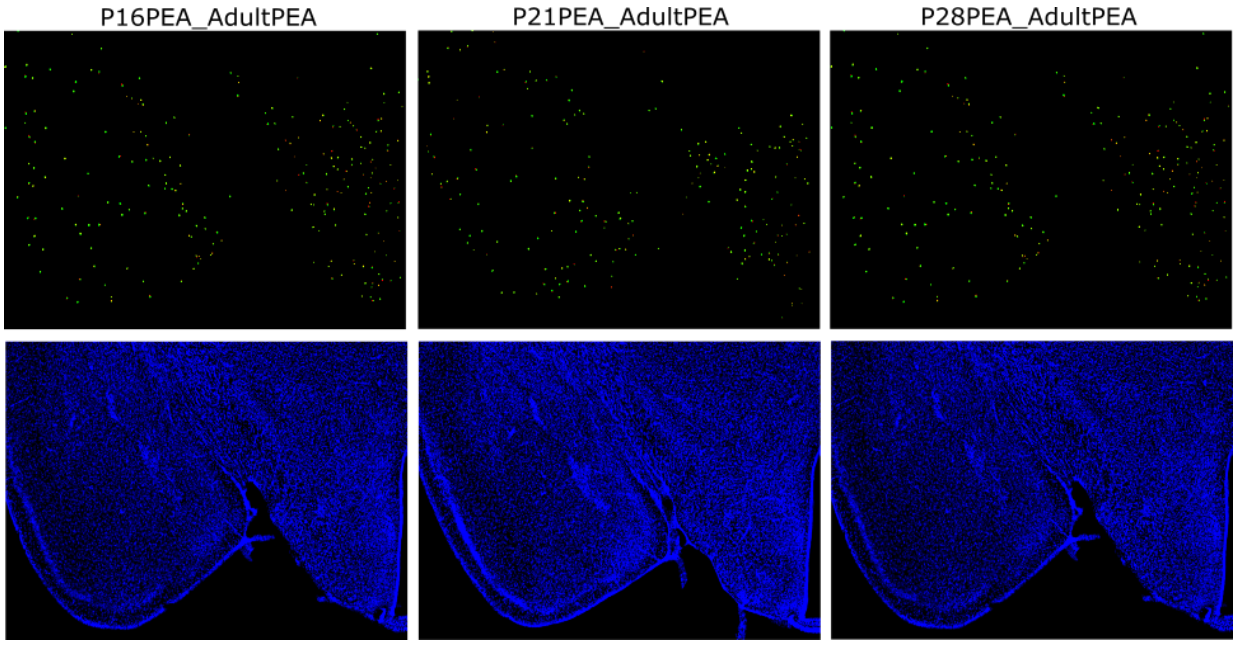

Figure 10: Representative Coronal section images of cFos and tdTomato double positive cells, and DAPI staining of P16, 21, 28 PEA exposed animals: *Red: TRAP2; R26Rtdt, Green: cFos, Blue: DAPI. Scale Bar, 500-µm, Bregma-1.10. Top Panel - Images of TRAP2; R26Rtdt and cFos double-positive cells in a coronal section of the mouse brain, Bregma, -1.31, exposed to PEA at P16 (Left), P21 (Middle) and P28 (Right) and re-exposed to PEA in the adult stage (Adult_PEA, P50-60). Bottom Panel - Images of DAPI staining of samples in the top panel for comparison of anatomical areas showing double-positive cells.*

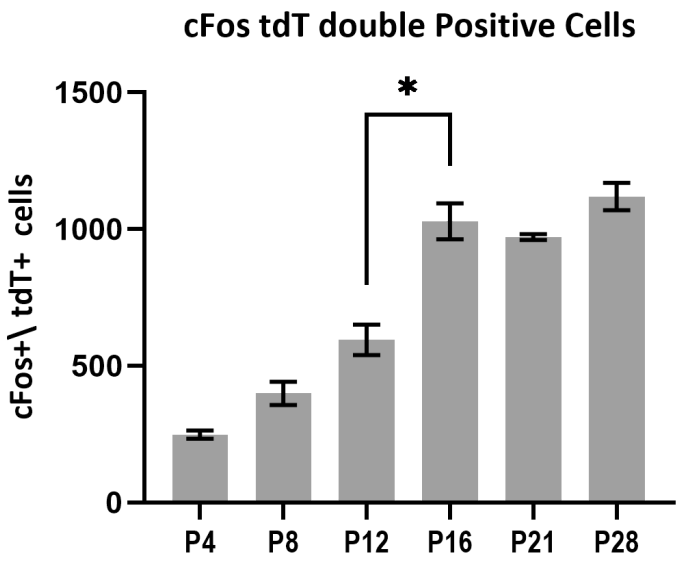

Figure 11: Bar graph showing cFos and tdTomato double positive cell number for a set of selected brain sections: *Bar graph shows the number of cFos+/tdT+ double positive cell number for a set of selected brain sections of animals exposed to PEA on P4, 8, 12, 16, 21, and 28 (x-axis). Data are shown as mean ± SD. *p < 0.05. n = 2.*

P12 to P16 also show a similar significant increase in double-positive cells. However, no significant increase in double-positive cells is observed from P16 onwards. Thus, indicating that the circuits most likely mature sometime between P12 and P16.

Chapter 4 Conclusions

The images in Fig. 5- 10 qualitatively indicate that the age of maturation of the innate odor processing circuits is between P12 and P16, as there is no more significant difference seen in the TRAP2 (Red, indicates the cells active in response to early postnatal PEA exposure) from P16 onwards. Also, the yellow (cFos and tdT double-positive) signals seem to have a similar trend. Indicating that the maturing circuit (tdT signals) and the mature circuit (cFos Signal) approach a maximum overlap between P12 and P16 and thus are most likely established within that period

The quantification of cFos and tdT double-positive cells across 3 selected sections of 2 animals for each age shows a significant difference in overlap only across the ages P12 and P16, indicating that the age of circuit maturation potentially lies between postnatal age P12 and P16.

Chapter 5 Discussion

In this study using TRAP2; R26Rtdt mouse line and the activity of neurons as a handle, the development of innate odor identity and valence establishment circuit was explored in the whole mouse brain. Using the early postnatal PEA exposure behavioral exposure, the cells potentially involved in innate aversive odor identity and valence establishment were permanently labeled and later examined to reveal the approximate time of circuit maturation during the critical period. The images indicate Postnatal Day 12 as a clear and qualitative measure of circuit maturation. Quantifying the proportion of cells permanently labeled during the first postnatal exposure to the DAPI nuclear marker indicates the level of circuit maturation at that age. The proportion of cFos and tdTomato overlapping cells to cFos-labeled cells will indicate the extent of overlap between the developing and the mature circuit in response to PEA exposure. The IEG cFos positive cells in response to adult PEA exposure is a representation of the active neuronal population of the mature circuit. In contrast, the tdTomato-positive cells in response to early postnatal exposure is a representation of the active neuronal population of the developing circuit. An overlapping population indicates cells that formed and remained a part of the circuit as it developed and matured. Although 100% is never expected, an increasing overlap percentage approaching an asymptote will indicate the age at which the circuit becomes mature and stable. It shows the difference in overlapping cFos-positive and tdTomato-positive cell population for two animals but lacks information on the anatomical regions that the detected cell populations belong to. A much more detailed analysis of the current dataset is needed to confirm the time of circuit maturation. Another interesting piece of data will be the proportion of overlapping tdTomato (Red) and cFos (Green) positive cells for each age.

4.1. Potential caveats and challenges of Methodologies:

Using the cFos activity marker as a handle for exploring circuit maturation, however, completely misses (i) inhibitory cells, (ii) cells that underwent apoptosis, (iii) non-neuronal cell populations that contribute to the circuit but do not express IEGs, and (iv) other relevant IEGs (Arc, Npas4, etc.) expressing cell population. Cells that are active and express cFos following PEA exposure need not necessarily be a part of the intended circuit, and it is also not necessary that the same population of active cells is always involved in processing the stimulus every time, which explains why a 100% overlap is never expected. Because innately recognized stimuli invariably lead to stereotyped behaviors, it is expected to have a stable cortical representation in at least the valence encoding areas of the brain. A subpopulation of neurons stably representing the odor identity should ideally also exhibit stable firing. Neurons that were active before 4OHT injection and not in response to PEA exposure, may be expressing some levels of cFos and thus have a chance of being labeled with tdTomato.

The current major challenge is the quantification of the overlapping population of cells through the whole brain within a given ABBA-registered annotation. Such quantification will help discover the neuronal population of the developing circuit within specific anatomical regions that show maximum overlap with the mature circuit in that anatomical region. An optimization and standardization of the cell detection parameters are needed for rigorously quantifying the overlapping cell population across all relevant anatomical regions. Due to time constraints and the tedious data acquisition process, the current data set only shows the trend for 3 selected brain slices for 2 animals of each age.

4.2. Future Experiments and Analysis

A detailed quantification of cFos and tdTomato double-positive cell detection for each nucleus in the whole brain for across all ages and both experimental and control groups will be performed. The comparison shall reveal information about the spatiotemporal maturation of all nuclei and differences in overlap between experimental and control groups. A difference in the overlap between the experimental and control groups shall raise further interesting questions about the cortical representation of innate odor identity and its development in various nuclei of the brain.

For learned odors, the memory engram related to the odor scene is recapitulated from signals relayed from the olfactory bulb via the downstream connections to the hippocampus. This engram then activates the valence circuit that drives an attractive or an aversive behavior. But are such memory engrams of odor scenes necessary for innate odor perception? If not, then how are the developmental programs of learned and innately recognized odors different? Depending on the relative developmental periods of memory engram-forming regions and valence and identity encoding regions the influence of genetic landscape, early postnatal exposure, and experience-dependent plasticity on the establishment of innate odor perception will play out. A more nuanced exploration of the data, using activity marking as a handle to study circuit maturation, will shed light on the development of innate odor identity and valence establishment regions of the brain.

References

1. Phelps EA, LeDoux JE. (2005) Contributions of the amygdala to emotion processing: from animal models to human behavior. Neuron 48:175–87
2. McDonald AJ. (1998) Cortical pathways to the mammalian amygdala. Prog. Neurobiol. 55:257–332
3. Price JL. (2003) Comparative aspects of amygdala connectivity. Ann. N. Y. Acad. Sci. 985:50–58
4. Alheid GF, de Olmos JS, Beltramino CS. (1995). Amygdala and extended amygdala. In The Rat Nervous System, ed. G Paxinos, pp. 443–93. San Diego: Academic. 2nd ed.
5. Allen Brain Atlas, Mouse Brain P56 Coronal, https://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas, Accessed Feb 27, 2025
6. Inokuchi K, Imamura F, Takeuchi H, Kim R, Okuno H, et al. (2017). Nrp2 is sufficient to instruct circuit formation of mitral cells to mediate odor-induced attractive social responses. Nat. Commun. 8:15977
7. Kobayakawa, K., Kobayakawa, R., Matsumoto, H. et al.. (2007) Innate versus learned odour processing in the mouse olfactory bulb. *Nature* 450, 503–508
8. Root CM, Denny CA, Hen R, Axel R. (2014) The participation of cortical amygdala in innate, odour-driven behaviour. Nature. 515(7526):269-73.Epub.
9. Qiu Q, Wu Y, Ma L, Yu CR.(2021). Encoding innately recognized odors via a generalized population code. Curr Biol.31(9):1813-1825.e4. Epub.
10. Gadziola MA, Tylicki KA, Christian DL, Wesson DW (2015). The olfactory tubercle encodes odor valence in behaving mice. J Neurosci.35(11):4515-27.
11. Qiang Qi, Yunming Wu, Limei Ma, Wenjing Xu, Max Hills Jr, Vivekanandan Ramalingam, C Ron Y, (2021) Acquisition of innate odor preference depends on spontaneous and experiential activities during critical period eLife 10:e60546.
12. Buck L, Axel R (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 65(1):175-87.
13. Jiang Y, Gong NN, Hu XS, Ni MJ, Pasi R, Matsunami H (2015). Molecular profiling of activated olfactory neurons identifies odorant receptors for odors in vivo. Nat Neurosci.(10):1446-54.
14. Bullitt E (1990). Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol. 296(4):517-30.
15. Stiles J, Jernigan TL(2010). The basics of brain development. Neuropsychol Rev.(4):327-48.
16. Goodman CS, Shatz CJ (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell.72 Suppl:77-98.
17. Kolodkin AL, Tessier-Lavigne M (2011). Mechanisms and molecules of neuronal wiring: a primer. Cold Spring Harbor Perspectives in Biology. 3(6):a001727.

18. Winnubst J, Lohmann C (2012). Synaptic clustering during development and learning: the why, when, and how. Front Mol Neurosci. 5:70.

19. Tau GZ, Peterson BS (2010). Normal development of brain circuits. Neuropsychopharmacology. 35(1):147-68.

20. Seng, C., Luo, W., & Földy, C. (2022). Circuit formation in the adult brain. *European Journal of Neuroscience*, 56(3), 4187–4213.

21. Feil R, Wagner J, Metzger D, Chambon P (1997). Regulation of cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem. Biophys. Res. Commun. 237:752–757.

22. Imai, T., Suzuki, M., and Sakano, H. (2006). Odorant receptor-derived cAMP signals direct axonal targeting. *Science* 314, 657–661.

23. Serizawa, S., Miyamichi, K., Takeuchi, H., Yamagishi, Y., Suzuki, M., and Sakano, H. (2006). A neuronal identity code for the odorant receptor-specific and activity-dependent axon sorting. *Cell* 127, 1057–1069.

24. Nakashima, A., Ihara, N., Shigeta, M., Kiyonari, H., Ikegaya, Y., and Takeuchi, H. (2019). Structured spike series specify gene expression patterns for olfactory circuit formation. *Science* 365:eaaw5030.

25. Howe JR, Chan CL, Lee D, Blanquart M, Lee JH, Romero HK, Zadina AN, Lemieux ME, Mills F, Desplats PA, Tye KM, Root CM (2024). Control of innate olfactory valence by segregated cortical amygdala circuits. bioRxiv [Preprint].

Source: https://www.molbiolcell.org/info-for-authors