

Acetylcholine Supports Olfactory Perceptual Learning through Adult Neurogenesis

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B.S., Majors: Biology, Psychology, and Biomedical Engineering

Minor: Mathematics, Tufts University, May 2010

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M.A.T., Biology Secondary Education, Brown University, December 2015

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Neuroscience at Brown University.

Providence, Rhode Island

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This dissertation by Arielle Nicole Price Schilit Nitenson is accepted in its present form
by the Department of Neuroscience as satisfying the dissertation requirement for the
degree of Doctor of Philosophy.

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EDUCATION

Brown University, Providence, RI: Ph.D. Expected January 2017 GPA: 4.00

Degree: Neuroscience

Dissertation: Acetylcholine Supports Olfactory Perceptual Learning through Adult Neurogenesis

Advisor: Kevin Bath, Ph.D.

Brown University, Providence, RI: M.A.T. December 2015 GPA: 4.00

State of Rhode Island and Providence Plantations, Department of Education, Certificate No: 67416

Degree: Secondary Education (Biology)

Brown University, Providence, RI: Sc.M. May 2015 GPA: 4.00

Degree: Neuroscience

Tufts University, Medford, MA: B.S. May 2010 GPA: 3.65

Honors: *Magna Cum Laude, Dean's List*

Majors: Biology, Psychology, Biomedical Engineering

Minor: Mathematics

School for International Training, Botswana: Study Abroad Fall 2008 GPA: 4.00

Program: Ecology and Conservation

RESEARCH EXPERIENCE

Graduate Student, Brown University Summer 2012 - Present

Department of Neuroscience: Bath Laboratory

Examined the effects of cholinergic modulation on olfactory perceptual learning and newly born cell density using pharmacological and optogenetic manipulations. Side projects examined other mechanisms underlying olfactory sensory plasticity, such as loss of Fragile X Mental Retardation Protein (FMRP), the protein missing in patients with Fragile X Syndrome, and early life stress.

Rotation Student, Brown University Summer 2011 - 2012

Department of Neuroscience: Paradiso, Barnea and Bath Laboratories

Studied the primate visual system, murine olfactory system, and early life stress, respectively.

Postbaccalaureate Fellow, National Institutes of Health Summer 2010 - 2011

National Human Genome Research Institute: Yang Laboratory

Studied the molecular mechanisms of planar cell polarity, a pathway that organizes cell behavior in mouse embryonic development.

Research Assistant, Tufts University Spring 2009 - 2010

Department of Psychology: Miczek/DeBold Laboratory

Investigated inter-male mouse aggression as a function of alcohol and baclofen, a GABA_B receptor agonist that disinhibits serotonergic projections of the dorsal raphe nucleus to the cortex in low-to-moderate doses.

Research Assistant, Lehigh University Summer 2009
Department of Biological Sciences: Swann Laboratory
 Independently studied the effect of sexual experience on a male Syrian hamster's ability to differentiate receptive and unreceptive females.

Ecology and Conservation Researcher Fall 2008
Center for African Resource: Animals, Communities and Land Use, Kasane, Botswana
 Conducted a behavioral, home-range, and foraging pattern study of the banded mongoose.

Research Assistant, Harvard Medical School Summer 2008
Department of Biological Chemistry and Molecular Pharmacology: Ahmad Laboratory
 Researched *Drosophila melanogaster* histone variants and chromatin patterns in spermatogenesis.

TEACHING AND RELATED WORK EXPERIENCE

Teacher, Brown School for Professional Studies Summer 2016
 STEM II is a Summer program for rising 9th and 10th graders. I taught "The Secret Lives of Animals: A View into their Brains and Behaviors," a hands-on, field trip and lab-based course where we explored animal behavior and its evolution from a genetic, neural and hormonal view.

Student Teacher, Classical High School Fall 2015
State of Rhode Island and Providence Plantations, Department of Education, Certificate No: S67416
 Taught AP Biology to approximately 60 juniors and seniors under the mentorship of Mr. Scott MacBeth. Encouraged career development by matching several students with summer lab opportunities at Brown. Provided educational support for many students taking the Biology SAT II exam by pairing them with free tutors. Professional development opportunities included conducting over 100 Classical staff and faculty teaching observations; researching the effects of reading guide assignments on time spent reading, reading comprehension, understanding of chapter organization and test grades; and attending the National Association of Biology Teachers conference in Providence, RI.

Teacher, Brown Summer High School Summer 2014
 Co-taught a course on sustainability to Providence-area high school students through Brown's MAT program. Students learned what it means to be sustainable from a hands-on Biological and Chemical perspective. The class concluded with a final poster session, held for government officials, which provided suggestions to make Providence more sustainable.

Teaching and Presentation Consultant, Brown University Fall 2013 - Present
 Served as a **Teaching Consultant** (Fall 2013 - Spring 2014) and an **Experienced Teaching Consultant** (Summer 2014 - Present). Conducted teaching observations in Brown University classrooms and gave feedback to teachers and teaching assistants. Observed practice presentations and facilitated conversations on effective public speaking techniques to prepare speakers for their talks.

Workshop Leader, Brown University Fall 2013 - Present
 Led several workshops for graduate students on how to prepare for the National Science Foundation Graduate Research Fellowship Program application and for graduate students, postdoctoral fellows, and faculty members on teaching for the first time, preparing for the first days of class, interactive classrooms and grading strategies.

Teaching Assistant, Neural Systems, Brown University Spring 2013
 Taught students the variety of ways the nervous system converts sensory signals into

electrical signals, the schemes that the nervous system uses to represent sensory inputs, the mechanisms employed by the nervous system to produce behaviors and the experimental tools used by systems neuroscientists.

Teaching Assistant, Organisms and Populations Lab, Tufts University Spring 2008

Taught students to use proper laboratory techniques and aided them in acquiring a basic knowledge of microevolution; plant evolution and physiology; and the effects of environmental conditions on animal growth, physiology, and anatomy.

Mathematics Tutor, Tufts Literacy Corps, Medford, MA

Fall 2006 - Spring 2008

Taught mathematics to 4th and 5th grade classes. Tutored 4th and 8th grade students one-on-one.

PEER-REVIEWED PUBLICATIONS

Schilit Nitenson, A., Poeta, D., Bahar, R., Rachofsky, C., Manzano-Nieves, G., Mandairon, N. and Bath, K. (2016). Acetylcholine regulates olfactory perceptual learning through adult neurogenesis (In preparation).

Schilit, S. and **Schilit Nitenson, A.** (2016). My identical twin sister sequenced our genome. *Journal of Genetic Counseling* 1-3. As a follow-up to this manuscript, I was interviewed by the *MIT Technology Review* for an article titled, "Do Your Family Members Have a Right to Your Genetic Code?"

Schilit Nitenson, A., Stackpole, E., Truszkowski, T., Midroit, M., Fallon, J. and Bath, K. (2015). Fragile X Mental Retardation Protein regulates olfactory sensitivity but not odorant discrimination. *Chemical Senses*, 40(5): 345-350.

Bath, K., **Schilit, A.** and Lee, F. (2013). Stress effects on BDNF expression: effects of age, sex, and form of stress. *Neuroscience* 149-156.

Andre, P., Wang, Q., Wang, N., Gao, B., **Schilit, A.**, Halford, M., Stacker, S., Zhang, X. and Yang, Y. (2012). The Wnt coreceptor Ryk regulates Wnt/planar cell polarity by modulating the degradation of the core planar cell polarity component Vangl2. *The Journal of Biological Chemistry* 1-17.

Takahashi, A., **Schilit, A.**, Kim, J., DeBold, J., Koide, T. and Miczek, K. (2012) Behavioral characterization of the escalated aggression induced by GABA_B receptor activation in the dorsal raphe nucleus. *Psychopharmacology* 1-12.

SELECTED ORAL PRESENTATIONS

Schilit Nitenson A. May 2016. Acetylcholine regulates olfactory perceptual learning through adult neurogenesis. Presented at Brown University, Providence, RI.

Schilit Nitenson A. July 2015. The deceitful brain: A story of tricks, lies and illusions. Presented for SPARK, a middle school science program, Providence, RI. *Gave a 75-minute lecture to over 100 rising 9th graders and participated in a research panel for over 100 rising 7th and 8th graders.*

Schilit Nitenson A. and Bath K. October 2014. Olfactory discrimination: modulating sensitivity to changes in the chemical environment. Presented at Brown University, Providence, RI.

Schilit Nitenson A. October 2014. Neuroscience graduate student data blitz. Presented at Brown University, Providence, RI.

Schilit Nitenson A. February 2014. Stop and smell the roses. Presented at the Open Graduate Education Program Event, Providence, RI.

Schilit Nitenson A. and Bath K. December 2013. The effects of cholinergic modulation on olfactory perceptual learning. Presented at Brown University, Providence, RI.

Schilit A. and Bath K. April 2013. Mechanisms underlying olfactory sensory plasticity. Presented at Brown University, Providence, RI.

POSTER PRESENTATIONS

Schilit Nitenson A. and Bath K. September 2016. Acetylcholine supports olfactory perceptual learning through adult neurogenesis. Presented at the Brown University Neuroscience Retreat, Bristol, RI.

Schilit Nitenson A. and Bath K. August 2016. Early life stress inhibits olfactory perceptual learning. Presented at Society for Behavioral Neuroendocrinology, Montreal, Quebec, Canada.

Schilit Nitenson A. May 2016. Directed reading guide (DRG) assignments increase student test scores, reading comprehension and understanding of unit organization. Presented at the Teacher Research Project Conference, Providence, RI.

Schilit Nitenson A., Stackpole E., Fallon J., and Bath K. November 2014. Mice lacking fragile X mental retardation protein have decreased olfactory sensitivity and intact spontaneous olfactory discrimination abilities. Presented at Society for Neuroscience, Washington, DC.

Schilit Nitenson A. and Bath K. August 2014. Acetylcholine is necessary for olfactory perceptual learning. Presented at the Brown University Neuroscience Retreat, Bristol, RI. *Awarded best poster.*

Schilit A. and Bath K. November 2013. Setting the tone: The effects of cholinergic modulation on olfactory perceptual learning. Presented at Society for Neuroscience, San Diego, CA.

Schilit A. and Bath K. August 2012. The effect of early life stress on spontaneous olfactory discrimination. Presented at the Brown University Neuroscience Retreat, Bristol, RI.

Takahashi A., **Schilit A.,** Koide T., DeBold J., and Miczek K. November 2011. Escalated aggression by GABA_B receptor activation in the dorsal raphe nucleus: Role of prefrontal cortex and amygdala. Presented at Society for Neuroscience, Washington, DC.

Andre P., **Schilit A.,** Song H., and Yang Y. November 2010. The role of planar cell polarity in endochondral bone formation. Presented at the National Human Genome Research Institute Scientific Retreat, Cambridge, MD. *Awarded best poster.*

FUNDING AND AWARDS

Reginald D. Archambault Award for Teaching Excellence

Fall 2016

The Archambault Teaching Award recognizes, rewards and promotes teaching excellence in Brown University summer programs. The award is given based upon three main criteria: overarching objectives and discipline specific learning goals; content including teaching and learning activities; and feedback, assessment and evaluation. I received this award along with my teaching associate, Dr. Victoria Heimer-McGinn, for our Summer 2016 course, "The Secret Lives of Animals: A View into their Brains and Behaviors."

Invited Speaker

Spring 2016

Asked to be the student speaker at the "Celebration of PhD Student and Postdoctoral Fellow Achievement Event," a reception for students who received their own funding for the 2015-2016 academic year.

Sigma Xi – Brown Chapter

Spring 2015 & 2016

Nominated to be an associate (2015) and full member (2016) of Sigma Xi, The Scientific Research Society. Sigma Xi is the international honor society of science and engineering. *Awarded the **Brown Chapter of Sigma Xi Prize** (April 2016), for excellence in research, academic achievement and high potential for future contributions to science.*

Conference Travel Award

Summer 2013 - 2016

Received funding from Brown's Office of Graduate and Postdoctoral Studies, available for students in years 2-5 who have been invited/accepted to present their research. I was given \$500 and \$650 to attend Society for Neuroscience (SfN) 2013 (San Diego, CA) and 2014 (Washington, DC), respectively. I was awarded \$650 to attend Society for Behavioral Neuroendocrinology (SBN) 2016 (Montreal, Quebec, Canada). In addition, I received \$600 from Brown's International Travel Fund for SBN.

ETS Recognition of Excellence Award

Summer 2013

Scored in the top 15% of Praxis II – Biology: Content Knowledge (0235) test takers

Open Graduate Education Program Award

Spring 2013

The Open Graduate pilot program accepts approximately nine Brown doctoral students each year to pursue a master's degree in a secondary field. Tuition for the master's degree is waived, and accepted students are guaranteed summer and sixth year funding in order to complete both degrees. In order to further encourage interdisciplinary work and career advancement, professional development opportunities and community support are available. In 2014, I was interviewed for *The Chronicle of Higher Education's* article, "Brown U. Tests Approach to Interdisciplinary Graduate Work."

National Science Foundation Graduate Research Fellowship Program

Summer 2013 - 2016

Grant No. DGE 0228243

Ruth L. Kirschstein National Research Service Award

September 2011 - 2012

National Institutes of Health Predoctoral Training Grant T32MH020068 (PI: Diane Lipscombe)

Special Dean's Award

Spring 2011

Received a research award for my successful application to Brown's Biology Portal, an application process for conducting Biomedical research at Brown for students with exceptional qualifications in terms of academic credentials, research experience and promise for achievement.

PROFESSIONAL DEVELOPMENT

Brown Executive Scholars Training (BEST) Program, Brown University

Spring 2015

Participated in a 12-week mentored education and training experience to learn about leadership roles in higher education administration.

The Harriet W. Sheridan Center for Teaching and Learning

Fall 2012 – Present

Complete four yearlong teaching certificate programs and attended several workshops at the Sheridan Center, Brown's resource for teaching and learning education.

Certificate I: The Sheridan Teaching Seminar – Reflective Teaching Fall 2012 – Spring 2013

Learned and refined fundamental teaching and assessment strategies as well as communication skills based on how students learn.

Certificate II: The Course Design Seminar – Principles and Practice Fall 2013 – Spring 2014

Developed strategies and practices for course design, implementation and assessment. Designed a course syllabus.

Certificate III: The Professional Development Seminar

Fall 2014 – Spring 2015

Created a teaching portfolio, cover letter and CV. Gained experience preparing for a job market interview or presentation.

Certificate IV: The Teaching Consultant Program

Fall 2013 – Spring 2014

Applied peer observation, discussion facilitation and feedback expertise by conducting teaching observations across the university and leading feedback sessions.

Professional Development Workshops, Brown University

Fall 2014 and 2015

NASA Endeavor Leadership in STEM Education Series

Fall 2014

Learned to incorporate Engineering design problems into classroom lesson plans.

Population Connections: Activities for People and the Planet

Fall 2015

Reviewed hands-on activities in Population Ecology, Conservation and Environmental Science.

SERVICE

Panelist at CareerLAB, Brown University

Fall 2016

Invited panelist for “Applying to Graduate School in STEM,” a panel hosted by CareerLAB for undergraduate students.

Neuroscience Graduate Student Representative, Brown University

Fall 2014 – 2015

Served as one of two student members on the Brown Neuroscience Graduate Program and Brown-National Institutes of Health Graduate Partnership Program admissions committees. In addition, the two Neuroscience Graduate Student Representatives planned the 2015 Brown Neuroscience Recruitment Weekend. Responsibilities included coordinating a faculty data blitz, graduate student host assignments, interview schedules, transportation, restaurant reservations, laboratory tours and hotel reservations for 32 prospective students.

PREFACE

Learning to perceptually discriminate chemical signals in our environment (olfactory perceptual learning) is critical for the survival of most species. Specifically, this olfactory behavior is crucial for a myriad of behaviors, such as social bonding, navigation, copulation, predator avoidance and foraging behaviors. Multiple mechanisms have been implicated in olfactory perceptual learning, including the modulation of neurogenesis and manipulation of cholinergic activity. However, the relative contribution of these mechanisms to olfactory perceptual learning has remained unclear. In my dissertation, I explore these gaps in the literature and answer our main question:

Does acetylcholine support olfactory perceptual learning through adult neurogenesis?

After confirming that repeated odorant enrichment is sufficient to support olfactory perceptual learning (Chapter 2), we verified that our enrichment protocol could induce a long-term olfactory memory (Chapter 3). Using a combination of pharmacological and optogenetic approaches, we then found that cholinergic activity is both necessary (Chapter 4) and sufficient (Chapter 5) to support olfactory perceptual learning, and that this process is dependent upon the presence of newly born cells in the olfactory bulb. To our knowledge, this study is the first to directly link cholinergic activity and neurogenesis to support olfactory perceptual learning (Chapter 6).

Disturbances in olfactory sensory function are highly co-morbid with several neurological and psychiatric disorders, including Anxiety Disorder, Major Depressive Disorder, Alzheimer's Disease and Parkinson's Disease. Since olfactory dysfunction may represent a biomarker of neurobiological disturbances, we hope that our work will eventually contribute to understanding the neurobiological basis of disorder as well as inform future research and clinical interventions.

ACKNOWLEDGEMENTS

Almost six years ago, I received the following email informing me of my acceptance to Brown's Neuroscience Graduate Program:

Jerome Sanes <jerome_sanes@brown.edu>
To: Arielle Schilit <arielle.schilit@gmail.com>

Thu, Mar 3, 2011 at 9:38 PM

Dear Arielle,

As we spoke earlier today, the Neuroscience Graduate Program Admission Committee at Brown has enthusiastically recommended to the Graduate Deans that you should receive an offer for Fall 2011 matriculation. We found your accomplishments and promise outstanding, and we believe that you are a wonderful match to the research and academic environment at Brown.

We reviewed more than 190 applications for admission to our neuroscience program and as you know you were one of a very selective group of 27 (22 during your visit) applicants that we interviewed.

We feel it is very important that each student admitted to our program has substantial choice when it comes to choosing her or his research laboratory. Therefore we do not over-admit and rather extend offers on a rolling basis.

Once you receive the official offer, you have until April 15th to tell us of your decision, a general practice for all US universities. However, if you reach a decision before this date, we would very much appreciate hearing from you. This helps us make decisions about whether to extend additional offers to other well-qualified applicants.

Following our recommendations, you will soon receive E-mail notification from the Embark system that an admissions decision has occurred, with an accompanying hotlink. Once you log on to the Embark system, I understand that there's an area to view the decision letter. You should see, maybe not immediately, two letters: one from Peter Weber, the Dean of the Graduate School, congratulating you and offering admission, and a second letter from Elizabeth Harrington, the Associate Dean of Graduate and Post-doctoral Studies (Biomed), that describes the financial aspects of the admissions offer. The Embark system also has a mechanism for you to accept or decline the offer.

Once again congratulations, and we hope to see you soon.

Also, as I mentioned in our conversation, please feel free to contact me, David Sheinberg (Director, NSGP), your student contact, or any other faculty member of student in our program for questions you might have to help you make your decision.

Yours,

Jerome Sanes
Co-Director, Neuroscience Graduate Program

I was ecstatic, stunned, nervous, but mostly grateful for the chance to study at Brown.

However, I knew my acceptance was an opportunity, not a guarantee for a successful

defense six years later. There are so many wonderful people that need to be acknowledged for their hard work and dedication; without them, this dissertation would not be possible.

I'd like to begin by thanking my advisor, Kevin Bath for taking a chance on me. As the first graduate student in the lab, I was nervous to join a community I knew nothing about. However, the "small lab feel" worked to my benefit. I received hands-on training directly from my PI, an opportunity that many graduate students sadly don't get to experience. Kevin has given me the freedom to work on a project different from the focus of the lab, which has allowed me to study a topic that has turned into a very exciting and fruitful research project. In addition, Kevin's flexibility has given me the chance to investigate the many wonderful programs Brown Graduate School has to offer, satisfying my need to explore many interests and professional development opportunities while in graduate school.

Although the Bath lab began small, it certainly grew throughout the course of my graduate school career. Each member has brought a unique perspective to the lab, so I'd like to individually thank all past and present Bath lab members (students listed in bold directly helped me collect data for my dissertation): Zach Aguilar, **Ryan Bahar**, Alexa Cohen, Meghan Gallo, Haley Goodwill, Kaitlyn Hajdarovic, Hailey Huddleston, Kathleen Huntzicker, Angelica Johnsen, Patrick LaChance, Sarah Lee, Ezra Lichtman, Shirley Lin, Chelsea Lopez, Gabriella Manzano Nieves, Taylor Moss, Matt Pescosolido,

Casey Poore, **Carolyn Rachofsky**, Sana Teramoto and Torrey Truszkowski. I'd especially like to thank my fellow graduate students, Gabi and Haley. They have both been amazing friends, inside and outside the lab. Whether I needed help coding, a coffee break or someone to talk to during difficult times in my life, they were always there. In addition, I would like to acknowledge the Burwell lab, especially Devon Poeta for giving me a surgery refresher and microscope training. The Burwell lab has been so accommodating to the needs of my project; I am glad our labs were positioned so close to one another.

I would also like to recognize my committee members, Justin Fallon and Chris Moore, for their extensive support and advice throughout the years. I truly believe my dissertation is stronger due to their suggestions, and I thank them for taking the time to improve me as a scientist. In addition, I would like to thank my external reader, Nathalie Mandaïron, for not only editing my manuscript and flying all the way from France to attend my defense, but also for her tremendous contributions to our field which have informed almost every experiment I conducted during my Ph.D.

I truly believe the Department of Neuroscience has some of the best past and present administrators that have made my life so much easier these last few years. A huge thanks to: Rosanna Cabral, Denise Camara, Mary-Ellen Flinn, Scott Lessard, Elyse Netto, Scott Rathburn, Barbara Senerchia, Heather Shalvey and Carol Viveiros. I would also like to thank all the Neuroscience directors that have shaped my graduate career: Anne Hart, Diane Lipscombe, Jerome Sanes and David Sheinberg. Furthermore, I'd like to

acknowledge all NSGP faculty and Animal Care Facility staff for their efforts the past several years. Finally, I am so appreciative to have met amazing students while at Brown. I could not have done it without my NSGP and GPP friends, especially the Entering Class of 2011. I am lucky to have made so many great friendships while at Brown that will last a lifetime.

During my graduate career, I have been fortunate to meet remarkable people and experience top-notch professional development through the following programs/departments: the Brown Executive Scholars Training (BEST) program, Open Graduate Education (OGE) Program, School for Professional Studies, Sheridan Center and Teacher Education Program (TEP, through which I received my M.A.T.). In particular, Dan Bisaccio, Vanessa Ryan and Peter Weber have played an integral role in shaping my graduate experience; without their help, I may not have been successful completing an M.A.T. alongside my Ph.D. These individuals have provided me with the resources I needed to succeed, always offering a helping hand and guidance.

In these final sections, I must thank my family. Without their support and love, this dissertation would not be possible. I am grateful to my in-laws, Jay, Linda and Sarah Nitenson for their constant support and encouragement. I couldn't have married into a better family, and I'm so glad I live close by to an inviting home with such wonderful people. Thanks to my mom, Susan Price and her husband, Marty Maier for their love and care throughout this journey. From their visits that restored my sanity during intense

periods of time, to Marty's help keeping my computer alive throughout the dissertation writing process, I am appreciative of everything they do.

This dissertation is written in memory of my dad, Robert Schilit, who passed away on June 7, 2012. Although he was only alive through my first year of graduate school, his unconditional love, infinite support and tremendous efforts to get me to where I was at the beginning of graduate school set me up on a path to success that would not have been possible without him. I am so proud to be his daughter.

I'd like to thank my sister, Sam Schilit for always encouraging me. As identical twins and "womb mates," she has literally been there since day one. As a graduate student herself, Sam has been enormously helpful throughout the years, editing my grant applications, posters, talks and manuscripts. More importantly, she has reinvigorating me every time I needed a push through difficult times. She has and will always be a great friend, dropping everything to give me a hug during the bad times, and reinforcing my successes during the good times.

I am grateful to my husband, Adam Nitenson for experiencing every good and bad day with me. Little did I know when we met in 2006 that we'd end up sharing an office in graduate school, seeing each other constantly, and yet be so happily married. He has kept me sane the last few years, taking me on frozen yogurt breaks when I needed to breathe and always lending a hand or an ear when I needed help or someone to listen. However, I want to thank him most for already being a great dad to our baby girl, due in February.

We are both so lucky to have him. Finally, although I haven't met her yet, I need to thank my daughter who kept me company for the majority of my dissertation writing time. My love for her is already so strong, and I am honored to dedicate this dissertation to her.

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APPENDIX: BATH LAB PUBLICATIONS BEFORE JANUARY 18, 2017

Bath KG, Schilit A, Lee FS (2013) Stress effects on BDNF expression: effects of age, sex, and form of stress. *Neuroscience* **239**: 149-156.

Schilit Nitenson A, Stackpole EE, Truszkowski TL, Midroit M, Fallon JR, Bath KG (2015) Fragile X mental retardation protein regulates olfactory sensitivity but not odorant discrimination. *Chem Senses* **40**: 345-350.

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CHAPTER 1: INTRODUCTION

1.1 The Olfactory System

Main Olfactory System

Main Olfactory Epithelium

The main olfactory epithelium (MOE) is a large structure in the nasal cavity capable of perceiving volatile chemicals, although some non-volatile compounds can be detected as well (Burger, 2005; Saito et al., 2009; Nara et al., 2011). Olfactory sensory neurons (OSNs) in the MOE express olfactory receptors (ORs), trace-amine associated receptors (TAARs) and occasionally guanylyl cyclase D (GC-D) (Buck and Axel, 1991; Juilfs et al., 1997; Liberles and Buck, 2006). Although the main olfactory system was classically thought of as the olfactory system for non-pheromonal cues, recent evidence suggests that some pheromonal cues are perceived by this system as well (Schaal et al., 2003; Liberles and Buck, 2006; Kobayakawa et al., 2007; Stowers and Logan, 2010; Yoshikawa et al., 2013). While my research focuses on the main olfactory system, it is important to recognize that there are four chemosensory organs that contribute to olfaction (Figure 1.1). The section below will detail how odorants activate OSNs in the MOE, and how information is typically processed through this system.

Olfactory Transduction

In order to perceive olfactory cues, chemicals in the environment stimulate receptors on the dendrites of OSNs. Several cilia emanate from each dendrite and contain multiple ORs, seven transmembrane G protein-coupled receptors where odorants bind to begin olfactory signal transduction (O'Dowd et al., 1989; Boekhoff et al., 1990; McIntyre et al., 2012). Interestingly, OSN axons also contain ORs, where they contribute to axon guidance (Wang et al., 1998; Barnea et al., 2004). In addition to OSNs, the MOE contains two other cell types: supporting cells and basal cells. While OSNs express olfactory marker protein (OMP), supporting cells and basal cells can be identified by their expression of numerous other markers (Hempstead et al., 1983; Danciger et al., 1989; Holbrook et al., 1995; Guo et al., 2010; Holbrook et al., 2011). Basal cells are stem cells that generate all populations of olfactory epithelium cells (including OSNs) throughout life (Moulton and Beidler, 1967; Graziadei and Graziadei, 1979). In addition to self-renewal, they produce multipotent proliferating progenitors that turn into transit amplifying cells, immediate neuronal precursors, immature neurons and finally, mature neurons (Graziadei et al., 1973). The majority of these differentiating cells are lost in the first three weeks post-birth. However, those that survive and turn into OSNs remain connected to the olfactory bulb (OB) for at least three months (Moulton et al., 1967; Hinds et al., 1984; Mackay-Sim and Kittel, 1991ab).

In mice, each OSN stochastically expresses only one of approximately 1000 functional OR genes, although the number is species-dependent, with humans at approximately 400 and the African Elephant at approximately 2000 functional OR genes (Buck and Axel,

1991; Chess et al., 1994; Niimura and Nei, 2003; Niimura, 2012; Niimura et al., 2014).

Functional receptor expression is necessary for suppressing the transcription of other receptors, ensuring that all neurons stably express a single functional receptor throughout life (Serizawa et al., 2003, Lewcock and Reed, 2004; Shykind et al., 2004; Shykind, 2005). In addition, the olfactory system uses combinatorial receptor coding, where one odorant can stimulate multiple ORs, and one OR can recognize multiple odorants (Malnic et al., 1999; Figure 1.2).

Once an odorant binds an OR, the receptor interacts with a guanosine triphosphate (GTP)-binding protein (G_{olf}). Adenylyl cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), opening cyclic nucleotide gated (CNG) channels, depolarizing the cell and generating an action potential (Pace et al., 1985; Sklar et al., 1986; Nakamura and Gold, 1987; Jones and Reed, 1989; Breer et al., 1990; Dhallan et al., 1990). This information is sent via OSN axons to the main olfactory bulb (MOB; Figure 1.3). Each OR generates its own cAMP level signature, which correlates with the anterior-posterior location of its glomeruli (Imai et al., 2006; Serizawa et al., 2006). In contrast, dorsal-ventral projections are determined by OSN anatomical locations (Norlin et al., 2001; Cho et al., 2007; Takeuchi et al., 2010).

Main Olfactory Bulb

Each glomerulus consists of a single OR species. In fact, OR proteins help target their OSNs to the correct glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998; Feinstein et al., 2004). Although there are approximately 1800

glomeruli in each OB (Royet et al., 1988), OSNs expressing a specific OR typically only project to two spatially invariant glomeruli per bulb, one lateral and one medial (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Nagao et al., 2000). A small subset of ORs are represented by only one glomerulus per bulb along the OB ventral midline (Strotmann et al., 2000).

Glomeruli form excitatory synaptic connections with mitral and tufted cells, the second order projection neurons of the MOB (Shepard et al., 2004). The MOB contains many layers, or laminae. In order from the bulbar surface to the center, these layers are the olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL) and granule cell layer (GCL) (Allison, 1953; Mori, 1987; Figure 1.4A). While mitral and tufted cells both give rise to a single primary dendrite that terminates within a glomerulus, they are genetically distinct (Greer and Shepherd, 1982) and have anatomical and functional differences. Mitral cell somata reside in the MCL while their secondary dendrites extend into the deeper half of the EPL. Tufted cell somata are located in the GL and EPL, while their secondary dendrites project to the superficial half of the EPL. While both mitral and tufted cells release glutamate, some tufted cells are also dopaminergic (Halasz et al., 1977; Cotman et al., 1987). Both cell types make dendrodendritic connections with granule cells, although they target slightly different populations (Mori, 1987). Functionally, tufted cells are important for low concentration odorant processing, while mitral cells only respond to strong stimuli. These cell types also relay temporally distinct odorant information to the

olfactory cortex (OC), as tufted cells have a higher temporal resolution (Igarashi et al., 2012).

Although mitral/tufted cells relay information out of the bulb, three types of interneurons reside with their processes strictly in the OB: periglomerular cells, granule cells, and short axon cells.

Periglomerular cells have small somata in the periglomerular region of the GL. Within a glomerulus, periglomerular cell dendrites intermingle and synapse with OSN axons, mitral/tufted cell dendrites and themselves (Pinching and Powell, 1971a; Smith and Jahr, 2002; Schoppa and Urban, 2003; Murphy et al., 2005). Their axons are long, spreading up to five glomeruli away (Pinching and Powell, 1971ab). Periglomerular cells may release dopamine or gamma-aminobutyric acid (GABA). These neurotransmitters may be localized in the same or different periglomerular cells (Mugnaini et al., 1984; Baker, 1988). Granule cell somata reside in the GCL. These GABAergic cells are the most abundant neuron in the OB (Ribak et al., 1977; Halasz et al., 1978; Mori, 1987). Granule cells are axonless; they make dendrodendritic synapses with mitral/tufted cells (Golgi, 1885; Rall et al., 1966). Importantly, mitral/tufted cells make excitatory synapses on granule cells, while granule cells make inhibitory synapses on mitral/tufted cells. When mitral/tufted cell dendrites become depolarized, they release glutamate. A cascade of events occurs in the granule cell dendritic spine that triggers a calcium influx, ultimately leading to GABA release and an inhibitory synapse back onto the mitral/tufted cell dendrite (Phillips et al., 1963; Rall and Shepherd, 1968; Nicoll, 1969; Wellis and Kauer,

1993, 1994; Isaacson and Strowbridge, 1998). This is crucial for mitral/tufted cells to participate in lateral and self-inhibition. The third type of inhibitory interneuron is the short axon cell. These cells are similar to periglomerular cells, but extend their processes into multiple glomeruli, forming the interglomerular circuit (Kosaka and Kosaka, 2008; Kiyokage et al., 2010). MOB connections do not exist in a bubble. Rather, these cellular circuits also receive and integrate information from higher areas of the brain in the form of centrifugal afferents, influencing the olfactory information processed.

Higher Order Brain Regions

Mitral/tufted cell axons form the lateral olfactory tract, which transmits information to the OC, cortical areas and limbic structures that receive direct synaptic input from the OB. These structures include the nucleus of the lateral olfactory tract, piriform cortex, olfactory tubercle, amygdala, insula, entorhinal cortex (EC), and portions of the olfactory peduncle (anterior olfactory nucleus, tenia tecta, dorsal peduncular cortex) (Le Gros Clark and Meyer, 1947; Lohman, 1963; Powell et al., 1965; White, 1965; Scalia, 1966; Heimer, 1968; Price and Powell, 1971; Price, 1973; Scalia and Winans, 1975; Haberly and Price, 1977; Skeen and Hall, 1977; De Olmos et al., 1978; Scott et al., 1980; Ojima et al., 1984; Figure 1.4B). While mitral cells axons project to nearly all olfactory cortical areas, tufted cells axons are restricted to the anterior olfactory cortex including the anterior olfactory nucleus (Haberly and Price, 1977; Skeen and Hall, 1977; Miyamichi et al., 2011).

The three-layer piriform cortex is the largest olfactory cortical region. Layer Ia, the superficial portion of the superficial plexiform layer, contains afferent fibers from the ipsilateral OB via the lateral olfactory tract. In contrast, layer Ib, the deeper portion of the superficial plexiform layer, contains efferent projections from the basal forebrain, brainstem, thalamus, and hypothalamus, as well as projections from other parts of the piriform cortex (Price, 1973; Haberly and Price, 1978; Luskin and Price, 1983).

Piriform cortex pyramidal cells send projections to the neocortex, orbitofrontal cortex, insula, thalamus, hypothalamus, hippocampus and amygdala (Price, 1985). The extensive portion of the brain involved in olfactory processing demonstrates the importance of olfaction as well as the multitude of characteristics this information contains: perception of flavor, odorant localization, learning and memory and emotional valence (Shipley and Adamek, 1984; Zald and Pardo, 1997; Eichenbaum, 1998; Ikemoto, 2007; Kikuta et al., 2010). These olfactory cues can leave a long lasting impression, as it appears that memory-related synaptic plasticity occurs in the piriform cortex (Roman et al., 1987; Stripling et al., 1988).

Additional Chemosensory Organs

Although my dissertation focuses on the main olfactory system, for completeness, I briefly review the three anatomically segregated chemosensory organs that along with the MOE comprise the olfactory system: the vomeronasal organ (VNO), septal organ of Maser (SO) and Grüneberg ganglion (GG) (Figure 1.1).

Vomeronasal Organ

The VNO is a cigar-shaped structure located at the base of the nasal septum (Jacobson et al., 1998). This structure is segregated into apical and basal layers. Vomeronasal sensory neurons (VSNs) located in the apical layer express primarily type 1 vomeronasal receptors (V1Rs), while those in the basal layer contains type 2 vomeronasal receptors (V2Rs) (Dulac and Axel, 1995; Herrada and Dulac, 1997; Pantages and Dulac, 2000). Formyl peptide receptors (FPRs) are also expressed mainly in a small subset of apical layer VSNs (Liberles et al., 2009; Rivière et al., 2009). V1Rs and V2Rs transduce information via the transient receptor potential cation channel, subfamily C, member 2 (TRP2, the homologue of the human TRPC2 pseudogene) (Liman et al., 1999). VSNs expressing V1Rs project to the anterior accessory olfactory bulb (AOB), while VSNs expressing V2Rs project to the posterior AOB (Jia and Halpern, 1996; Halem et al., 2001). Integration occurs early in this circuit, with VSNs expressing the same receptor projecting their axons to multiple glomeruli, and each glomerulus receiving projections from multiple VSNs expressing different receptor types (Belluscio et al., 1999). Information is further processed in several brain regions including the medial and posteromedial cortical amygdalae, the bed nucleus of the stria terminalis and the hypothalamus (Winans and Scalia, 1970; Barber and Field, 1975; Scalia and Winans, 1975; Davis et al., 1978; De Olmos et al., 1978; Mohedano-Moriano et al., 2008; Martinez-Marcos, 2009).

Classically, the VNO was thought of as the pheromone-detecting organ, capable of mediating the neuroendocrine system and innate behavioral responses like social

behavior. As an example, in 2002, Stowers et al. genetically ablated TRP2 in mice to examine VNO-mediated behaviors. They found that TRP2 and therefore VNO function is necessary for conspecific gender identification as well as gender-specific behaviors. Behavioral responses are mediated by a variety of ligands binding receptors on VSNs. In addition to small volatile molecules and bodily fluids such as urine, the VNO also detects a variety of water-soluble molecules (Leinders-Zufall et al., 2000; Luo et al., 2003; Kimoto et al., 2007; He et al., 2008). Although it is clear that the VNO plays a role in pheromone detection, it is now known that other chemosensory organs contribute as well.

Septal Organ of Maser

The SO is located in the ventral portion of the nasal septum, posterior to the VNO (Broman, 1921). This organ contains receptors similar to those found in the MOE, such as ORs and GC-D cells that project to the ventromedial portion of the MOB (Ma et al., 2003). Although the role of the SO is largely unknown, it may be involved in transmitting information about nasal airflow (Grosmaître et al., 2007).

Grüneberg Ganglion

The GG is a small olfactory organ located at the tip of the nose (Grüneberg, 1973). Its neurons express one type of V2R, OR and guanylyl cyclase G (GC-G), as well as many TAARs (Fleischer et al., 2007; Chao et al., 2010; Fleischer and Breer, 2010). GG neurons project to a cluster of glomeruli with a necklace-like layout in the dorsal portion of the MOB called necklace glomeruli (NG) (Roppolo et al., 2006; Fleischer and Breer, 2010). Due to the maturity of these neurons at birth as well as their projection site, these neurons

are thought to detect alarm pheromones and be important for maternal interaction (Shinoda et al., 1989; Fuss et al., 2005; Fleischer et al., 2007; Brechbuhl et al., 2008, 2013).

1.2 Centrifugal Inputs

Although the OB is the first relay station in the brain for olfactory processing, centrifugal fibers employ exquisite control in this region by synapsing in several MOB layers, influencing sensory perception and integrating odorant information with the state of the brain. Centrifugal afferents to the MOB emerge bilaterally from the anterior olfactory nucleus, locus coeruleus and raphe nucleus, while ipsilateral afferents stem from the piriform cortex, nucleus of the lateral olfactory tract, tenia tecta, dorsal peduncular cortex, basal forebrain and hypothalamus (Price and Powell, 1970; Davis et al., 1978; De Olmos et al., 1978; Haberly and Price, 1978; Macrides et al., 1981). These centrifugal fibers can be grouped into two categories, fibers that emerge from the olfactory cortex, and those that release neuromodulators.

The fibers originating from the olfactory cortex are feedback projections, as reciprocal connections exist between these cortical areas and the bulb. However, the centrifugal projections that release neurotransmitters come from the nucleus of the horizontal limb of the diagonal band of Broca (HDB), raphe nucleus and locus coeruleus in a unidirectional manner, releasing acetylcholine, serotonin and noradrenaline, respectively. These neuromodulatory systems are important for many reasons, including long-term synaptic plasticity (Bailey et al., 2000).

Olfactory Neuromodulation

Before detailing the roles of each neuromodulatory system below, it is important to note that these systems work synergistically; ascribing simple functions to each one is at best challenging, and more likely than not incomplete, as these neurotransmitters function very differently under varying OB parameters (Linster and Cleland, 2016). Therefore, the information below should be read as a starting point to understanding the complexities of olfactory neuromodulation.

Acetylcholine

Cholinergic fibers from the basal forebrain project via the HDB to the OB, where they primarily innervate the GCL and the periglomerular region of the GL (Macrides et al., 1981; Carson, 1984; Ichikawa and Hirata, 1986; Zaborszky et al., 1986; Nickell and Shipley, 1988; Kasa et al., 1995; Gomez et al., 2005; Figure 1.5). These fibers generally terminate on periglomerular and granule cell spines, releasing acetylcholine to depolarize these cells (Le Jeune and Jourdan, 1993; Kasa et al., 1995). Cholinergic projections additionally innervate the piriform cortex, primarily targeting layers II and III (Luskin and Price, 1982; Lysakowski et al., 1989).

Acetylcholine has been shown to play a significant role in olfactory tuning and odorant discrimination (Linster et al., 2001; Mandairon et al., 2006a; Chaudhury, et al., 2009; Ma and Luo, 2012). In 2001, Linster et al. stereotactically injected 192 IgG saporin, a lesioning agent selective for basal forebrain cholinergic neurons, into the HDB of male

Sprague-Dawley rats, increasing perceptual generalization between chemically related odorants. Similarly, the bilateral infusion of nicotinic acetylcholine receptor antagonist mecamylamine hydrochloride (MLA) or muscarinic antagonist scopolamine to the OBs disrupts this spontaneous discrimination. However, neostigmine, an acetylcholinesterase inhibitor sharpens discrimination abilities (Mandairon et al., 2006a). Complementing these results, mitral cell recordings from anesthetized rats administered drugs to the OB revealed neostigmine's enhancing effect, and MLA and scopolamine attenuating influence on mitral cell odorant receptive field size (Chaudhury et al., 2009). Finally, Ma and Luo (2012) optogenetically activated HDB neurons, sharpening mitral cell olfactory tuning curves while increasing periglomerular and granule cell receptive fields. Therefore, from these and other studies, cholinergic input from the HDB is thought to sharpen mitral cell olfactory tuning by increasing and broadening the responsiveness of granule cells as well as enhancing periglomerular cell inhibition of mitral cells (Heimer et al., 1990; Castillo et al., 1999; Linster and Cleland, 2002; Chaudhury et al., 2009; Ma and Luo, 2012). In addition to olfactory tuning and odorant discrimination, cholinergic modulation is involved in conspecific recognition, working memory, associative learning and short-term memory (Kendrick et al., 1992; Ravel et al., 1992; Roman et al., 1993; Ravel et al., 1994; Levy et al., 1997; Fletcher and Wilson, 2002; Linster and Cleland, 2002; Kroon and Carobrez, 2009; Devore and Linster, 2012). However, no studies have specifically examined the role of acetylcholine in olfactory perceptual learning (Linster et al., 2001; Fletcher and Wilson, 2002; Mandairon et al., 2006a; Chaudhury, et al., 2009).

Contrast enhancement is an operation used by the olfactory systems to sharpen an odorant's representation by separating out the overlap between signals, mainly accomplished through lateral inhibition between mitral cells and inhibitory interneurons (Yokoi et al., 1995; Linster and Hasselmo, 1997; Cleland and Sethupathy, 2006; Urban and Arevian, 2009). The combination of granule and periglomerular inhibition enhances the selectivity of mitral cells by making them less sensitive to weak chemicals in the environment, while maintaining sensitivity to more potent signals (Elaagouby and Gervais, 1992; Mandaïron et al., 2006a; Tsuno et al., 2008; D'Souza and Vijayaraghavan, 2012). This leads to sparser mitral cell activation, increasing the separation in spatial coding at the level of the OB and improving odorant spontaneous discrimination (Chaudhury et al., 2009). GABAergic interneurons inhibit mitral cells via center-surround lateral inhibition, enhancing the contrast between neighboring mitral cell receptive fields, and allowing animals to distinguish chemically related odorants. Since nicotinic receptors are typically found in the glomerular and mitral cell layers, they influence the responsiveness of inhibitory periglomerular and sharpen mitral cell olfactory tuning by minimizing mitral cell receptive field size (Castillo et al., 1999; Chaudhury et al., 2009; Figure 1.6). Support for cholinergic modulation of olfactory discrimination has also come from recent modeling and *in vivo* studies, in which increasing cholinergic activity at the level of the glomeruli enhances receptive field contrast and increases the selectivity of mitral cell firing (Linster and Hasselmo, 1997; Cleland and Sethupathy, 2006; Mandaïron et al., 2006a; Chaudhury et al., 2009; Linster and Cleland, 2009). Acetylcholine can function similarly in the piriform cortex, influencing long-term potentiation, and ultimately sharpening odorant receptive field size

to better discriminate similar odorants (Hasselmo and Barkai, 1995; Patil et al., 1998; Linster and Hasselmo, 2001).

Synchronization is important for odorant discrimination, processing and learning (Stopfer et al., 1997; Nusser et al., 2001). Gamma oscillations (approximately 50-100 Hz) appear in the OB as a result of mitral-granule cell dendrodendritic interactions (Becker and Freeman, 1968; Gray and Skinner, 1988; Neville and Haberly, 2003). When mitral cells become activated, they synchronously activate the granule cells with which they form synapses. The granule cells then release GABA onto the activated mitral cell as well as neighboring inactive mitral cells. This negative feedback loop is responsible for OB oscillations (Rall and Shepherd, 1968; Freeman, 1975; Bathellier et al., 2006; Brea et al., 2009). Beta oscillations (approximately 15-40 Hz) arise in the OB and piriform cortex, as well as the dentate gyrus (DG) and EC (Chapman et al., 1998; Ravel et al., 2003). These oscillations are believed to be more involved in odor learning than gamma oscillations (Martin et al., 2004; Martin and Ravel, 2014). Cholinergic inputs alter the synchronization of firing, and this process serves to regulate olfactory discrimination and learning (Stopfer et al., 1997; Nusser et al., 2001; Cleland and Linster, 2012). Muscarinic acetylcholine receptors located mainly in the GCL influence granule cell excitability and therefore dendrodendritic inhibition of mitral cells, enhancing network synchrony and gamma oscillations (Ghatpande et al., 2006; Pressler et al., 2007; Li and Cleland, 2013; Figure 1.7).

Although my dissertation only covers cholinergic neuromodulation, I introduce dopamine, serotonin and noradrenaline in the following sections as a comparison to the work done in the cholinergic system.

Dopamine

Since the OB receives inputs from all major neuromodulatory systems except dopamine, it is important to note that dopamine still plays a role in olfaction. Dopamine is intrinsic to the OB, localized to a subset of juxtaglomerular neurons (periglomerular, short axon and external tufted cells) (Halasz et al., 1981; Davis and Macrides, 1983; Kosaka et al., 1985; Gall et al., 1987; McLean and Shipley, 1988; Shipley and Ennis, 1996). It modulates synaptic transmission between OSN axons and mitral/tufted cells via D2 receptor action leading to presynaptic inhibition (Nickell et al., 1991; Berkowicz and Trombley, 2001; Ennis et al., 2001). Dopaminergic modulation affects odorant sensitivity, discrimination and associative learning (Doty and Risser, 1989; Coopersmith et al., 1991; Doty et al., 1998; Zhang et al., 2000; Rosenkranz and Grace, 2002; Yue et al., 2004). Unlike the OB, which receives centrifugal projections from only serotonergic, noradrenergic and cholinergic neuromodulatory systems, the olfactory cortex receives projections from all four systems (Dahlstroem and Fuxe, 1964; Broadwell and Jacobowitz, 1976; Haberly and Price, 1978).

Serotonin

Serotonin-containing fibers reach the OB from the dorsal and median raphe nuclei, where they terminate within all bulbar layers, especially the GL and within glomeruli (De

Olmos et al., 1978; Halasz et al., 1978; Macrides et al., 1981; Shipley and Geinisman, 1984; McLean and Shipley, 1987; Won et al., 1998). The raphe nuclei also project to the piriform cortex, with dense innervation to layers I and III (De Olmos and Heimer 1980; Datiche et al., 1995). In both the OB and OC, serotonin can act in an excitatory or inhibitory fashion, depending on the site of its action and the receptors involved (Sheldon and Aghajanian, 1990, 1991; Gellman and Aghajanian, 1993). Serotonergic modulation functions in the olfactory system to influence short-term memory and associative learning, potentially by enhancing the signal-to-noise ratio of odorant input (McLean et al., 1993; Moriizumi et al., 1994; Letty et al., 1997; Marchetti et al., 2000; Fletcher and Chen, 2010).

Serotonin may influence memory acquisition in pups by acting synergistically with noradrenaline; serotonin and noradrenaline must bind to 5-HT_{2A} and β ₁ receptors on mitral cells, respectively, increasing mitral cell cAMP, and ultimately leading to long-term changes in plasticity (Langdon et al., 1997; Yuan et al., 2003). It also has effects in adulthood. Several studies have determined that serotonin has both inhibitory and excitatory effects on mitral cells, through directly binding to the mitral cell and by acting on neighboring inhibitory interneurons, respectively. Therefore, serotonin can modulate odorant processing at the level of mitral, granule or periglomerular cells (Bloom et al., 1964; Hardy et al., 2005; Petzold et al., 2009).

Noradrenaline

Noradrenergic fibers from the locus coeruleus terminate mostly in the GCL and IPL, with

moderate projections to the MCL and EPL, and minimal innervation of the GL (Macrides et al., 1981; Shipley et al., 1985; McLean et al., 1989; Gomez et al., 2005). All OB layers express both α and β adrenergic receptors (Pieribone et al., 1994; Woo and Leon, 1995). Noradrenergic fibers also project to the piriform cortex, where they densely innervate layers I and III (Fallon and Moore, 1978; Loughlin et al., 1982; Datiche and Cattarelli, 1996; Shipley and Ennis, 1996). In olfaction, noradrenaline is associated with odor detection and discrimination, mother-infant attachment, associative learning, perceptual learning and potentially odorant habituation (Levy et al., 1990; Kendrick et al., 1992; Doucette et al., 2007; Guerin et al., 2008; Mandairon et al., 2008; Escanilla et al., 2010; Moreno et al., 2012; Vinera et al., 2015).

In pups, β adrenergic receptors on granule and mitral cells are thought to mediate olfactory learning (Wilson and Leon, 1988; Yuan et al., 2003). However, early studies (Bloom et al., 1964; Salmoiraghi et al., 1964; Mouly et al., 1995; Jiang et al., 1996; Ciombor et al., 1999) along with recent behavioral and computational modeling studies in adult rats have hypothesized that noradrenergic modulation (via mitral and granule cell $\alpha 1$ receptor activation) alters 1) signal-to-noise ratios by reducing spontaneous firing, 2) oscillatory dynamics and synchrony and 3) enhanced detection of low concentration odorant (Escanilla et al., 2010; Linster et al., 2011). As with serotonin, it is important to note that noradrenaline's mechanisms of action depend upon many parameters, including receptor subtypes activated and odorant concentration (Collins et al., 1984; Nai et al., 2009, 2010; Pandipati et al., 2010).

Overall, it appears that noradrenergic fibers are more important for adjusting the signal-to-noise ratio to perceive low concentration odorants, while cholinergic modulation is more important for discriminating similar odorants (Escanilla et al., 2012). In cholinergic modulation, the GL is important for contrast enhancement and normalization, while the deeper layers also modulate contrast enhancement, as well as spike timing and synchrony. Although it is known that acetylcholine acts on both OB nicotinic and muscarinic receptors, we still do not fully understand the separate influences of these receptors (Castillo et al., 1999; Pressler et al., 2007). However, it is believed that nicotinic and muscarinic receptors have unique yet complimentary roles, synergistically improving odorant processing.

1.3 Adult Neurogenesis

Although neurogenesis was classically thought to only occur in the developing brain (Ramón y Cajal, 1928), it is now clear that neurogenesis occurs throughout adulthood. Adult neurogenesis is widespread in non-mammalian vertebrates (Lindsey and Tropepe, 2006; Kaslin et al., 2008; Barker et al., 2011). However, it is restricted to primarily two regions in the adult mammalian brain: the SVZ of the lateral ventricles (where OB interneurons arise) and the subgranular zone (SGZ) of the hippocampus (which leads to an increase in DG neurons) (Altman and Das, 1965; Altman, 1969; Figure 1.8). Adult neurogenesis has been implicated in learning and circuit plasticity. Disruptions in neurogenesis lead to impaired cognitive functioning and the development of symptoms associated with a variety of neurological and psychiatric disorders. The mechanisms

regulating adult neurogenesis remain largely unknown, with specific interest in how and why select cells survive.

Olfactory Bulb Adult Neurogenesis

Although most rodent postnatal olfactory neurogenesis occurs within the first three weeks after birth, this process continues throughout the life of the animal (Altman, 1969; Kaplan and Hinds, 1977; Corotto et al., 1993; Lois and Alvarez-Buylla, 1994). Although I will review adult born neurogenesis that influences the MOB below, there is evidence that newly born cells are present in the AOB as well, where they are believed to be linked to reproductive social behaviors (Bonfanti et al., 1997; Oboti et al., 2009, 2011; Nunez-Parra et al., 2011; Wu et al., 2013).

New neurons are born during adulthood in the SVZ, a structure originating from the lateral ganglionic eminence, near the anterior horn of the lateral ventricles (Altman, 1969; Luskin, 1993). SVZ stem cells are glial fibrillary acidic protein (GFAP)-positive radial glia-like cells (type B cells) (Alvarez-Buylla and Lim, 2004). These cells give rise to distal-less homeobox 2 (Dlx2) expressing proliferating transit-amplifying cells (type C cells), which in turn generate polysialic acid neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX)-positive neuroblasts (type A cells) (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1999; Laywell et al., 2000; Garcia et al., 2004). Interestingly, the latter are capable of releasing GABA, which provides a feedback mechanism to influence the further division of type B cells (Liu et al., 2005).

Neuroblasts migrate tangentially up to 5 mm in rodents along a chain of cells called the rostral migratory stream (RMS) to the OB (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). They travel within tubular structures formed by GFAP-positive astrocytes, detach from these chains, and continue migrating radially (Lois and Alvarez-Buylla, 1994; Jankovski and Sotelo, 1996; Lois et al., 1996). Migration takes four to 10 days, with the peak number of neurons arriving to the GCL around the tenth day (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Hu and Rutishauser, 1996; Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). These cells then begin to differentiate into two types of inhibitory interneurons, granule and periglomerular cells, and incorporate themselves into the preexisting bulbar network (Carleton et al., 2003; Lledo et al., 2006; Whitman and Greer, 2007; Figure 1.9).

OB granule cells become mature as early as two weeks after birth, faster than both periglomerular cells and hippocampal granule cells which can take four weeks to develop their mature dendritic morphology (Petreanu and Alvarez-Buylla, 2002; van Praag et al., 2002; Belluzzi et al., 2003; Lledo et al., 2006). Newly born neurons first make GABAergic synaptic connections before they receive glutamatergic inputs. However, OB granule cells have delayed excitability compared to periglomerular cells. Periglomerular cells create mature voltage-dependent sodium currents prior to establishing synaptic connections, while granule cells can only fire these action potentials once their synaptic integration into the circuit is complete (Belluzzi et al., 2003; Carleton et al., 2003; Lledo et al., 2006).

A staggering 30,000 to 80,000 new cells are born each day in the SVZ, representing approximately 1% of OB inhibitory interneurons (Kaplan et al., 1985; Biebl et al., 2000; Peterson and Peterson, 2000; Alvarez-Buylla et al., 2001; Peterson, 2002; Winner et al., 2002). Most of these cells become granule cells, although approximately 4% become periglomerular cells and 2% astrocytes (Curtis et al., 2009; Lledo and Valley, 2016). Although about half of these newborn cells are eliminated within the first few months, with 6-10% replacement per month in the GCL, those cells that survive to three months typically live for at least 16 more months (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002).

Hippocampal Adult Neurogenesis

In the hippocampus, cells from the EC project to granule cells, the only principal cell type in the DG. The DG consists of three layers: the molecular layer, the GCL and the hilus. DG granule cell axons, termed mossy fibers then project to cornu ammonis 3 (CA3) and synapse on pyramidal cells (Christie and Cameron, 2006; Amaral et al., 2007; Figure 1.10). Although the majority of granule cells in the DG are born during development, up to 10% of these cells can be added in adulthood (Imayoshi et al., 2008).

New granule cells arise from neural stem cells in the SGZ, an area situated between the GCL and hilus. Radial glial stem cells expressing GFAP divide to create neural progenitor cells, astrocytes and more stem cells (Bonaguidi et al., 2011). These neural progenitor cells, also known as transit-amplifying cells, express DCX, a marker of immature cells committed to a neuronal fate (Seri et al., 2001, 2004; Brown et al., 2003).

As these cells continue to differentiate, they move upward into the GCL, eventually extending dendritic arbors into the molecular layer and axons to CA3 (Stanfield and Trice, 1988; Markakis and Gage, 1999). Maturation typically occurs within about four weeks of mitosis (Cameron and McKay, 2001; van Praag et al., 2002; Brown et al., 2003; Deng et al., 2010).

Under standard conditions, approximately 1000 to 10,000 hippocampal neurons are born in the rodent brain each day representing less than 0.03% of the neurons in the DG, with the number depending on species and strain type (Kempermann et al., 1997; Cameron and McKay, 2001; Hayes and Nowakowski, 2002). However, it is estimated that only 10-50% of these cells survive to maturation and DG circuit integration at four weeks (Dayer et al., 2003; Sierra et al., 2010). Hippocampal neurogenesis can be bolstered or minimized by many environmental or endogenous factors, such as enrichment, stress, exercise, neurotransmitters and neurotrophins (Gould et al., 1997; Kempermann et al., 1997; van Praag et al., 1999; Fowler et al., 2002; Lledo et al., 2006; Kvajo et al., 2008; Zhao et al., 2008; Lieberwirth et al., 2016). In addition, this process is known to be important for spatial learning and memory (Drapeau et al., 2003; Snyder et al., 2005; Wojtowicz et al., 2008; Zhang et al., 2008; Ming and Song, 2011).

Just like hippocampal neurogenesis, OB neurogenesis is modulated by numerous internal and external factors, including but not limited to transcription factors, neurotransmitters, enrichment, exercise, age and stress (Conover et al., 2000; Caillé, 2004; Cooper-Kuhn et al., 2004; Hack et al., 2005; Kohwi et al., 2005; Hitoshi et al., 2007; Leuner et al., 2007;

Tanaka et al., 2007; Moreno et al., 2012; Chae et al., 2014). In particular, olfactory experience is crucial for the maturation and survival of newly born neurons, as demonstrated by both olfactory sensory deprivation and enrichment studies (Corotto et al., 1994; Rochefort et al., 2002; Yamaguchi and Mori, 2005; Mandaïron et al., 2006b; Bovetti et al., 2009). Through contrast enhancement, gain control and synchrony, these cells are thought to contribute to olfactory learning and memory (Lledo and Saghatelian, 2005; Kay and Stopfer, 2006; Cleland, 2010; Ming et al., 2011; Lepousez and Lledo, 2013).

Human Adult Neurogenesis

Due to methodological constraints, adult neurogenesis has been difficult to study in humans. However, in 1998, Eriksson et al. intravenously infused squamous cell carcinoma patients with bromodeoxyuridine (BrdU). In postmortem tissue analysis, they found human adult born neurons in the DG. Since then, numerous studies have tried to localize and quantify adult neurogenesis in the human brain. Given that adult neurogenesis has been extensively linked to neurological and psychiatric diseases in animal models, it is an intriguing avenue to explore for the treatment of human affective pathology (Parent et al., 1997; Galvan and Bredesen, 2007; Schloesser et al., 2009; Christian et al., 2010; Mu and Gage, 2011).

Human Olfactory Bulb Adult Neurogenesis

Even more controversial than the importance of human hippocampal adult neurogenesis is the existence and relevance of neurogenesis from the SVZ. Although the human SVZ

contains proliferative cells, and DCX and PSA-NCAM positive neuroblasts have been identified in a human RMS, neuronal migration and a significant rate of neurogenesis in the adult brain have been called into question (Kirschenbaum et al., 1994; Johansson et al., 1999; Roy et al., 2000; Palmer et al., 2001; Curtis et al., 2007; Sanai et al., 2011; Wang et al., 2011; Bergmann et al., 2012; Spalding et al., 2013). Nevertheless, several studies have shown the adult human brain incorporates newly born OB cells (Liu and Martin, 2003; Bédard and Parent, 2004; Curtis et al., 2012). In addition, the density of proliferating cells in the SVZ has been associated with affective pathology (Haughey et al., 2002; Curtis et al., 2003; Höglinger et al., 2004; Jin et al., 2004). It is possible these newly born cells are generated locally in the bulb, minimizing the need for long-range neuronal migration (Pagano et al., 2000; Bédard and Parent, 2004). In addition, it is possible that neuroblasts from the SVZ migrate to a location other than the OB, such as the striatum (Ernst et al., 2014). More work is needed to understand the generation of newly born cells in the SVZ and OB, as well as their possible role in influencing neurogenesis in other regions of the human brain.

Human Hippocampal Adult Neurogenesis

In non-human primates, adult neurogenesis studies have found low rates of hippocampal neurogenesis in the DG (Rakic, 1985; Kornack and Rakic, 1999). Therefore, even after a few papers found evidence for human adult neurogenesis, controversy still existed as to whether these new neurons are functionally relevant (Eriksson et al., 1998; Kukekov et al., 1999; Knöth et al., 2010). In 2013, Spalding et al. took advantage of the surge of atmospheric radiocarbon ^{14}C from 1945 to 1963 resulting from nuclear bomb testing

during the Cold War. Cleverly, the integration of ^{14}C into cellular DNA was used as a cell birth dating technique, through which it was inferred that human hippocampal adult neurogenesis is as prominent in humans as it is in middle-aged rodents. Numerous studies have now examined human hippocampal adult neurogenesis with respect to affective pathology, such as Alzheimer's disease, depression, anxiety, epilepsy, Huntington's disease, and Parkinson's disease, although the importance of hippocampal adult neurogenesis in human remains an active area of controversy (Boekhoorn et al., 2006; Liu et al., 2008; Lucassen et al., 2010; Low et al., 2011; Boldrini et al., 2013).

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Figures

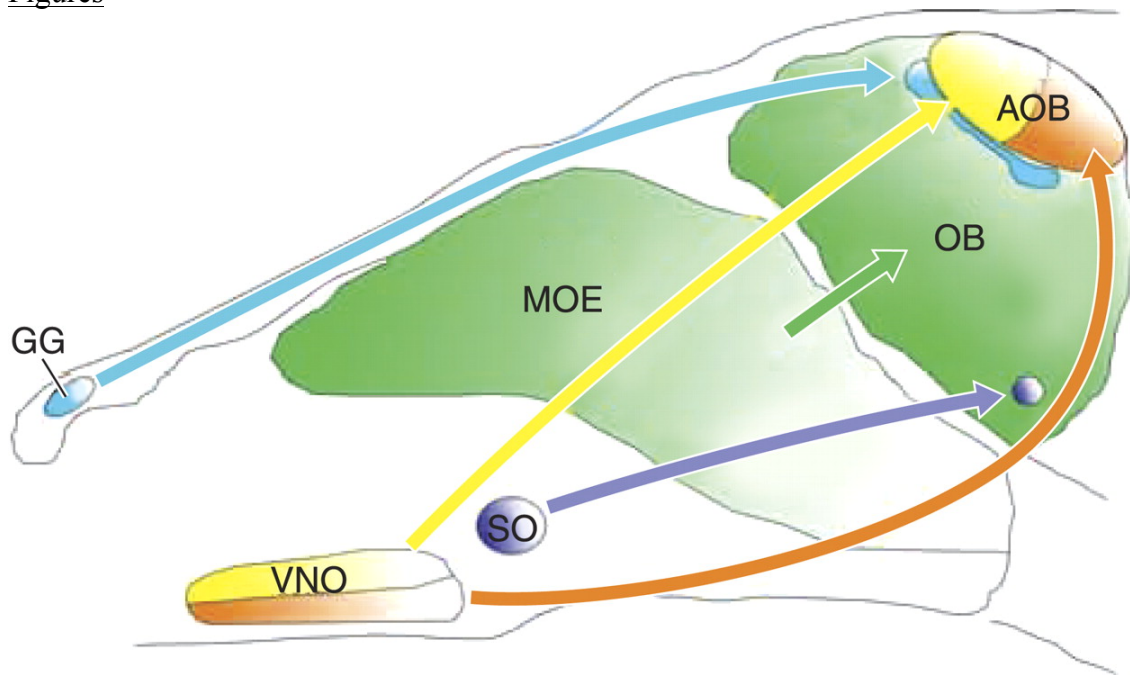


Figure 1.1. Four olfactory chemosensory organs. The olfactory system contains four chemosensory organs: the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the septal organ of Masera (SO) and the Grüneberg ganglion (GG). *Figure from Tirindelli et al., 2009.*

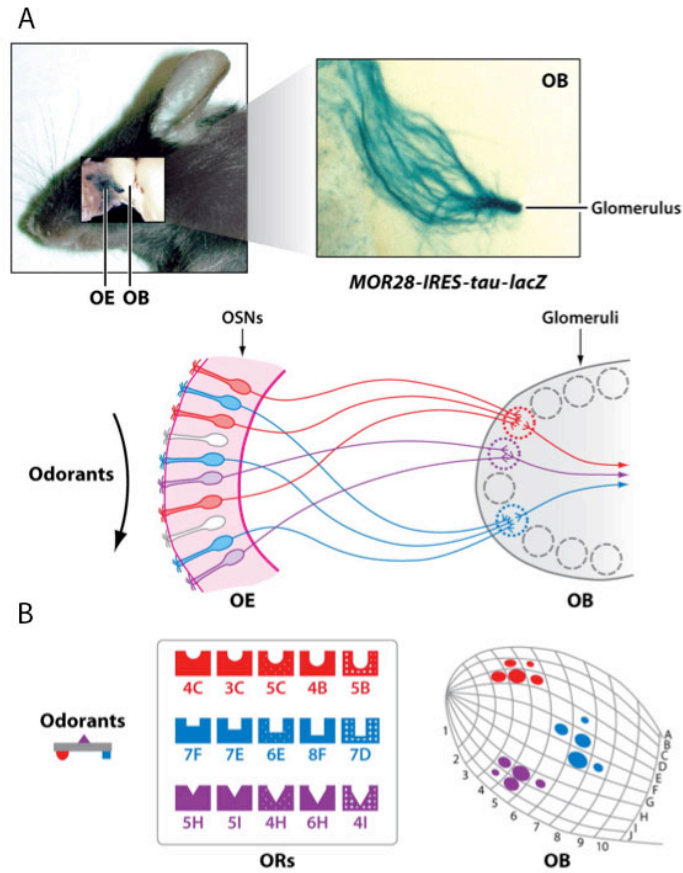


Figure 1.2. The murine olfactory system. A) Olfactory sensory neurons (OSNs) expressing the same olfactory receptor (OR) converge their axons on spatially conserved glomeruli. B) The olfactory system uses combinatorial receptor coding to start processing odorant information. OE = olfactory epithelium, OB = olfactory bulb. *Figure adapted from Mori and Sakano, 2011.*

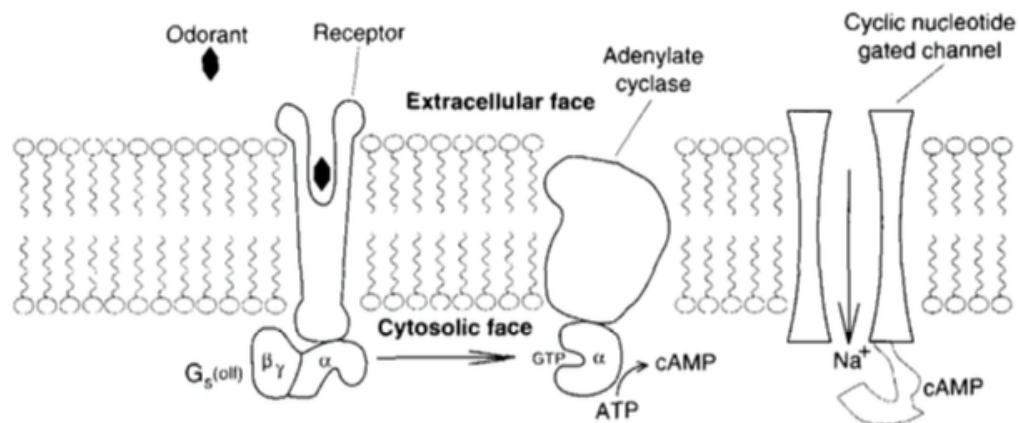


Figure 1.3. Olfactory transduction. When an odorant binds an olfactory receptor, the receptor interacts with a guanosine triphosphate (GTP)-binding protein (G_{olf}), releasing a GTP-coupled subunit, and allowing adenosine triphosphate (ATP) to be converted into cyclic adenosine monophosphate (cAMP). From there, cAMP helps open cyclic nucleotide gated (CNG) cation channels, depolarizing the cell. *Figure adapted from Buck and Axel, 1991.*

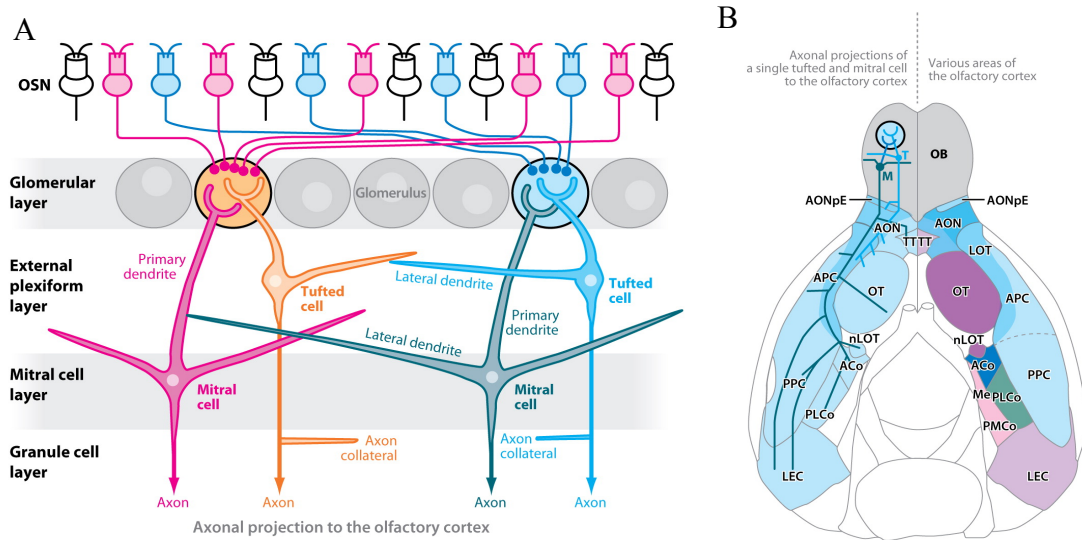


Figure 1.4 The olfactory bulb and cortex. A) Each glomerulus receives axons from olfactory sensory neurons (OSNs) with the same olfactory receptor (OR). These axons synapse with mitral and tufted cells, whose axons project to the olfactory cortex (OC). B) The OC, shown in light blue, covers a wide number of brain regions: ACo = anterior cortical amygdaloid nucleus, AON = anterior olfactory nucleus pars principalis, AONpE = anterior olfactory nucleus pars externa, APC = anterior piriform cortex, LEC = lateral entorhinal cortex, LOT = lateral olfactory tract, Me = medial amygdaloid nucleus, nLOT = nucleus of lateral olfactory tract, OT = olfactory tubercle, PLCo = posterolateral cortical amygdaloid nucleus, PMCo = posteromedial cortical amygdaloid nucleus, PPC = posterior piriform cortex, TT = tenia tecta. *Figure adapted from Mori and Sakano, 2011 and Luskin and Price, 1983. Abbreviations adapted from Mori and Sakano, 2011.*

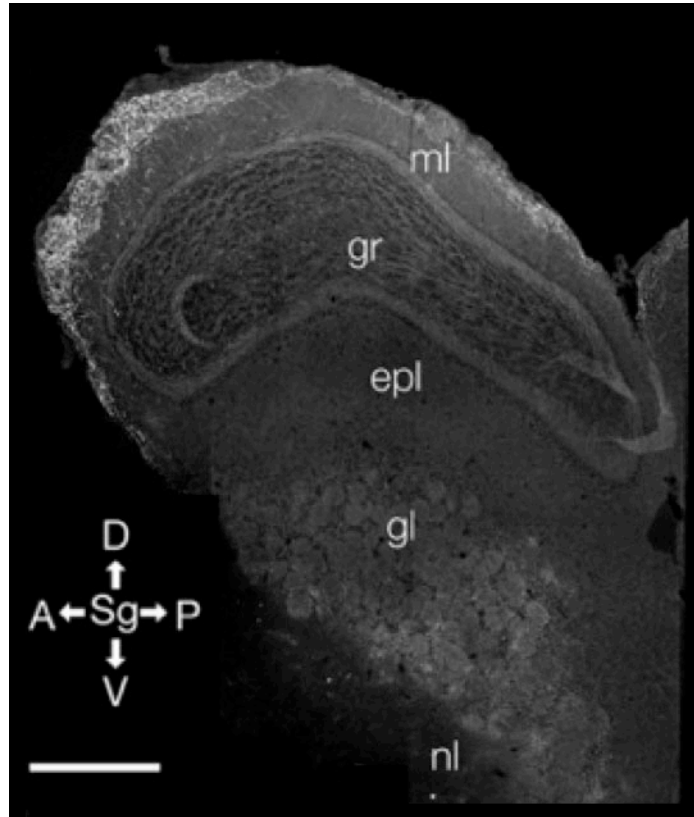


Figure 1.5. Cholinergic fibers innervate the olfactory bulb. The nucleus of the horizontal limb of the diagonal band of Broca (HDB) sends cholinergic fibers to the olfactory bulb (OB). In this parasagittal image from a ChAT-tauGFP mouse, bright areas represent dense cholinergic innervation. A = anterior, D = dorsal, epl = external plexiform layer, gl = glomerular layer, gr = granule cell layer, ml = mitral cell layer, nl = olfactory nerve layer, P = posterior, Sg = sagittal, V = ventral. *Figure adapted from Salcedo et al., 2011. Abbreviations adapted from D'Souza and Vijayaraghavan, 2014.*

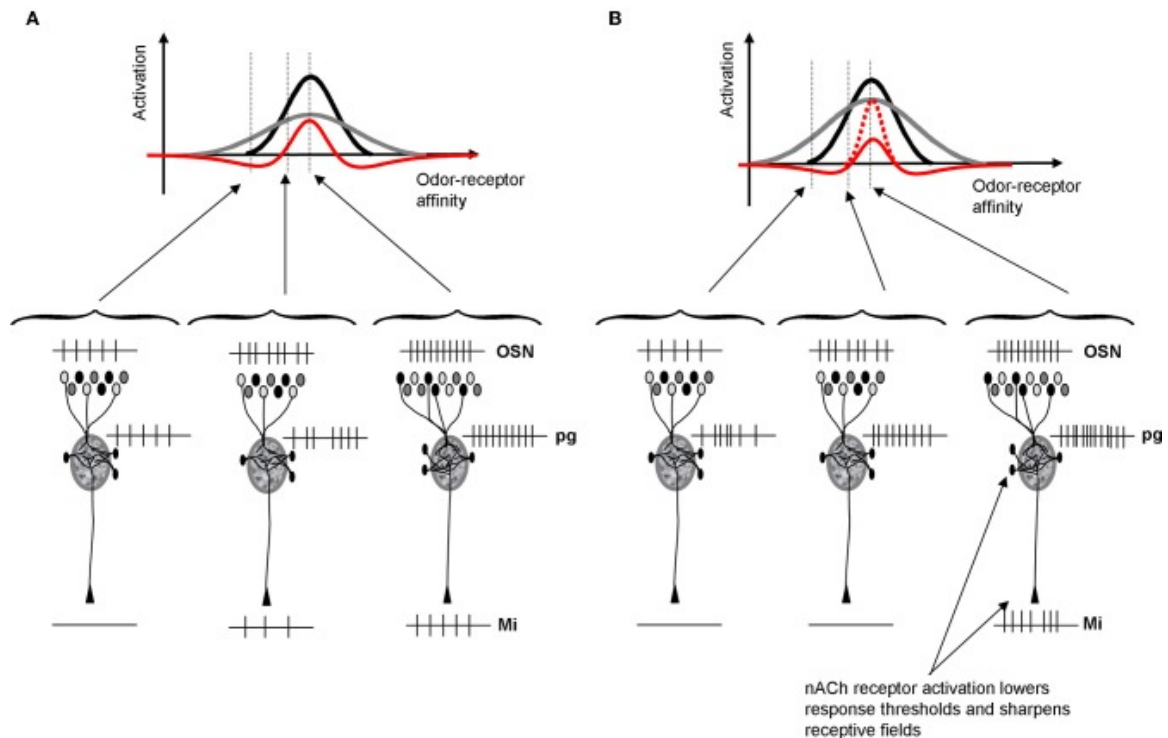


Figure 1.6. Acetylcholine influences contrast enhancement via nicotinic acetylcholine receptors. A) Olfactory sensory neurons (OSNs) send their axons to glomeruli in the olfactory bulb. These axons synapse with periglomerular (pg) and mitral (Mi) cells. Pg cells (whose activation is represented as a gray arch) become stimulated even at low odorant affinities, although their activation stays relatively low, even at high odorant affinities. Conversely, it takes a higher odor-receptor affinity for Mi cells to become activated, but their activation level is higher (black arch). Pg cells inhibit Mi cells, leading to contrast enhancement and the detection of only higher affinity odorants (red trace). B) Since nicotinic acetylcholine (nACh) receptors are typically found in the glomerular and mitral cell layers, they are perfectly located on pg and Mi cells to sharpen Mi receptive fields. Acetylcholine broadens pg cell receptive fields and sharpens Mi cell activation to high affinity odorants. Therefore, acetylcholine cumulatively induces greater contrast enhancement (dotted red trace), heightening odorant discrimination. *Figure from Devore and Linster, 2012.*

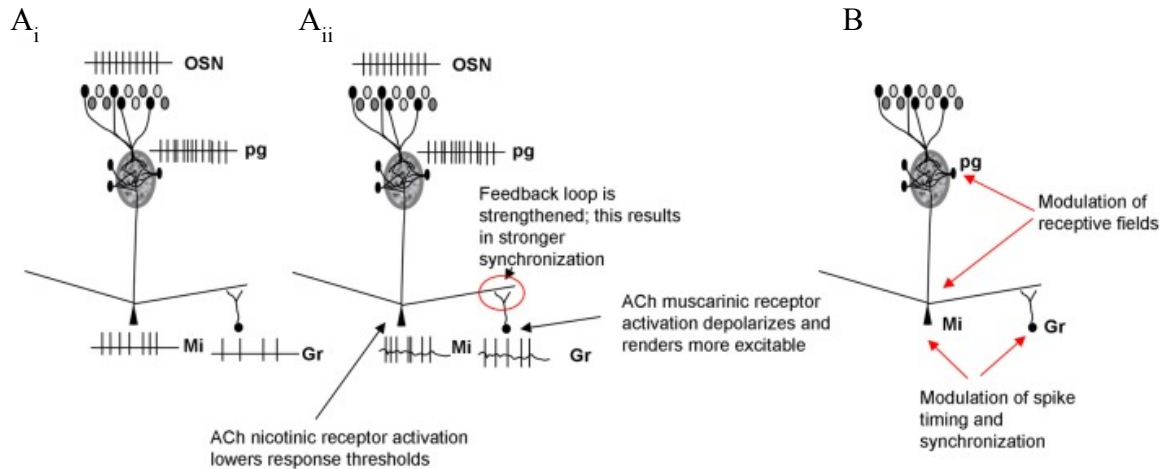


Figure 1.7. Acetylcholine impacts synchrony via nicotinic and muscarinic acetylcholine receptors. A_i) Olfactory sensory neurons (OSNs) converge their axons on glomeruli. These axons synapse with periglomerular (pg) and Mi (mitral) cells. When odorants activate Mi cells, granule cells (Gr) synchronously become activated via dendrodendritic interactions. A_{ii}) Nicotinic acetylcholine (ACh) receptors on Mi cells increase their activation to high affinity odorants while muscarinic ACh receptors located on Gr cells increase Gr cell responsiveness. The negative feedback loop between Mi cells releasing glutamate and Gr cells releasing GABA induces oscillations and synchrony. B) Summary of ACh's influences on contrast enhancement and synchrony. *Figure adapted from Devore et al., 2012.*

Hippocampal

Olfactory

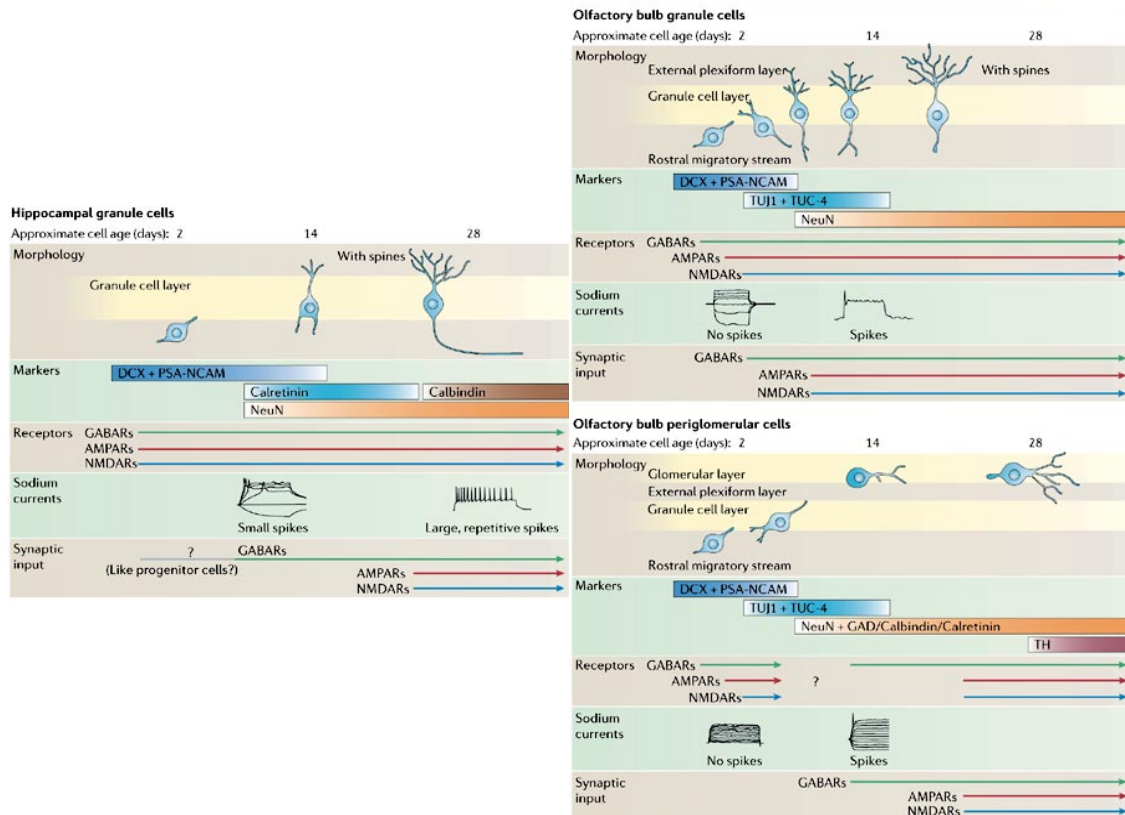


Figure 1.8. Adult neurogenesis. This figure summarizes the three types of neurons produced throughout life: hippocampal granule cells, olfactory bulb (OB) granule cells and OB periglomerular cells. Days represent the earliest time point at which morphological changes can occur, although maturation can take place for up to four months postnatally. Although there are many similarities between these cells [e.g. all three cell types express receptors prior to synaptic activation and receive gamma-aminobutyric acid (GABA) inputs before glutamate], there are also many differences. OB granule cells not only mature faster than periglomerular and hippocampal granule cells, but also only fire sodium-based action potentials after receiving synaptic input, unlike the other two cell types. In addition, more newly born cells are added daily to the OB than the hippocampus. Newly born olfactory neurons are inhibitory, while hippocampal granule cells are excitatory. Finally, hippocampal neurogenesis is localized to the dentate gyrus (DG), while newly born cells from the subventricular zone (SVZ) must migrate to the OB. AMPAR = alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, DCX = doublecortin, GABAR = GABA receptor, GAD = glutamate decarboxylase, NMDAR = N-methyl-D-aspartate receptor, NeuN = neuronal nuclear antigen, PSA-NCAM = polysialic acid neuronal cell adhesion molecule, TH = tyrosine hydroxylase, TUC4 = collapsin response mediator protein 4, TUJ1 = beta III-tubulin. *Figure and caption adapted from Lledo et al., 2006.*

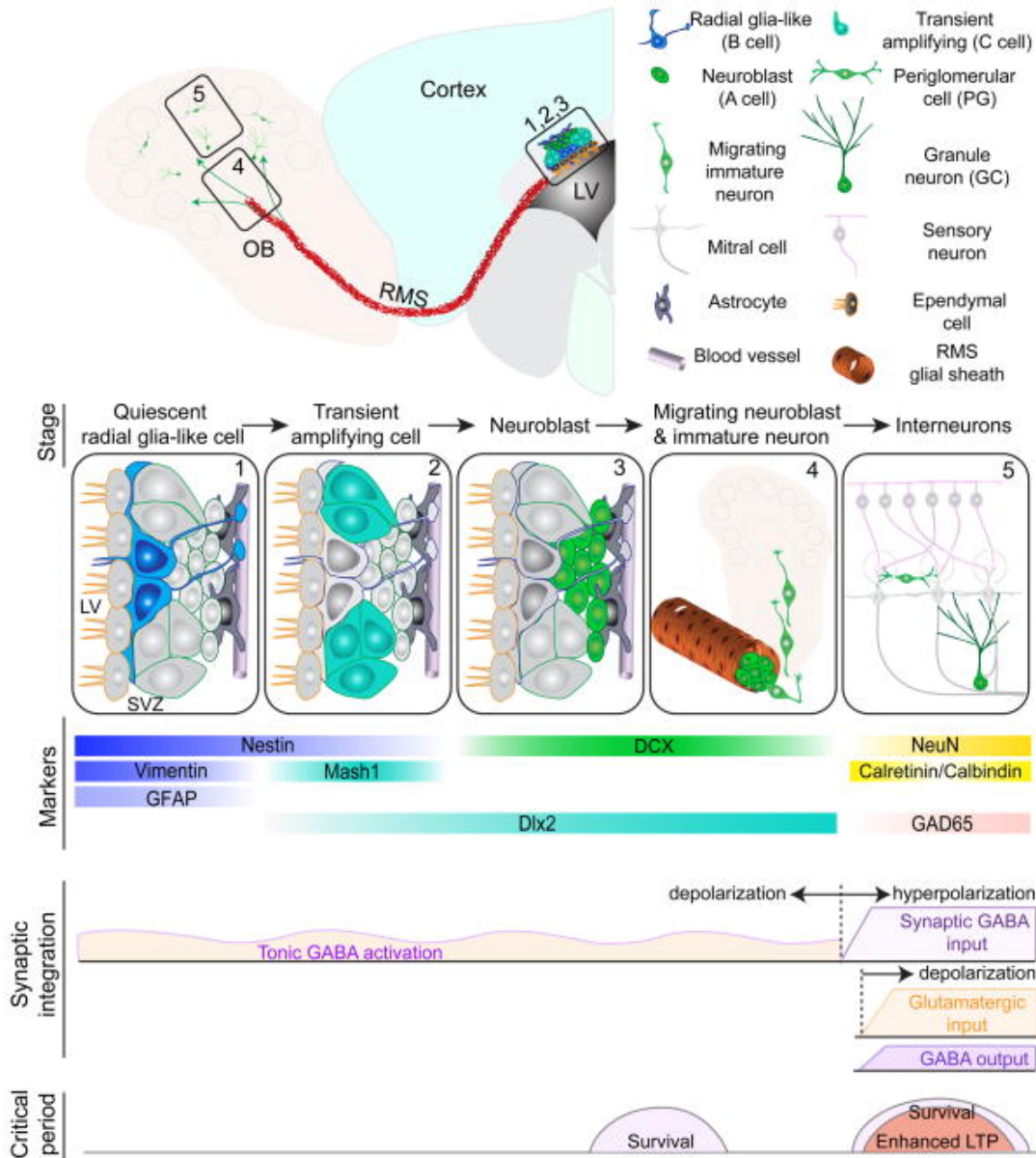


Figure 1.9. Olfactory neurogenesis. This figure summarizes the stages, markers, synaptic integration and critical periods radial glia-like cells must endure to successfully travel through the rostral migratory stream (RMS) and ultimately differentiate into mature olfactory bulb (OB) cells. DCX = doublecortin, GFAP = glial fibrillary acidic protein, LTP = long-term potentiation, NeuN = neuronal nuclei. *Figure from Ming and Song, 2011.*

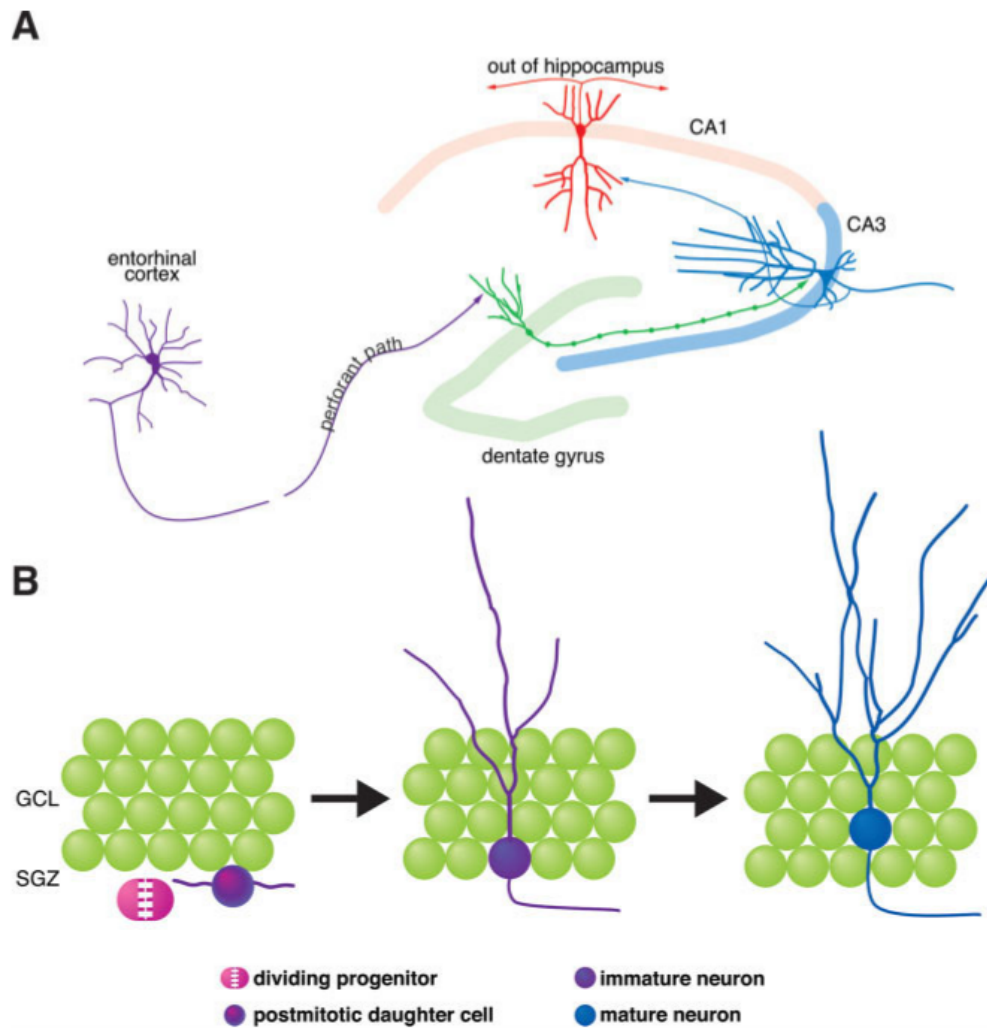


Figure 1.10. Hippocampal neurogenesis. A) Entorhinal cortex (EC) stellate cells project to granule cells in the dentate gyrus (DG), whose axons (mossy fibers) synapse on pyramidal cells within cornu ammonis 3 (CA3). These cells project to cornu ammonis 1 (CA1), the last stop in the hippocampus. B) Adult born granule cells begin in the subgranular zone (SGZ), moving up to the granule cell layer (GCL). As these cells mature, their dendrites project to the molecular layer while their axon projects to CA3. *Figure from Christie and Cameron, 2006.*

CHAPTER 2: REPEATED ODORANT ENRICHMENT IS SUFFICIENT TO SUPPORT OLFACTORY PERCEPTUAL LEARNING

Introduction

Olfactory discrimination, or the ability to detect differences between odorants, is partially accomplished at every level in the olfactory system: the main olfactory epithelium (MOE), olfactory bulb (OB) and cortex. In the MOE, odorants bind to olfactory receptors (ORs) on the dendrites of olfactory sensory neurons (OSNs). Since ORs can bind multiple odorants, and each odorant can bind to multiple ORs, combinatorial receptor coding in the MOE can significantly influence odorant discrimination (Malnic et al., 1999).

At the level of the OB, odorants with similar molecular properties map to similar regions of the bulb (Takahashi et al., 2004; Johnson and Leon, 2007; Johnson et al., 2009; Figure 2.1). Since the mitral-granule cell microcircuit can induce gamma oscillations and synchrony, as well as center-surround lateral inhibition, separating the perception of similar odorants via contrast enhancement, odorants that are similar but not identical should ideally be used to study odorant discrimination. (Rall and Shepherd, 1968; Yokoi et al., 1995; MacLeod and Laurent, 1996; Linster and Hasselmo, 1997; Nusser et al., 2001; Bathellier et al., 2006; Cleland and Sethupathy, 2006; Urban and Arevian, 2009; Giridhar et al., 2011).

Olfactory experience has not only been proven to enhance olfactory acuity and discrimination, but has also been associated with an increase in OB newborn cell density (Rocheffort et al., 2002; Fletcher and Wilson, 2003; Alonso et al., 2006; Mandairon et al., 2006a; Lazarini et al., 2009; Sultan et al., 2010). Specifically, odorant exposure promotes the selective survival of newly born OB granule cells, allowing for increased discrimination ability among odorants previously experienced (Moreno et al., 2012).

In addition to adult neurogenesis, olfactory experience modulates olfactory discrimination through synaptic plasticity. It is now known that the functional reciprocal dendrodendritic interactions between mitral and granule cells requires N-methyl-D-aspartate (NMDA) and supports long-term potentiation (LTP) (Wilson, 1995; Elaagouby and Gervais, 1996; Ennis et al., 1998; Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Mandairon et al., 2006b).

Non-olfactory cues, such as neuromodulatory systems, can also influence olfactory discrimination. The OB and cortex receive dense innervation from several neuromodulatory systems, including acetylcholine and noradrenaline. These neuromodulators are crucial for behavioral olfactory discrimination tasks (Mandairon et al., 2006c; Chaudhury et al., 2009; Escanilla et al., 2010; Moreno et al., 2012).

Throughout this dissertation, we will examine a specific kind of olfactory discrimination called olfactory perceptual learning. Olfactory perceptual learning is a passive learning

process where animals can learn to discriminate similar chemical signals without activating learning, memory or reward systems (Cleland et al., 2002; Wilson and Stevenson, 2003).

In order to understand olfactory discrimination, we should be able to predict olfactory perception based upon the olfactory bulb topographic map. Enantiomer pairs (in this dissertation, also referred to as odorant pairs) are two stereoisomers, or molecules that have the same molecular formula and constitution but different chirality. Some enantiomers activate very overlapping glomeruli as their counterparts, while others do not, a measure that correlates with behavioral discrimination (Linster et al., 2001).

However, enantiomer discrimination is largely species- and task-dependent, with neural activation pattern overlap of enantiomer pairs determined by method of statistical analysis (Friedman and Miller, 1971; Leitereg et al., 1971; Heth et al., 1992; Taniguchi et al., 1992; Laska and Teubner, 1999; Laska et al., 1999ab; Ma and Shepherd, 2000; Laska and Galizia, 2001; Linster et al., 2001, 2002; Rubin and Katz, 2001). Furthermore, implicit learning tasks provide a stronger correlation between olfactory perception and neural representation than their associative learning counterparts (Cleland et al., 2002). Therefore, after choosing potential enantiomer pairs, we employed our olfactory perceptual learning task to determine for ourselves if C57BL6/N mice could perceptually distinguish within enantiomer pairs.

We tested the ability of C57BL6/N mice to spontaneously discriminate between the members of three enantiomer pairs, + limonene and - limonene, + carvone and - carvone,

and + terpinen-4-ol and – terpinen-4-ol using a cross-habituation task (Table 7.1). In addition to the inability of C57Bl6/N mice to spontaneously discriminate within these three enantiomer pairs, these odorants were chosen due to their structural similarity. Adding a ketone group to limonene changes the odorant to carvone, while adding a hydroxyl group and removing a double bond from limonene's isopropyl group converts limonene into terpinen-4-ol (Linster et al., 2001; Figure 2.2). Although these changes may seem insignificant, these chemical structures are sufficiently different to produce minimally overlapping glomerular spatial activation patterns and perception (Johnson and Leon, 2000; Linster et al., 2001; Figure 2.3). By choosing enantiomer pairs with dissimilar receptive fields, we expected limonene enrichment to specifically affect limonene discrimination (Mandairon et al., 2008).

Methods

For more information on the methods used in Chapter 2 experiments, see “Subjects,” “Olfactory Cross-Habituation Tasks” and “Statistical Analysis” in Chapter 7.

Olfactory Enrichment

Mice were injected with saline intraperitoneally (IP; BD Insulin Syringe with the BD Ultra-fine needle, 0.3 cc, 30 gauge, ½”, Franklin Lakes, NJ) 20 minutes before each olfactory enrichment session. During enrichment, the mice were exposed to the limonene enantiomer pair. Two tea balls were draped on opposite sides of the home cage for one hour, one containing filter paper with 100 µl undiluted + limonene, the other with 100 µl undiluted – limonene (Mandairon et al., 2006d). Each session lasted for one hour, and was repeated once per day for ten consecutive days. Throughout the dissertation, every olfactory perceptual learning experiment will contain a 10-day olfactory manipulation period between the pretest and posttest. In this chapter, the manipulation is +/- limonene enrichment. For an overview of all manipulations, see “Olfactory Manipulation” in Chapter 7.

Olfactory Perceptual Learning Overview

Passively learning to discriminate new chemical signals has been termed olfactory perceptual learning (Wilson and Stevenson, 2003). We tested discrimination ability for three sets of enantiomer pairs over three consecutive days: + and – limonene, + and – carvone, and + and – terpinen-4-ol (Table 7.1). Each enantiomer pair was chosen based upon the perceptual similarity within pairs (see “Results” and Figure 2.6A). Furthermore,

carvone and terpinen-4-ol were selected as control enantiomer pairs, as the glomerular patterns activated by these enantiomer pairs are quite different than those glomeruli activated by limonene, a measure that correlates with perception (Linster et al., 2001). For each trial, a tea ball containing the odorant was draped over an upper corner of the cage. Each one-minute trial was followed by a five-minute inter-trial interval. During the first four trials, mice were exposed to the + form of the odorant (+ limonene, + carvone or + terpinen-4-ol). On the fifth trial, mice were presented with the - form of the odorant (- limonene, - carvone, - terpinen-4-ol, respectively). Odorant investigation time was recorded. For detailed methods, see “Olfactory Perceptual Learning” in Chapter 7.

Spontaneous Olfactory Discrimination Overview

We used a spontaneous olfactory discrimination task to evaluate the baseline discrimination abilities of our C57Bl6/N mice (Table 7.2). After four habituation trials with an aliphatic acid odorant, mice were presented with three novel odorant trials in a counter-balanced and randomized order. By choosing odorants that were systematically different than the habituation odorant, these odorants were highly similar (S1), moderately similar (S2), or dissimilar (D) to the habituated odorant. This task was repeated with five different odorant sets over five days. Odorant presentation, trial length and inter-trial interval were conserved among olfactory perceptual learning and spontaneous olfactory discrimination experiments. For detailed methods, see “Spontaneous Olfactory Discrimination” in Chapter 7.

Bromodeoxyuridine Overview

Thymidine autoradiography was originally used in the discovery of adult neurogenesis, a technique that incorporates tritiated thymidine ($[^3\text{H}]$ -thymidine) into the DNA of replicating cells (Altman and Das, 1965). Nowadays, 5-bromo-2'-deoxyuridine, or BrdU is commonly used to study newly born cells (Gratzner, 1982). BrdU is a thymidine analog that incorporates itself into DNA (by substituting bromouracil for thymidine) during the S phase of the cell cycle (Miller and Nowakowski, 1988). This technique is ideal for the stereological quantification of newly born cells, although it must be done in fixed tissue (Scharfman et al., 2005). In our study, we injected mice with BrdU (IP: 50mg/kg) three times at two-hour intervals on the day following the final pretest. Enrichment followed eight days later. Thirty-nine days post-BrdU injection, mice were sacrificed, and we used immunohistochemistry and stereological counting to test for the effects of enrichment on newborn cell survival only in the OB granule cell layer, as enrichment has been shown to have no effect on newborn cell survival in the glomerular layer (Moreno et al., 2009; Figure 7.2A). We also analyzed newly born cell differentiation using BrdU NeuN (a neuronal nuclei marker) double labeling (Figure 7.2B). For detailed methods, see “Bromodeoxyuridine Injections,” “Perfusion and Histology Preparation” and “Immunohistochemistry” in Chapter 7.

Timeline

At the beginning of the experiment, mice were tested on their ability to behaviorally discriminate within three enantiomer pairs. After three pretest days, mice were injected with BrdU. Eight days after newly born cells were labeled, we began olfactory enrichment and saline administration. Olfactory enrichment lasted for ten days, followed

by a three-day washout period and three posttest days. For a baseline adult neurogenesis comparison, one group of mice was injected with saline, but did not undergo olfactory enrichment. Finally, we assessed the general bulbar affects of limonene enrichment by completing a five-day spontaneous olfactory discrimination task. Mice were sacrificed 39 days after the BrdU injection for histology (Figure 2.4). For an overview of all timelines, see “Experiment Timelines” in Chapter 7.

Results

To assess olfactory perceptual learning, we employed an olfactory cross-habituation task (Cleland et al., 2002; Mandaïron et al., 2006b; Bath et al., 2008; Moreno et al., 2009).

With this task, we can measure discrimination ability before and after an exposure period to a pair of perceptually similar odorants (Mandaïron et al., 2006bd). Cross-habituation tasks consist of repeated odorant exposure over multiple trials leading to attenuated investigation time (time sniffing within 1 inch of the odorant) across trials, indicating successful habituation. When a new odorant is introduced, if investigation time increases (dishabituation or discrimination), this indicates successful perceptual differentiation between the novel and the original habituation odorant. If investigation time does not increase for the new odorant (cross-habituation), the animal has failed to perceptually discriminate between the habituation and novel odorant (Figure 2.5).

Prior to enrichment, mice could not discriminate between the + and - form of any tested odorants (Limonene - $F(1,10)=0.347$, $p=0.569$; Carvone - $F(1,11)=0.045$, $p=0.837$; Terpinen-4-ol - $F(1,11)=1.511$, $p=0.245$; Figure 2.6A). Eight days later, mice began olfactory enrichment to + and - limonene for one hour a day on ten consecutive days. Three days after enrichment, we conducted the posttest for all three enantiomer pairs. We found that mice only learned to discriminate between + and – limonene, the enriched pair of odorants, as evidenced by a significant increase in investigation between the fourth habituation (Hab4) and enantiomer trials ($F(1,11)=9.750$, $p=0.010^{**}$; Figure 2.6B). Mice continued to cross-habituate within the other two enantiomer pairs (Carvone - $F(1,11)=3.557$, $p=0.086$; Terpinen-4-ol - $F(1,11)=0.621$, $p=0.447$; Figure 2.6B). Improved

performance between + and – limonene required the enrichment phase, as non-enriched saline-injected mice continued to cross-habituate within all three enantiomer pairs (Posttest statistics: Limonene - $F(1,10)=.451$, $p=0.517$; Carvone - $F(1,10)=7.087$, $p=0.024^*$ (significant decrease); Terpinen-4-ol - $F(1,10)=0.708$, $p=0.420$; Figures 2.6C and 2.6D). We then used a spontaneous olfactory discrimination task to assess the general discrimination abilities of these mice (Table 7.2). Consistent with our hypothesis, enriched and non-enriched mice did not show deficits in general olfactory discrimination ($F(3,9)=7.886$, $p=0.007^{**}$; Figure 2.6E).

It has been previously shown that olfactory enrichment is associated with enhanced survival of newly born cells in the OB (Moreno et al., 2009). To confirm this result, we injected mice with BrdU on the day following the final pretest (Figure 7.2A). We allowed labeled cells eight days to migrate from their birthplace in the subventricular zone (SVZ) to the OB (Winner et al., 2002) prior to beginning enrichment. The OB granule cell layers of enriched mice had a significantly higher density of BrdU positive cells than non-enriched mice ($F(1,13)=10.425$, $p=0.007^{**}$; Figure 2.6F). Odor enrichment did not change the percentage of cells committing to a neural phenotype, as assessed by BrdU NeuN double labeling ($F(1,13)=0.002$, $p=0.969$; Figures 2.6F and 7.2B). All F-statistics are represented in Table 2.1.

Discussion

Olfaction supports basic processes such as navigation, copulation, social bonding, predator avoidance and foraging behaviors. To accomplish this, the olfactory system must sense and discriminate countless potential chemical signals in the environment and rapidly adapt in order to detect and discriminate between new and highly similar odorants. Understanding the basic mechanisms that govern olfactory perceptual learning has the potential to shed light on some of the most basic yet critical learning processes supporting survival and adaptation.

Odorant enrichment has been shown to enhance discrimination ability between perceptually similar odorants that activate common glomerular regions in the OB (Mandairon et al., 2006bde, 2008). In addition, repeated odorant exposure also modifies the responsiveness of the specific main olfactory bulb primary neurons activated by the enriching odorant (Buonviso et al., 1998; Buonviso and Chaput, 2000). Therefore, it follows that changes in olfactory perceptual discrimination (olfactory perceptual learning) are associated with experience-dependent shifts in OB inhibitory tone (e.g. enhanced activation of inhibitory interneurons within regions responsive to experienced odorants) (Mandairon et al., 2008). This increase in inhibitory tone has been implicated as a critical process underlying changes in discrimination ability for odorants with overlapping activation maps, such as + and - limonene.

New neurons are born during adulthood in the subventricular zone (SVZ) of the lateral ventricles, where OB interneurons arise (Altman, 1969). In olfactory neurogenesis, newly

born cells integrate into preexisting OB circuits and differentiate into periglomerular and granule cells, inhibitory interneurons that support olfactory perceptual learning (Moreno et al., 2009). Since limonene enrichment causes olfactory perceptual learning between + and – limonene as well as an increase in newly born OB granule cells, it is possible that these newly born cells are necessary for implicit olfactory learning.

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Figures and Table

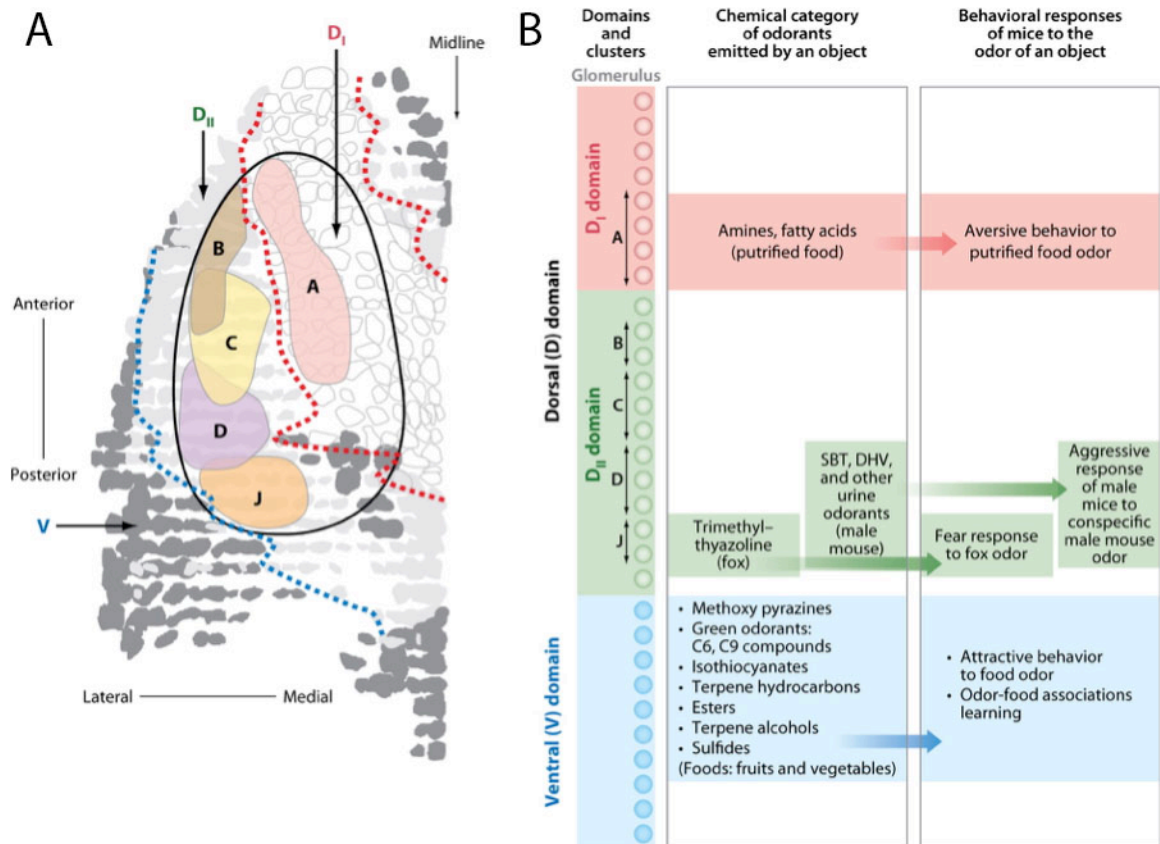


Figure 2.1. Odorants with common molecular features activate similar olfactory bulb regions. A) In this schematic of a left olfactory bulb, A, B, C, D and J represent clusters of glomeruli activated by odorants with similar molecular features. This bulb is divided into three sections, two dorsal (D_I and D_{II}), and one ventral (V), each separated by a red dotted line and with the glomeruli in each region drawn as a differently shaded circle (no shading, light shading, heavy shading, respectively). B) Information about odorants mapping to each individual domain. DHB = dehydro-exo-brevicomine, SBT = 2-sec-butyl-dihydrothiazole. *Figure adapted from Mori and Sakano, 2011.*

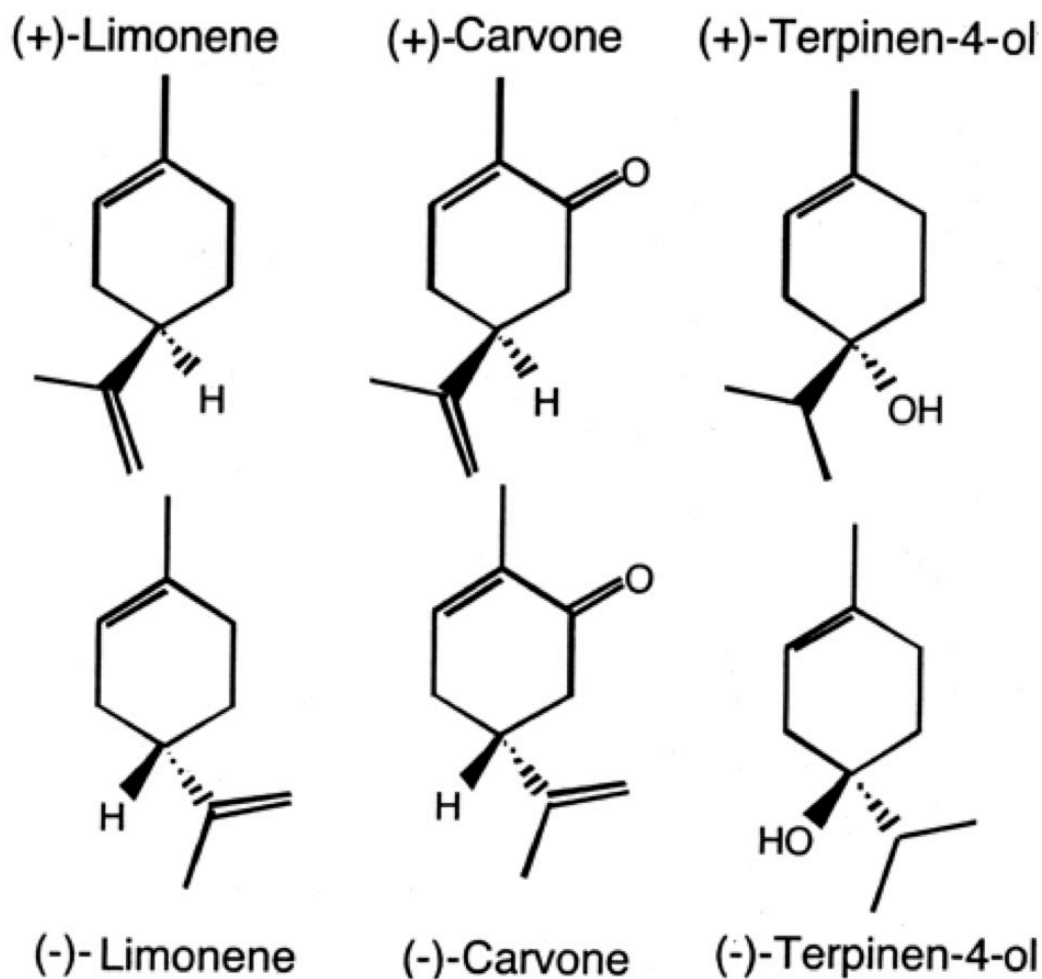


Figure 2.2. Chemical structures of odorant pairs used throughout the dissertation. We used three enantiomer pairs in the olfactory perceptual learning task: + and – limonene, + and – carvone, + and – terpinen-4-ol. *Figure adapted from Linster et al., 2001.*

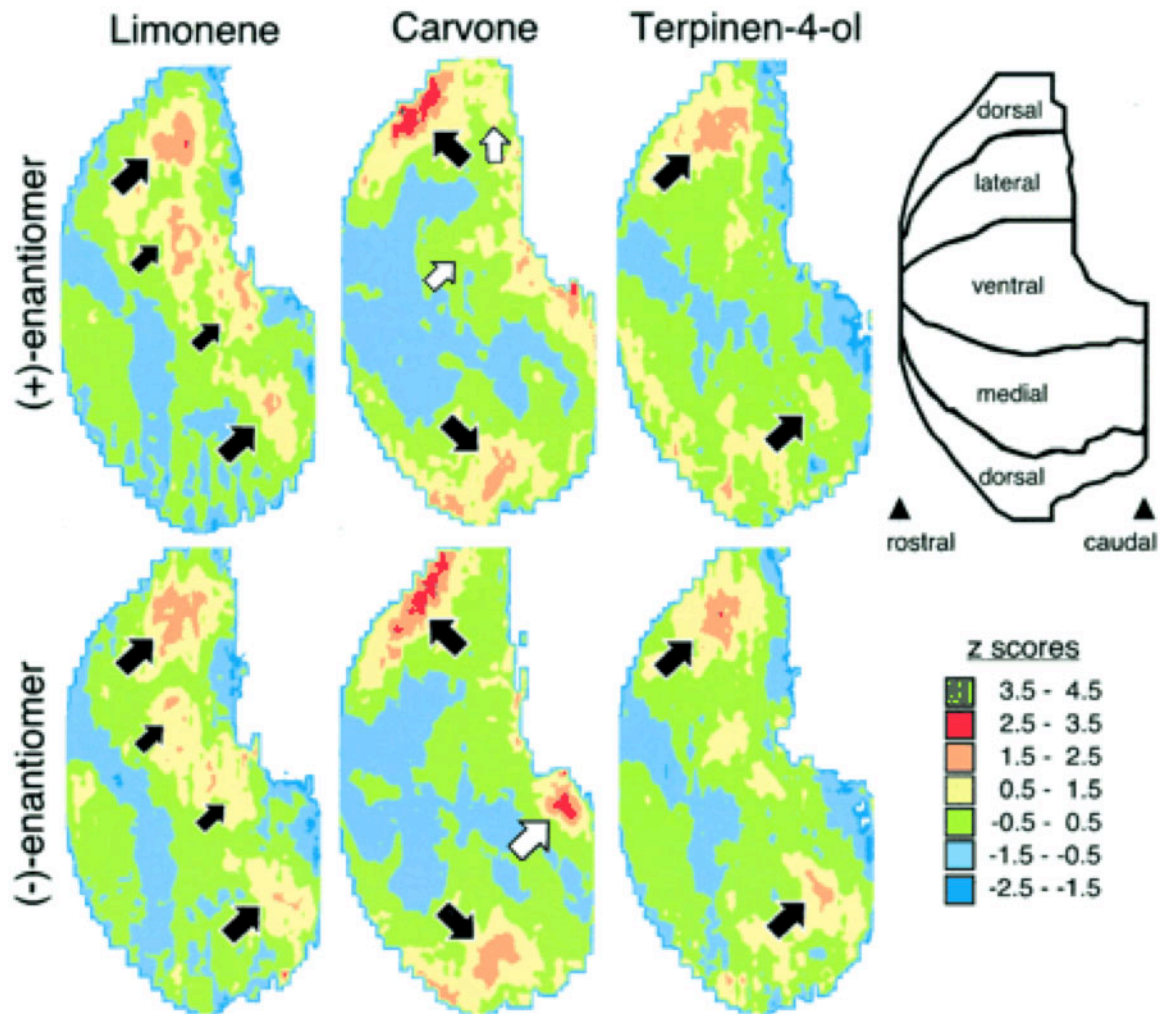


Figure 2.3. Bulbar activation from odorant pairs used throughout the dissertation. Using [^{14}C]2-deoxyglucose (2-DG) uptake, glomerular activity was assessed in the six odorants used throughout the olfactory perceptual learning task. After deriving z-scores, a heat map was created, where glomerular responses can be assessed from lowest (blue) to highest (green) activity. Black arrows indicate areas of high activity where each odorant within an odorant pair substantially overlaps. White arrow indicate activation regions that differ between + and – carvone. *Figure adapted from Linster et al., 2001.*

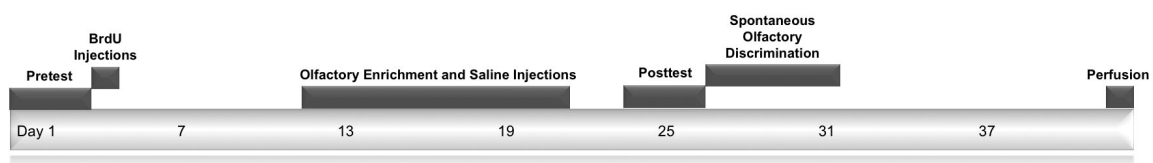


Figure 2.4. Olfactory enrichment experiment timeline.

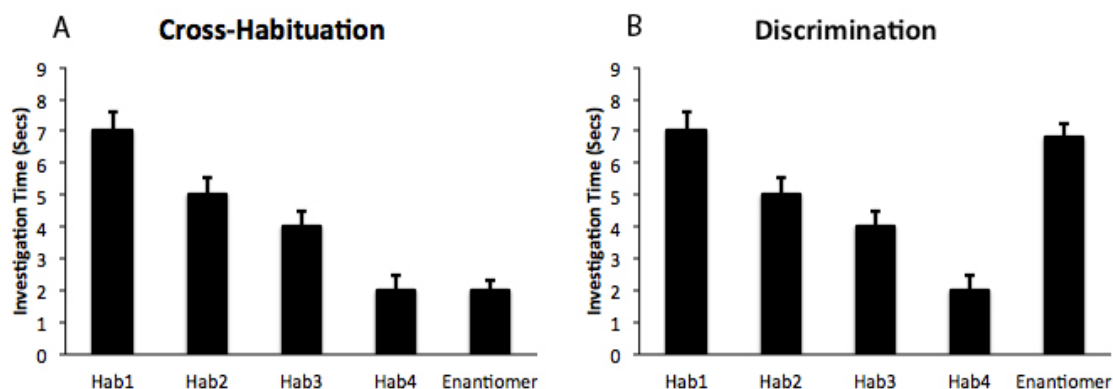


Figure 2.5. Example habituation, cross-habituation and discrimination graphs. A) If investigation time significantly decreases between the first habituation trial (Hab1) and the fourth (Hab4), this indicates that the mice habituated to the habituation odorant, (the + form of the odorant pair tested). If investigation time stays low for the enantiomer trial (i.e. investigation time for the enantiomer trial is not significantly higher than Hab4), this indicates cross-habituation. In this case, mice cannot perceptually distinguish the + and – form of the odorant pair tested. B) If investigation time significantly increases between Hab4 and the enantiomer trial, this suggests that mice perceive the enantiomer as novel, and are perceptually able to distinguish the odorants within the tested odorant pair. Hab2 = habituation trial 2, Hab3 = habituation trial 3.

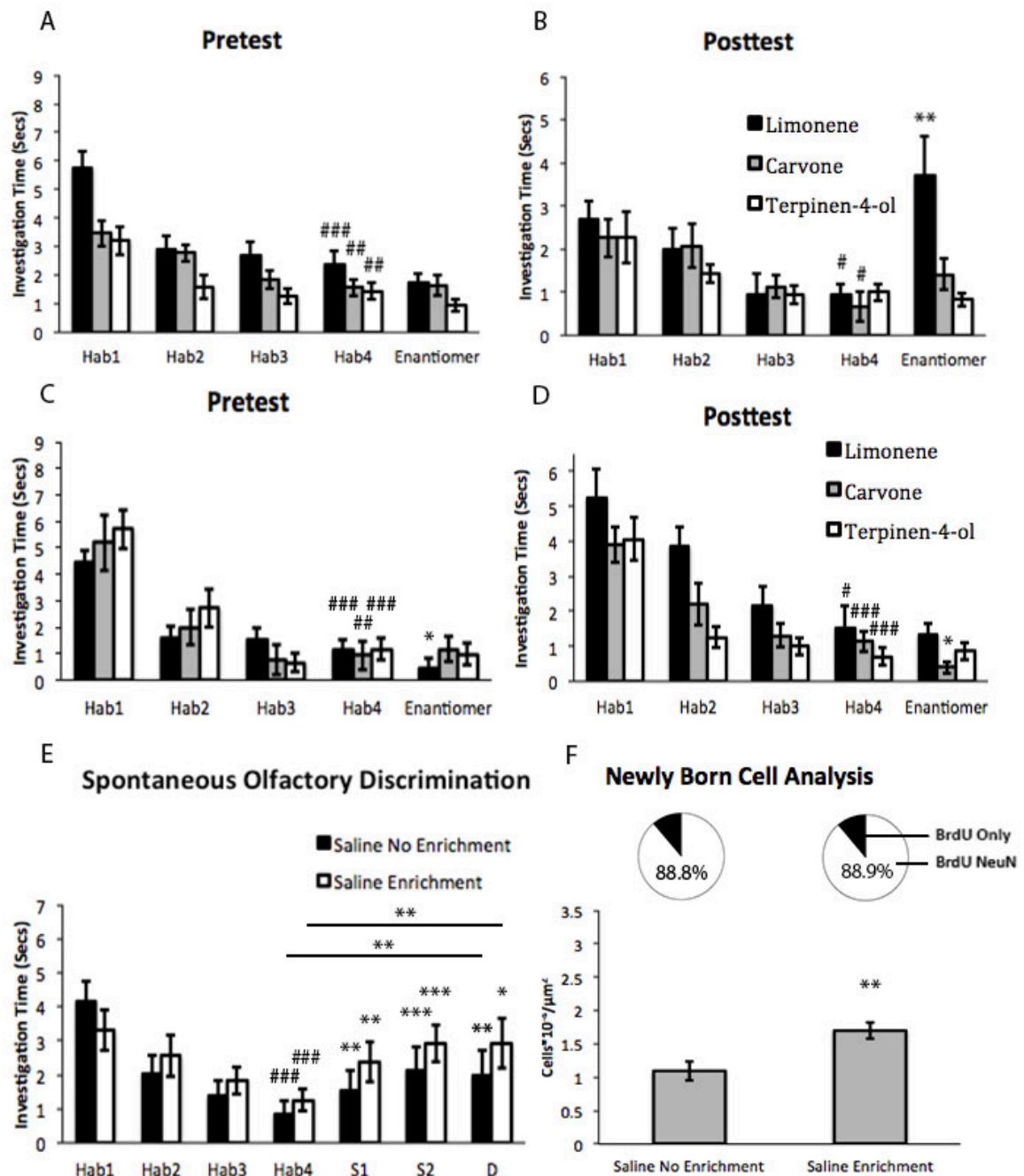


Figure 2.6. Mice learn to distinguish similar odorants after olfactory enrichment to those odorants. A) Mice cannot distinguish limonene, carvone nor terpinen-4-ol enantiomer pairs prior to olfactory enrichment. B) Mice showed evidence of +/- limonene discrimination ($F(1,11)=9.750$, $p=0.010^{**}$), but not +/- carvone nor +/- terpinen-4-ol after olfactory enrichment. C) Mice cannot distinguish any tested enantiomer pairs in the original exposure or D) 21 days later (in the absence of olfactory enrichment). E) Saline-

injected mice show intact habituation and discrimination between the habituation odorant (Hab4), and three novel odorants: S1 (moderately similar odorant), S2 (slightly similar odorant), and D (structurally and perceptually different odorant). Upon further analysis of the Hab4 pairwise comparisons to S1, S2 and D, all three tests were significant, even after Bonferroni corrections. F) Enriched mice had significantly more BrdU positive cells than non-enriched mice ($F(1,13)=10.425$, $p=0.007^{**}$), although both groups had the same percent of newly born neurons. Data are represented as mean $\pm$ SEM. Hab1 – 4 = habituation trials 1 – 4; $^{\#}p\leq 0.05$, $^{##}p\leq 0.01$, $^{###}p\leq 0.001$ statistically significant habituation between Hab1 and Hab4; $^{*}p\leq 0.05$, $^{**}p\leq 0.01$, $^{***}p\leq 0.001$ statistically significant discrimination between Hab4 and Enantiomer, S1, S2 or D. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen. See Table 2.1 for all F-statistics.

Table 2.1. Olfactory enrichment statistics.

Group	Task	Behavior	Odorant Pair	Statistics
Saline Enrichment	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,10)=25.498, p≤0.001 ^{###}
			Carvone	F(1,11)=10.248, p=0.008 ^{##}
			Terpinen-4-ol	F(1,10)=12.882, p=0.005 ^{##}
		Discrimination	Limonene	F(1,10)=0.347, p=0.569
			Carvone	F(1,11)=0.045, p=0.837
			Terpinen-4-ol	F(1,11)=1.511, p=0.245
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,11)=8.668, p=0.013 [#]
			Carvone	F(1,11)=9.317, p=0.011 [#]
			Terpinen-4-ol	F(1,11)=4.474, p=0.058
		Discrimination	Limonene	F(1,11)=9.750, p=0.010 ^{**}
			Carvone	F(1,11)=3.557, p=0.086
			Terpinen-4-ol	F(1,11)=0.621, p=0.447
	Spontaneous Olfactory Discrimination	Habituation		F(1,11)=28.897, p≤0.001 ^{###}
		Discrimination		F(3,9)=8.302, p=0.006 ^{**}
		Hab4 – S1		F(1,11)=13.642, p=0.004 ^{**}
		Hab4 – S2		F(1,11)=28.081, p≤0.001 ^{***}
		Hab4 – D		F(1,11)=14.626, p=0.003 ^{**}
Saline No Enrichment	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,11)=46.959, p≤0.001 ^{###}
			Carvone	F(1,10)=12.618, p=0.005 ^{##}
			Terpinen-4-ol	F(1,9)=22.343, p=0.001 ^{###}
		Discrimination	Limonene	F(1,10)=7.863, p=0.019 [*]
			Carvone	F(1,10)=0.927, p=0.358
			Terpinen-4-ol	F(1,10)=0.360, p=0.562
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,10)=9.565, p=0.011 [#]
			Carvone	F(1,10)=35.746, p≤0.001 ^{###}
			Terpinen-4-ol	F(1,10)=22.405, p=0.001 ^{###}
		Discrimination	Limonene	F(1,10)=.451, p=0.517
			Carvone	F(1,10)=7.087, p=0.024 [*]
			Terpinen-4-ol	F(1,10)=0.708, p=0.420
	Spontaneous Olfactory Discrimination	Habituation		F(1,11)=88.293, p≤0.001 ^{###}
		Discrimination		F(3,9)=7.886, p=0.007 ^{**}
		Hab4 – S1		F(1,11)=11.327, p=0.006 ^{**}
		Hab4 – S2		F(1,11)=23.084, p=0.001 ^{***}
		Hab4 – D		F(1,11)=8.429, p=0.014 [*]
Both	BrdU			F(1,13)=10.425, p=0.007 ^{**}
	BrdU NeuN			F(1,13)=0.002, p=0.969

[#]p≤0.05, ^{##}p≤0.01, ^{###}p≤0.001 statistically significant habituation between Hab1 and Hab4 (habituation trials 1 and 4); ^{*}p≤0.05, ^{**}p≤0.01, ^{***}p≤0.001 statistically significant discrimination between Hab4 and Enantiomer, S1 (moderately similar), S2 (slightly similar), or D (structurally and perceptually different) odorants. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen

CHAPTER 3: OLFACTORY ENRICHMENT INDUCES A LONG-TERM OLFACTORY MEMORY

Introduction

In the previous chapter, we confirmed that mice successfully distinguish odorants within an odorant pair after 10 days of olfactory enrichment to those odorants. Therefore, we naturally wondered how long that olfactory memory lasts.

Although olfactory memory can be measured behaviorally, it can also be examined by other methods. Odorant exposure has been shown to modify the responsiveness of neurons in the olfactory bulb (OB) via immediate early gene expression, electrophysiological recording and [^{14}C] 2-deoxyglucose uptake, just to name a few. These changes in responsiveness correlate with olfactory perception (Mandairon et al., 2008). There have been several studies that have examined “long-term” olfactory memory retention, although most of these studies utilize associative learning tasks, measure olfactory memory only a few days after training concludes, or both (Freeman and Schneider, 1982; Wilson et al., 1985; Baba et al., 1997; Buonviso et al., 1998; Wilson and Leon, 1988; Buonviso and Chaput, 2000; Busto et al., 2009; Kermen et al., 2010; Sultan et al., 2010). In this chapter, we will refer to long-term memory as the olfactory memory trace remaining after 90 days, as we found discrimination learning at this time point, and no other studies have examined long-term olfactory memory after this period of time.

Although both passive (untrained) and active (trained) tasks can measure some degree of odorant similarity, the results are heavily influenced by the behavioral paradigm. Passive olfactory tasks can give a clear behavioral readout of the spatial overlap in OB odorant-evoked activity patterns (Johnson et al., 1999), a measure that can be artificially altered through the overtraining and activation of learning, memory and reward systems elicited by active tasks (Cleland et al., 2002). In addition, passive learning should be just as effective as active learning since odorant enrichment appears to shape odor perception independently of motivation (Escanilla et al., 2008). Importantly, both forms of learning modulate mitral cell responsiveness (Buonviso et al., 1998; Doucette and Restrepo, 2008). With passive learning, we can explore discrimination abilities from a more natural approach than active learning tasks, without demanding the extra resources required for a training period. We are able to better represent the optimal functioning of the system without worry that we are measuring reward-motivated behaviors (Cleland et al., 2002).

Therefore, we employed our olfactory perceptual learning task to determine how long an olfactory memory is retained after passive olfactory enrichment. As far as we are aware, only one other study has examined olfactory memory months after learning. However, in that study, testing followed an associative learning task (Sultan et al., 2010). The present experiment will test for olfactory memory retention until the memory is forgotten.

Methods

For more information on the methods used in Chapter 3 experiments, see “Subjects,” “Olfactory Cross-Habituation Tasks” and “Statistical Analysis” in Chapter 7.

Olfactory Enrichment

See “Olfactory Enrichment” in Chapter 2 methods, disregarding the saline injection step. For an overview of all manipulations, see “Olfactory Manipulation” in Chapter 7.

Olfactory Perceptual Learning

See “Olfactory Perceptual Learning Overview” in Chapter 2 methods. For detailed methods, see “Olfactory Perceptual Learning” in Chapter 7.

Timeline

In order to determine how long mice remember odorant discrimination after olfactory perceptual learning, we repeated our olfactory perceptual learning paradigm. Mice were tested on their ability to discriminate + limonene and – limonene (as well as the other two odorant pairs) in a pretest, and reassessed after 10 days of limonene olfactory enrichment. Limonene posttests were administered three days after olfactory enrichment ended (normal timeline, immediate posttest), as well as one month, three months and six months after the immediate posttest. Carvone and terpinen-4-ol posttests were also conducted, but only in the immediate posttest (Figure 3.1). For an overview of all timelines, see “Experiment Timelines” in Chapter 7.

Results

As predicted by Chapter 2, when we repeated our olfactory perceptual learning pretest paradigm, mice could not discriminate within any of the three tested enantiomer pairs (Limonene - $F(1,10)=3.552$, $p=0.089$; Carvone - $F(1,10)=0.686$, $p=0.427$; Terpinen-4-ol - $F(1,11)=1.511$, $p=0.245$; Figure 3.2A and Table 7.1). Similarly, after 10 days of + and – limonene enrichment and a three-day waiting period, mice learned to discriminate within the + and – limonene enantiomer pair, but not within the other two sets of enantiomers (Limonene - $F(1,11)=10.078$, $p=0.009^{**}$; Carvone - $F(1,11)=1.309$, $p=0.277$; Terpinen-4-ol - $F(1,11)=0.621$, $p=0.447$; Figure 3.2B). For this chapter, we will term this posttest “immediate posttest,” as several other posttests were performed at later time points. After the immediate posttest, we no longer tested the carvone nor the terpinen-4-ol odorant pairs, as no olfactory discrimination occurred within these pairs. In order to determine how long mice remembered +/- limonene discrimination, we retested our mice 30 days after the immediate posttest (one month posttest) and every 90 days after the immediate posttest (three and six month posttests) until the olfactory memory was no longer present. Interestingly, mice were able to discriminate + and – limonene one month and three months after the immediate posttest, although this memory was non-existent at the six month time point (One month – $F(1,10)=6.730$, $p=0.027^{*}$; Three month – $F(1,10)=11.932$, $p=0.006^{**}$; Six month - $F(1,9)=0.027$, $p=0.873$; Figure 3.2C). All F-statistics are represented in Table 3.1.

Discussion

In this chapter, we have displayed the first evidence for a passive olfactory task evoking a memory that lasts for several months. However, it is unknown how this memory is formed and retained for long periods of time: by adult neurogenesis, long-term potentiation (LTP), neuromodulation, or a combination of these processes.

Adult Neurogenesis

In 2002, Rochefort et al. determined that olfactory enrichment not only improves olfactory memory, but also increases the number of OB newly born cells. Several other studies have pointed to this association as well (Alonso et al., 2006; Mandaïron et al., 2006a; Lazarini et al., 2009; Sultan et al., 2010). In fact, Moreno et al. (2009) found that adult neurogenesis is required for olfactory perceptual learning. The survival of newly born cells is dependent on olfactory exposure 15 to 45 days after birth (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002; Yamaguchi and Mori, 2005; Mandaïron et al., 2006b), and these newly born cells must be present during memory acquisition for a long-term olfactory memory trace to exist (Sultan et al., 2010). Furthermore, their survival as well as olfactory memory is dependent upon memory consolidation (Kermen et al., 2010). Therefore, it is likely that adult neurogenesis contributes to long-term olfactory memory, although future studies using bromodeoxyuridine (BrdU) and a mitotic blocker during olfactory enrichment would shed more light on this mechanism (see Chapter 6 for a similar experiment using optogenetics instead of olfactory enrichment).

Long-term Potentiation

In addition to adult neurogenesis, LTP may be important for the long-term olfactory memory examined in this study. In 2006c, Mandaïron et al. demonstrated that daily OB infusions of N-methyl-D-aspartate (NMDA) enhanced odorant discrimination, while rats injected with the NMDA antagonist, MK-801 showed learning deficits. NMDA is important for the reciprocal dendrodendritic interactions between mitral and granule cells (Wilson, 1995; Elaagouby and Gervais, 1996; Isaacson and Strowbridge, 1998; Schoppa et al., 1998). As newly born inhibitory interneurons become integrated into the existing bulbar network, synaptic plasticity may work in conjunction with adult neurogenesis, especially for memory acquisition (Staubli et al., 1989; Kermen et al., 2010).

Neuromodulation

The OB and cortex receive dense innervation from several neuromodulatory systems. In particular, cholinergic modulation has been shown to modulate OB neuron responsiveness and influence olfactory behavior (Elaagouby et al., 1991; Elaagouby and Gervais, 1992; Castillo et al., 1999; Linster and Cleland, 2002; Mandaïron et al., 2006d; Chaudhury et al., 2009; Ma and Luo, 2012). In recent collaborative work, Moreno et al. (2012) determined that noradrenergic modulation supports olfactory perceptual learning through adult neurogenesis. Although we will pose a similar question with respect to acetylcholine in future chapters, we have not explored the role of neuromodulatory systems on long-term olfactory memory. Future work exploring adult neurogenesis, synaptic plasticity and neuromodulation as potential mechanisms underlying long-term olfactory memory could provide insight into this phenomenon.

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Figures and Table

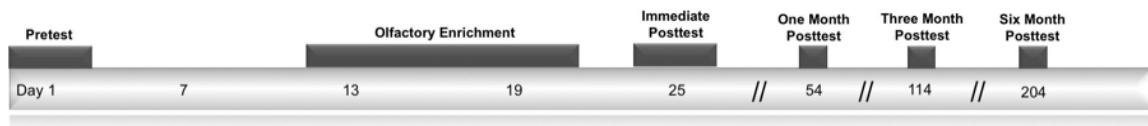


Figure 3.1. Long-term experiment timeline. // = time break.

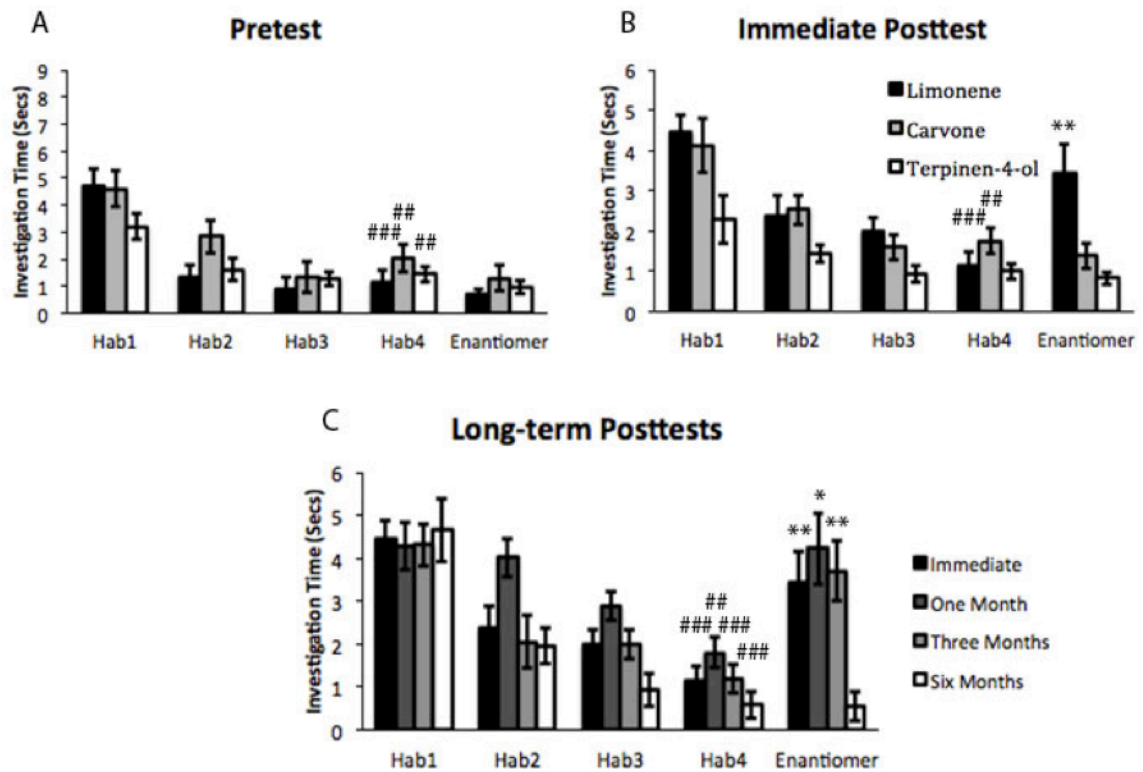


Figure 3.2. Mice can perceptually distinguish + and – limonene at a three-month posttest, but not at six months. A) Mice cannot distinguish limonene, carvone nor terpinen-4-ol enantiomer pairs prior to olfactory enrichment. B) Mice were able to show evidence of +/- limonene discrimination ($F(1,11)=10.078$, $p=0.009^{**}$) in the immediate posttest, but not +/- carvone nor +/- terpinen-4-ol. C) Mice can distinguish + and – limonene in the immediate posttest ($F(1,11)=10.078$, $p=0.009^{**}$), one-month posttest ($F(1,10)=6.730$, $p=0.027^{*}$) and three-month posttest ($F(1,10)=11.932$, $p=0.006^{**}$), but not the six-month posttest ($F(1,9)=0.027$, $p=0.873$). Data are represented as mean $\pm$ SEM. Hab1 – 4 = habituation trials 1 – 4; # $p\leq 0.05$, ## $p\leq 0.01$, ### $p\leq 0.001$ statistically significant habituation between Hab1 and Hab4; * $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$ statistically significant discrimination between Hab4 and Enantiomer. See Table 3.1 for all F-statistics.

Table 3.1. Long-term olfactory perceptual learning statistics.

Group	Task	Behavior	Odorant Pair	Statistics
Long-term Enrichment	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,9)=30.937, p≤0.001 ^{###}
			Carvone	F(1,11)=11.310, p=0.006 ^{##}
			Terpinen-4-ol	F(1,10)=12.882, p=0.005 ^{##}
		Discrimination	Limonene	F(1,10)=3.552, p=0.089
			Carvone	F(1,10)=0.686, p=0.427
			Terpinen-4-ol	F(1,11)=1.511, p=0.245
	Immediate Posttest	Habituation	Limonene	F(1,11)=28.658, p≤0.001 ^{###}
			Carvone	F(1,11)=15.130, p=0.003 ^{##}
			Terpinen-4-ol	F(1,11)=4.474, p=0.058
		Discrimination	Limonene	F(1,11)=10.078, p=0.009 ^{**}
			Carvone	F(1,11)=1.309, p=0.277
			Terpinen-4-ol	F(1,11)=0.621, p=0.447
	One Month Posttest	Habituation	Limonene	F(1,10)=14.720, p=0.003 ^{##}
		Discrimination	Limonene	F(1,10)=6.730, p=0.027 [*]
	Three Month Posttest	Habituation	Limonene	F(1,10)=50.650, p≤0.001 ^{###}
		Discrimination	Limonene	F(1,10)=11.932, p=0.006 ^{**}
	Six Month Posttest	Habituation	Limonene	F(1,10)=50.312, p≤0.001 ^{###}
		Discrimination	Limonene	F(1,9)=0.027, p=0.873

[#]p≤0.05, ^{##}p≤0.01, ^{###}p≤0.001 statistically significant habituation between Hab1 and Hab4 (habituation trials 1 and 4); ^{*}p≤0.05, ^{**}p≤0.01, ^{***}p≤0.001 statistically significant discrimination between Hab4 and Enantiomer.

CHAPTER 4: PHARMACOLOGICAL BLOCKADE OF SELECT CHOLINERGIC RECEPTORS INHIBITS OLFACTORY PERCEPTUAL LEARNING

Introduction

Odorant discrimination is accomplished through multiple processes, including the activity of cholinergic afferents to the olfactory bulb (OB). Cholinergic inputs from the basal forebrain project via the nucleus of the horizontal limb of the diagonal band of Broca (HDB) to the OB, where they innervate primarily the glomerular (GL) and granule cell layers (GCL) (Shepherd et al., 2004). Cholinergic activity is thought to sharpen mitral cell olfactory tuning by expanding the responsiveness of granule cells while enhancing periglomerular inhibition of mitral cells (Heimer et al., 1990; Castillo et al., 1999; Linster and Cleland, 2002; Chaudhury et al., 2009; Ma and Luo, 2012). This makes mitral cells less sensitive to low affinity odorants while retaining sensitivity to higher affinity chemicals (Elaagouby and Gervais, 1992; Mandaïron et al., 2006a; Tsuno et al., 2008; D'Souza and Vijayaraghavan, 2012). Since mitral cells are more tightly activated by higher affinity odorants, this increases the separation in spatial coding at the level of the OB and improves spontaneous odorant discrimination (Chaudhury et al., 2009). Support for cholinergic modulation of olfactory discrimination has also come from recent modeling and *in vivo* studies, in which increasing cholinergic activity at the level of the glomeruli via nicotinic acetylcholine receptors enhances receptive field contrast and increases the selectivity of mitral cell firing (Linster and Hasselmo, 1997; Cleland and

Sethupathy, 2006; Mandaïron et al., 2006a; Chaudhury et al., 2009; Linster and Cleland, 2009). Some have also argued that cholinergic inputs alter the synchronization of firing via muscarinic acetylcholine receptors, and that this process serves to regulate olfactory discrimination and learning (Stopfer et al., 1997; Nusser et al., 2001; Pressler et al., 2007; Cleland and Linster, 2012; Li and Cleland, 2013).

Although cholinergic modulation has been shown to affect odorant discrimination, no studies have specifically examined the role of acetylcholine in olfactory perceptual learning. These studies either use associative learning (classical conditioning, reward or punishment), altered cholinergic activity during the olfactory discrimination task, or both (Linster et al., 2001; Fletcher and Wilson, 2002; Mandaïron et al., 2006a; Chaudhury, et al., 2009). Our study is the first to use an implicit learning task and only alter cholinergic modulation during an olfactory manipulation period (in this chapter, olfactory enrichment), not during the posttest. To determine if the activity of cholinergic receptors during olfactory enrichment is necessary for olfactory perceptual learning, mice were administered the anticholinergic agent Benztropine Mesylate (Cogentin) prior to each olfactory enrichment session. In a posttest, we then assessed their ability to discriminate within enantiomer pairs.

Methods

For more information on the methods used in Chapter 4 experiments, see “Subjects,” “Olfactory Cross-Habituation Tasks” and “Statistical Analysis” in Chapter 7.

Cogentin

Cogentin, a drug that combines the tropine portion of atropine and the benzohydryl portion of diphenhydramine, was first synthesized (and patented) by Phillips in 1952. Since then, it has been used in the treatment of Parkinson’s disease. Cogentin is a selective M1 muscarinic acetylcholine receptor antagonist, an effect largely restricted to the central nervous system (Freedman et al., 1988; Bolden et al., 1992). It also serves as a dopamine reuptake inhibitor (Farnebo et al., 1970).

To determine if the activity of cholinergic receptors during olfactory enrichment is necessary for olfactory perceptual learning, mice were administered Cogentin prior to each enrichment session.

Although Cogentin has a lethal dose (LD₅₀) of 65 mg/kg when administered IP in mice, the onset and half-life of this drug have not been determined for this species and injection location (Haley et al., 1963). However, as a comparison from the human literature, Cogentin acts within 15 minutes of intramuscular (IM) or intravenous (IV) administration and has a half-life of four to eight hours (Aschenbrenner and Venable, 2009).

Olfactory Enrichment

Mice were injected with Cogentin (25 mg/kg in saline; Enzo Life Sciences, Farmingdale, NY) intraperitoneally (IP; BD Insulin Syringe with the BD Ultra-fine needle, 0.3 cc, 30 gauge, ½", Franklin Lakes, NJ) 20 minutes before each enrichment session. During each session, mice were exposed to undiluted + and - limonene. Two tea balls were draped on opposite sides of the home cage for one hour, one containing filter paper with 100 µl + limonene, the other with 100 µl – limonene (Mandairon et al., 2006b). Enrichment lasted for one hour per day on ten consecutive days. For an overview of all manipulations, see “Olfactory Manipulation” in Chapter 7.

Olfactory Perceptual Learning

See “Olfactory Perceptual Learning Overview” in Chapter 2 methods. For detailed methods, see “Olfactory Perceptual Learning” in Chapter 7.

Spontaneous Olfactory Discrimination

See “Spontaneous Olfactory Discrimination Overview” in Chapter 2 methods. For detailed methods, see “Spontaneous Olfactory Discrimination” in Chapter 7.

Bromodeoxyuridine

See “Bromodeoxyuridine Overview” in Chapter 2 methods. For detailed methods, see “Bromodeoxyuridine Injections,” “Perfusion and Histology Preparation” and “Immunohistochemistry” in Chapter 7.

Timeline

At the beginning of the experiment, mice were tested on their ability to behaviorally discriminate within three enantiomer pairs. After three pretest days, mice were injected with bromodeoxyuridine (BrdU) to label newly born cells. Olfactory enrichment and Cogentin (or saline as a control, Chapter 2) administration began eight days later. Olfactory enrichment lasted for ten days, followed by a three-day washout period to eliminate Cogentin prior to the posttest. Finally, we confirmed that no widespread olfactory damage had occurred as a result of the Cogentin injections by completing a five-day spontaneous olfactory discrimination task. Mice were sacrificed 39 days after the BrdU injection for histology (Figure 4.1). For an overview of all timelines, see “Experiment Timelines” in Chapter 7.

Results

Mice treated with Cogentin during limonene enrichment did not differ from control-treated mice (Figure 2.6A) in their habituation nor initial investigation to odorants (Habituation: Limonene - $F(1,16)=8.715$, $p=0.009^{##}$; Carvone - $F(1,15)=20.422$, $p\leq 0.001^{###}$; Terpinen-4-ol - $F(1,11)=19.265$, $p=0.001^{###}$; Discrimination: Limonene - $F(1,15)=2.118$, $p=0.166$; Carvone - $F(1,15)=0.000$, $p=0.990$; Terpinen-4-ol - $F(1,11)=0.239$, $p=0.634$; Figure 4.2A). However, at posttest, Cogentin-treated mice failed to discriminate between the + and – limonene enantiomers ($F(1,12)=0.588$, $p=0.458$; Figure 4.2B). To ensure that impairments in posttest performance were not due to general effects of Cogentin on olfactory sensory function, we employed another olfactory habituation task, spontaneous olfactory discrimination following the posttest (Cleland et al., 2002; Bath et al., 2008; See Chapter 7). Using this task, we demonstrated that mice previously treated with Cogentin showed normal olfactory discrimination ability, suggesting that impairments in perceptual learning were not due to general deficits in olfactory sensory function ($F(3,10)=5.482$, $p=0.017^*$; Figure 4.2C).

We again collected the brains of mice following all behavioral experiments and tested for the effects of Cogentin treatment on the survival of newly born cells in the OB. Cogentin-treated mice showed a reduction in the density of BrdU positive cell compared to saline-injected control animals receiving enrichment ($F(1,11)=14.987$, $p=0.003^{**}$; Figure 4.2D). There was no effect of treatment on the percentage of cells committing to a neural phenotype ($F(1,12)=0.557$, $p=0.470$; Figure 4.2D). All F-statistics are represented in Table 4.1.

Discussion

Although manipulating cholinergic activity has previously been shown to modulate odorant discrimination, we conducted a novel investigation on the role of acetylcholine in an implicit learning task called olfactory perceptual learning (Linster et al., 2001; Fletcher and Wilson, 2002; Mandairon et al., 2006a; Chaudhury, et al., 2009). We used the anticholinergic agent, Cogentin to antagonize cholinergic receptors during olfactory enrichment and assessed olfactory perceptual learning and discrimination.

Given the lack of limonene discrimination learning Cogentin-treated mice displayed after 10 days of olfactory enrichment (in comparison to saline-treated mice, Figure 2.6B), it appears that cholinergic modulation is necessary for olfactory perceptual learning. However, we cannot conclude that this is due specifically to cholinergic modulation within the OB. Since Cogentin non-specifically targets M1 acetylcholine receptors, it is possible that our result could in part be due to its dual role as a dopamine reuptake inhibitor. Therefore, in the next chapter, we will use optogenetics to specifically target HDB cholinergic fibers.

When examining the neural basis of olfactory perceptual learning, acetylcholine is a good candidate as it can affect perceptual learning through multiple mechanisms, including long-term potentiation (LTP), influences on the sensitivity of mitral cells, or adult neurogenesis (Cooper-Kuhn et al., 2004; Mechawar et al., 2004; Kaneko et al., 2006; Whitman and Greer, 2007; Sharma, 2013; Paez-Gonzalez et al., 2014). In fact, within seconds of odorant investigation, neurons in the HDB have altered firing rates (Devore et

al., 2016). HDB connections to the bulb have been shown to be critical for the acquisition, but not recall in olfactory discrimination tasks, possibly through the modulation of sustained attention (Himmelheber et al., 2000; Sarter et al., 2005; Chapuis and Wilson, 2013; Devore et al., 2014).

Granule and periglomerular inhibitory interneurons are continually produced at high rates throughout life (Altman and Das, 1965; Altman, 1969), and have been implicated in olfactory sensory function (Bath et al., 2008; Moreno et al., 2009; Moreno et al., 2012, Gheusi and Lledo, 2014) and plasticity (Mouret et al., 2008; Moreno et al., 2009; Nissant et al., 2009; Alonso et al., 2012). These cells support olfactory tuning via lateral inhibition (Mori et al., 1999; Restrepo et al., 2009; Tan et al., 2010). From our results, it is clear that Cogentin decreases the number of OB newly born granule cells after olfactory enrichment compared to saline-treated mice. However, we cannot conclude that this decrease in newly born neurons accounts for the lack of limonene olfactory discrimination. It is possible that antagonizing cholinergic receptors blocks learning due to the location of acetylcholine receptors on preexisting inhibitory interneurons.

There are two main classes of acetylcholine receptors: nicotinic and muscarinic. Nicotinic acetylcholine receptors are composed of five subunits. These ligand-gated ion channels are either homomeric or heteromeric; neuronal nicotinic acetylcholine receptors can be made from combinations of the following receptor subunits: $\alpha 2 - \alpha 7$, $\alpha 9$, $\alpha 10$, and $\beta 2 - \beta 4$ (McGehee and Role, 1995; McKay et al., 2007; Zoli et al., 2014; Fasoli and Gotti, 2015). Muscarinic acetylcholine receptors are metabotropic G protein-coupled receptors

(GPCRs) that come in five subtypes: M1 – M5 (Dale, 1914; Kubo et al., 1986; Bonner et al., 1987; Buckley et al., 1988). The M1, M2 and M4 subtypes are the main muscarinic acetylcholine receptors expressed in the brain (especially M1 and M2), while M3 and M5 are present, but at a much lower level (Levey et al., 1991).

Using quantitative *in vitro* autoradiography, it was determined that M1 and M2 acetylcholine receptors are expressed throughout the bulb, with highest levels in the external plexiform layer (EPL) followed by the deeper layers. In contrast, $\alpha 4\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptors are found in the more superficial layers, such as the GL, EPL and mitral cell layer (MCL) (Hunt and Schmidt, 1978; Le Jeune et al., 1995).

Muscarinic acetylcholine receptors in the GCL modulate mitral and granule cell dendrodendritic interactions, while nicotinic acetylcholine receptors exert their effects in the GL and MCL (Le Jeune and Jourdan, 1993; Castillo et al., 1999; Ghatpande et al., 2006; Pressler et al., 2007; Tsuno et al., 2008; D'Souza and Vijayaraghavan, 2012). In Chapter 6, we will explore whether cholinergic modulation affects olfactory perceptual learning through its effect on newly born cell survival or through a separate mechanism.

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Figures and Table

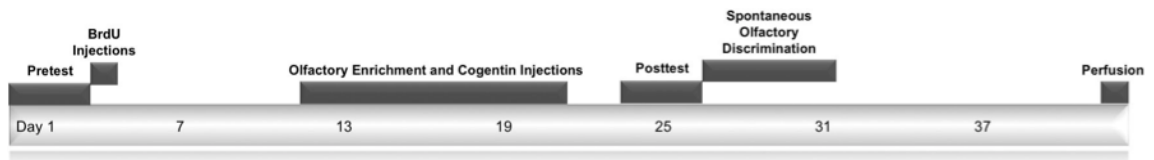


Figure 4.1. Pharmacological experiment timeline.

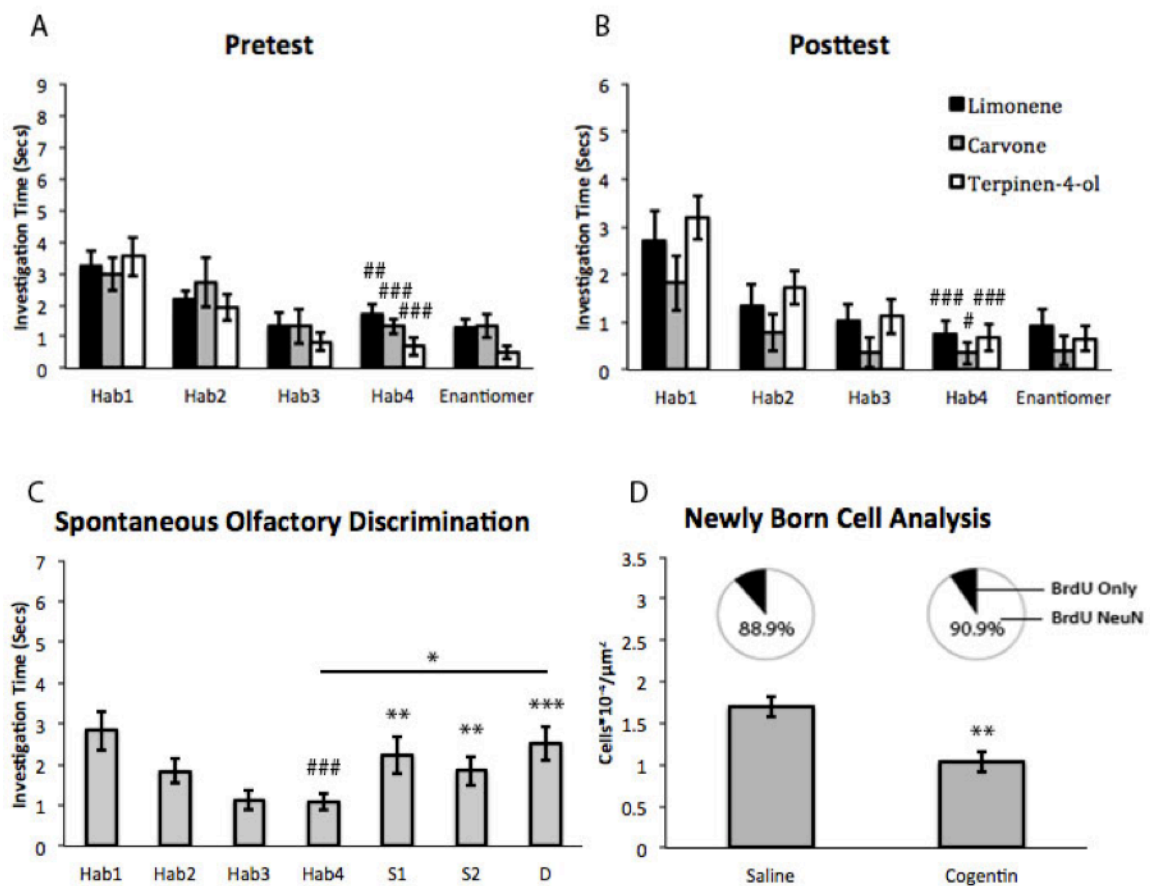


Figure 4.2. Pharmacologically blocking cholinergic receptors during olfactory enrichment inhibits olfactory perceptual learning. A) Mice randomly chosen to receive injections of the anticholinergic drug, Cogentin exhibited typical pretest data compared to mice chosen for the control saline group (Figure 2.6A). B) Mice exposed to Cogentin during a ten-day enrichment period showed normal habituation data, but no enantiomer discrimination. C) Mice injected with Cogentin can discriminate Hab4, S1, S2 and D ($F(3,10)=5.482$, $p=0.017^*$). After posthoc analysis, each Hab4 to S1, S2 or D pairwise comparison remained statistically significant. Hab1 – 4 = habituation trials 1 – 4, S1 (moderately similar odorant), S2 (slightly similar odorant), and D (structurally and perceptually different odorant); # $p\leq 0.05$, ## $p\leq 0.01$, ### $p\leq 0.001$ for statistically significant habituation between Hab1 and Hab4; * $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$ for statistically significant discrimination between Hab4 and Enantiomer, S1, S2 or D. D) Enriched Cogentin-treated mice had a statistically significant decrease in BrdU positive cell density compared to enriched saline-treated mice ($F(1,11)=14.987$, $p=0.003^{**}$). There were statistically the same percent of neurons out of the counted BrdU positive cells in saline- and Cogentin-treated mice. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen. Data are represented as mean $\pm$ SEM. See Table 4.1 for all F-statistics.

Table 4.1. Pharmacological experiment statistics.

Group	Task	Behavior	Odorant Pair	Statistics
Cogentin Enrichment	Olfactory	Habituation	Limonene	F(1,16)=8.715, p=0.009 [#]
	Perceptual		Carvone	F(1,15)=20.422, p≤0.001 ^{###}
	Learning Pretest		Terpinen-4-ol	F(1,11)=19.265, p=0.001 ^{###}
		Discrimination	Limonene	F(1,15)=2.118, p=0.166
			Carvone	F(1,15)=0.000, p=0.990
			Terpinen-4-ol	F(1,11)=0.239, p=0.634
	Olfactory	Habituation	Limonene	F(1,11)=26.072, p≤0.001 ^{###}
	Perceptual		Carvone	F(1,10)=8.590, p=0.015 [#]
	Learning Posttest		Terpinen-4-ol	F(1,11)=33.172, p≤0.001 ^{###}
		Discrimination	Limonene	F(1,12)=0.588, p=0.458
			Carvone	F(1,10)=0.454, p=0.516
			Terpinen-4-ol	F(1,9)=0.954, p=0.354
Both	Spontaneous	Habituation		F(1,12)=25.580, p≤0.001 ^{###}
	Olfactory	Discrimination		F(3,10)=5.482, p=0.017 [*]
	Discrimination	Hab4 – S1		F(1,12)=11.777, p=0.002 ^{**}
		Hab4 – S2		F(1,12)=7.378, p=0.010 ^{**}
		Hab4 – D		F(1,12)=19.150, p≤0.001 ^{***}
Both	BrdU			F(1,11)=14.987, p=0.003 ^{**}
	BrdU NeuN			F(1,12)=0.557, p=0.470

[#]p≤0.05, ^{##}p≤0.01, ^{###}p≤0.001 for statistically significant habituation between Hab1 and Hab4 (habituation trials 1 and 4); ^{*}p≤0.05, ^{**}p≤0.01, ^{***}p≤0.001 for statistically significant discrimination between Hab4 and Enantiomer, S1 (moderately similar), S2 (slightly similar), or D (structurally and perceptually different) odorants. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen. See Table 2.1 for saline-treated enriched mice behavioral statistics.

CHAPTER 5: OPTOGENETIC DRIVE OF CHOLINERGIC PROJECTIONS TO THE OLFACTORY BULB ENHANCES DISCRIMINATION OF ENANTIOMER PAIRS

Introduction

Previous work has identified a putative role for acetylcholine in the regulation of olfactory bulb (OB) neurogenesis. Preventing acetylcholine degradation via an acetylcholinesterase inhibitor has been shown to increase newly born cell density in the OB (Kaneko et al., 2006). Similarly, lesioning basal forebrain cholinergic cells decreases the density of OB newly born cells (Cooper-Kuhn et al., 2004). More recent work has found that cholinergic receptors are critical for regulating the proliferation and migration of newly born cells (Sharma, 2013; Paez-Gonzalez et al., 2014). While it is clear that acetylcholine can influence spontaneous discrimination or associative discrimination learning, the role of acetylcholine in perceptual learning is not known (Fletcher and Wilson, 2002; Chaudhury et al., 2009). In the previous chapter, we provided evidence that cholinergic modulation is necessary for olfactory perceptual learning. In this chapter, we will examine sufficiency.

Methods

For more information on the methods used in Chapter 5 experiments, see “Olfactory Cross-Habituation Tasks” and “Statistical Analysis” in Chapter 7.

Subject Overview

To determine if enhancing the activity of cholinergic projections to the OB is sufficient to improve perceptual discrimination, we utilized a ChAT ChR2 EYFP transgenic mouse model (referred to as ChR2 in this study), which selectively expresses channelrhodopsin2 in cholinergic neurons. For detailed methods, see “Subjects” in Chapter 7.

Surgery Overview

We surgically implanted an optical fiber over the nucleus of the horizontal limb of the diagonal band of Broca (HDB), which provides the primary cholinergic projections to the OB. For detailed methods, see “Surgery” in Chapter 7.

Laser Stimulation

For the olfactory manipulation period, each mouse received one hour of laser stimulation every day for 10 consecutive days (in lieu of olfactory enrichment). Stimulation was achieved by coupling a patch cord (Doric Lenses, Quebec, Canada) to a blue light diode laser (MBL-III-473, 100mW, OptoEngine, LLC, Midvale, UT). Each session began with a 10 second habituation period, followed by eight-second trains of light pulses with 23-second intervals between each train. For each train of light pulses, we randomly assigned a frequency (from 5-50 Hz, at 5 Hz intervals) using MED-PC IV (Med-Associates, Inc.,

Burlington, VT). Each pulse lasted for 15 ms (protocol adapted from Ma and Luo, 2012). For an overview of all manipulations, see “Olfactory Manipulation” in Chapter 7.

Olfactory Perceptual Learning

See “Olfactory Perceptual Learning Overview” in Chapter 2 methods. For detailed methods, see “Olfactory Perceptual Learning” in Chapter 7.

Spontaneous Olfactory Discrimination

See “Spontaneous Olfactory Discrimination Overview” in Chapter 2 methods. For detailed methods, see “Spontaneous Olfactory Discrimination” in Chapter 7.

Bromodeoxyuridine

See “Bromodeoxyuridine Overview” in Chapter 2 methods. For detailed methods, see “Bromodeoxyuridine Injections,” “Perfusion and Histology Preparation” and “Immunohistochemistry” in Chapter 7.

Timeline

Mice were implanted with an optical fiber aimed at the HDB. Seven days after surgery, mice underwent a pretest to compare the investigation time between the last habituation trial and enantiomer odorant presentation for all three enantiomer pairs. Mice were subsequently injected with bromodeoxyuridine (BrdU), followed by a 10-day laser stimulation protocol beginning eight days post-injection. A posttest was then conducted to determine if discrimination learning had occurred, followed by spontaneous olfactory

discrimination testing and perfusion (Figure 5.1). Fiber placements were later confirmed (Figure 5.4) and BrdU labeling was used to test for the potential effects of optogenetic stimulation on the density of OB BrdU positive granule cells as well as the percent of newly born cells differentiating into neurons (when the tissue was double labeled with BrdU and a neuronal nuclei marker, NeuN). For an overview of all timelines, see “Experiment Timelines” in Chapter 7.

Results

We surgically implanted ChR2 positive and negative mice with an optical fiber aimed at the HDB. At pretest, surgically-manipulated mice could not discriminate the enantiomers within any enantiomer pair (ChR2 +: Limonene - $F(1,11)=1.419$, $p=0.259$; Carvone - $F(1,11)=0.006$, $p=0.940$; Terpinen-4-ol - $F(1,10)=0.088$, $p=0.772$; ChR2 -: Limonene - $F(1,9)=2.360$, $p=0.159$; Carvone - $F(1,11)=0.896$, $p=0.364$; Terpinen-4-ol - $F(1,11)=0.009$, $p=0.925$; Figure 5.2A and 5.2C). However, following a 10-day optogenetic stimulation paradigm, ChR2 positive mice receiving light stimulation succeeded in discriminating within all enantiomer pairs tested (Limonene - $F(1,12)=14.380$, $p=0.003^{**}$; Carvone - $F(1,11)=5.187$, $p=0.044^*$; Terpinen-4-ol - $F(1,11)=18.879$, $p=0.001^{***}$; Figure 5.2B). As a control for potential effects of light presentation on performance, ChR2 negative mice did not show any benefit of light stimulation on discrimination ability, and continued to fail discrimination within all enantiomer odorant pairs at posttest (Limonene - $F(1,11)=1.220$, $p=0.293$; Carvone - $F(1,11)=3.163$, $p=0.103$; Terpinen-4-ol - $F(1,11)=1.146$, $p=0.307$; Figure 5.2D). Both ChR2 positive and negative mice displayed normal general olfactory function in a spontaneous olfactory discrimination task (ChR2 +: $F(3,10)=7.200$, $p=0.007^{**}$; ChR2 -: $F(3,9)=5.624$, $p=0.019^*$; Figure 5.2E). We confirmed there were no pretest differences between surgically and non-surgically manipulated mice (Figures 5.2AC and 5.3AC), nor effects of transgene insertion on olfactory perceptual learning and general olfactory function by testing a separate batch of ChR2 positive and negative mice on our olfactory habituation tasks before and after limonene enrichment [ChR2 + (Posttest: Limonene - $F(1,13)=4.724$, $p=0.049^*$; Carvone - $F(1,12)=0.074$, $p=0.791$; Terpinen-4-ol -

$F(1,13)=0.105$, $p=0.751$; Spontaneous Olfactory Discrimination: $F(3,11)=5.987$, $p=0.011^*$; ChR2 – (Posttest: Limonene - $F(1,10)=12.393$, $p=0.006^{**}$; Carvone - $F(1,10)=1.480$, $p=0.252$; $F(1,11)=0.526$, $p=0.484$; Spontaneous Olfactory Discrimination: $F(3,9)=16.214$, $p=0.001^{***}$); Figure 5.3].

Following behavioral testing, mice were sacrificed, fiber placements were confirmed (Figure 5.4) and BrdU labeling was used to test for the potential effects of optogenetic stimulation on the density of BrdU positive cells. We found that optical stimulation of cholinergic projections led to a significant increase in BrdU positive cell density in ChR2 positive mice compared with the ChR2 negative mice that also received light pulses ($F(1,16)=10.989$, $p=0.005^{**}$; Figure 5.2F). We did not detect any differences in the percentage of cells committing to a neuronal phenotype ($F(1,12)=0.029$, $p=0.867$; Figure 5.2F). All F-statistics are represented in Tables 5.1 and 5.2.

Discussion

From our pharmacological (Chapter 4) and optogenetic (Chapter 5) experiments, it is clear that we can modulate discrimination ability between highly similar odorants. This suggests that manipulating cholinergic receptors or fibers impacts the OB fine tuning system while mostly sparing the broad discrimination abilities of less similar odorant pairs (as assessed by our spontaneous olfactory discrimination task). Although cholinergic modulation has previously been shown to affect odorant discrimination, this experiment is the first to show that cholinergic modulation is sufficient for olfactory perceptual learning (Linster et al., 2001a; Fletcher and Wilson, 2002; Mandaïron et al., 2006; Chaudhury, et al., 2009).

Although acetylcholine could impact newly born cell proliferation, migration, differentiation or survival, we believe acetylcholine influences olfactory behavior through the survival of newly born cells in the OB. Although progenitor cells express several acetylcholine receptors (including the predominant $\alpha 3\beta 4$ nAChR), cell proliferation alone could not be responsible for acetylcholine's effect, as BrdU was injected eight days prior to olfactory enrichment (Chapters 2, 3, 4) or optogenetic stimulation (Chapter 5) (Sharma et al., 2013). Therefore, BrdU positive cells were arriving in the OB or had already arrived before cholinergic manipulation started. Even if we started enrichment immediately after the BrdU injections, it is unlikely that cell proliferation would be affected, as lesioning the basal forebrain with 192 IgG-saporin does not affect Ki-67-expressing proliferating cells (Calza et al., 2003). In addition, cholinergic modulation could not act solely on migrating cells, as ChAT-positive fibers are minimal in the rostral

migratory stream, limiting their exposure to acetylcholine during that time period (Kaneko et al., 2006). Through our BrdU NeuN double labeling experiments, it is clear that cholinergic modulation does not affect the percentage of newly born cells differentiating into a neural phenotype. Since cholinergic fibers innervate immature OB neurons, which express numerous types of muscarinic and nicotinic acetylcholine receptors like their mature counterparts, it appears that immature neurons can respond to cholinergic stimulation, a property necessary for acetylcholine's effects (Wada et al., 1989; Le Jeune et al., 1995; Kaneko et al., 2006; Whitman and Greer, 2007).

Therefore, it seems that enhanced odorant discrimination results from the selective survival of newborn cells in the OB regions that are sensitive to experienced odorants. For example, limonene enrichment increases interneuron survival in the part of the OB responsible for detecting limonene (Moreno et al., 2012). Since the glomeruli activated by limonene have dissimilar receptive fields to carvone and terpinen-4-ol, limonene enrichment potentially causes specific interneuron survival, not widespread survival that would influence carvone or terpinen-4-ol sensory perception (Linster et al., 2001b; Mandaïron et al., 2008). However, the ChR2 positive optogenetically-manipulated mice showed olfactory discrimination within all three enantiomer pairs. Since these results correlate with an increase in newly born OB granule cells compared to ChR2 negative controls, we predict that our HDB fiber stimulation protocol induced widespread newborn cell survival, and that this increase in newly born cells directly impacts olfactory discrimination. We will test this hypothesis in Chapter 6.

While neurogenesis has been implicated in olfactory perceptual learning (Mouret et al., 2008; Moreno et al., 2009; Nissant et al., 2009; Alonso et al., 2012), significant questions remain regarding the mechanisms regulating adult neurogenesis, with specific interest in how and why select newly born OB cells survive to support learning. In recent work, a number of potential mechanisms have been identified that regulate the proliferation, migration and survival of these newly born cells (Kaneko et al., 2006; Bath et al., 2008; Scotto-Lomassese et al., 2011). Since approximately half of new cells die before maturity, it is possible that factors regulating apoptosis contribute to the survival of newly born cells (Petreanu and Alvarez-Buylla, 2002). As an example, nicotinic receptor activation can promote cell survival through the phosphoinositide 3-kinase/ protein kinase B (PI3K/Akt) signaling pathway, specifically through both Akt and another downstream target, B-cell lymphoma 2 (Bcl-2) (Eves et al., 1998; Matsuzani et al., 1999; Kihara et al., 2001). In fact, odorant exposure increases OB Bcl-2 expression, which in turn increases newborn cell survival (Miwa and Storm, 2005). Bcl-2 can also be regulated by mitogen-activated protein kinase (MAPK) phosphorylation, a process that can inhibit its anti-apoptotic effects (Torcia et al., 2001). Interestingly, cholinergic modulation may also influence cell survival through the inhibition of pro-apoptotic factors, such as caspase-3, -8 and -9 (Garrido et al., 2001). It is also possible that cholinergic modulation impacts cell survival indirectly, potentially through regulating neurotrophic factors (Wrenn and Wiley, 1998; Berchtold et al., 2002; Bath et al., 2008). Future experiments will further explore how acetylcholine acts on newly born cells, through influencing the survival of immature interneurons, fostering their stable integration into these circuits, or by acting on the interneurons previously incorporated into the OB.

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Figures and Tables

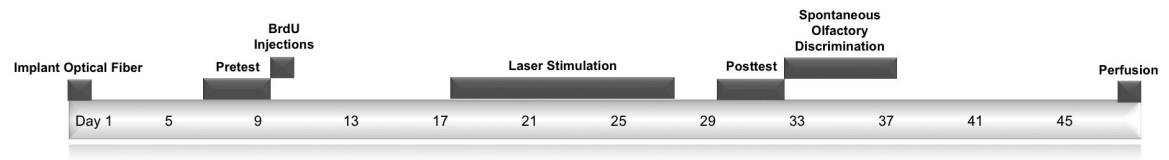


Figure 5.1. Main optogenetics experiment timeline.

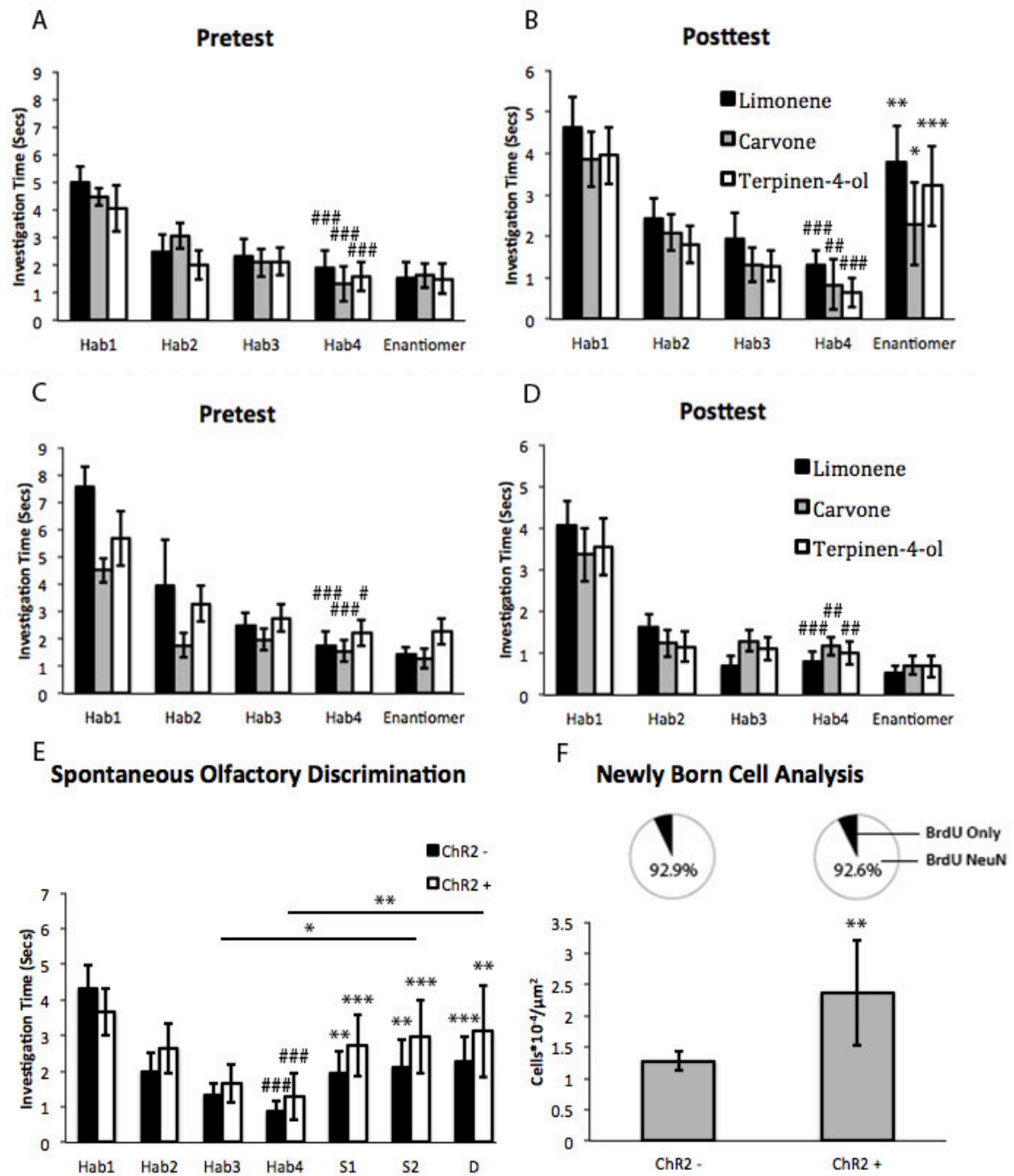


Figure 5.2. Optogenetically stimulating cholinergic fibers mimics enrichment learning within all odorant pairs tested. A) ChAT ChR2 EYFP (ChR2) + mice show normal habituation and cross-habituation after surgical implantation of an optical fiber. B) ChR2 + mice discriminate the odorants within all three odorant pairs post optogenetic stimulation (Limonene - $F(1,12)=14.380$, $p=0.003^{**}$, Carvone - $F(1,11)=5.187$, $p=0.044^{*}$, Terpinen-4-ol - $F(1,11)=18.879$, $p=0.001^{***}$). Mice continued to show normal habituation behavior. C) ChR2 - mice exhibit normal habituation and cross-habituation after optical

fiber implantation. D) ChR2 - mice do not discriminate the odorants within the three odorant pairs after optogenetic stimulation. E) Optogenetically-stimulated mice show intact habituation and discrimination between the habituation odorant (Hab4), and the three novel odorants: S1 (moderately similar odorant), S2 (slightly similar odorant), and D (structurally and perceptually different odorant). Upon further analysis of the Hab4 pairwise comparisons to S1, S2 and D, all three tests were significant, even after Bonferroni corrections. F) Optogenetically-stimulated ChR2 + mice had significantly more BrdU positive cells than ChR2 – mice ($F(1,16)=10.989$, $p=0.005^{**}$). There were statistically the same percent of neurons out of the counted BrdU positive cells in ChR2 + and ChR2 – mice. Data are represented as mean $\pm$ SEM. Hab1 – 4 = habituation trials 1 – 4; $^{\#}p\leq 0.05$, $^{\#\#}p\leq 0.01$, $^{\#\#\#}p\leq 0.001$ for statistically significant habituation between Hab1 and Hab4; $^{*}p\leq 0.05$, $^{**}p\leq 0.01$, $^{***}p\leq 0.001$ for statistically significant discrimination between Hab4 and Enantiomer, S1, S2 or D. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen. See Table 5.1 for all F-statistics.

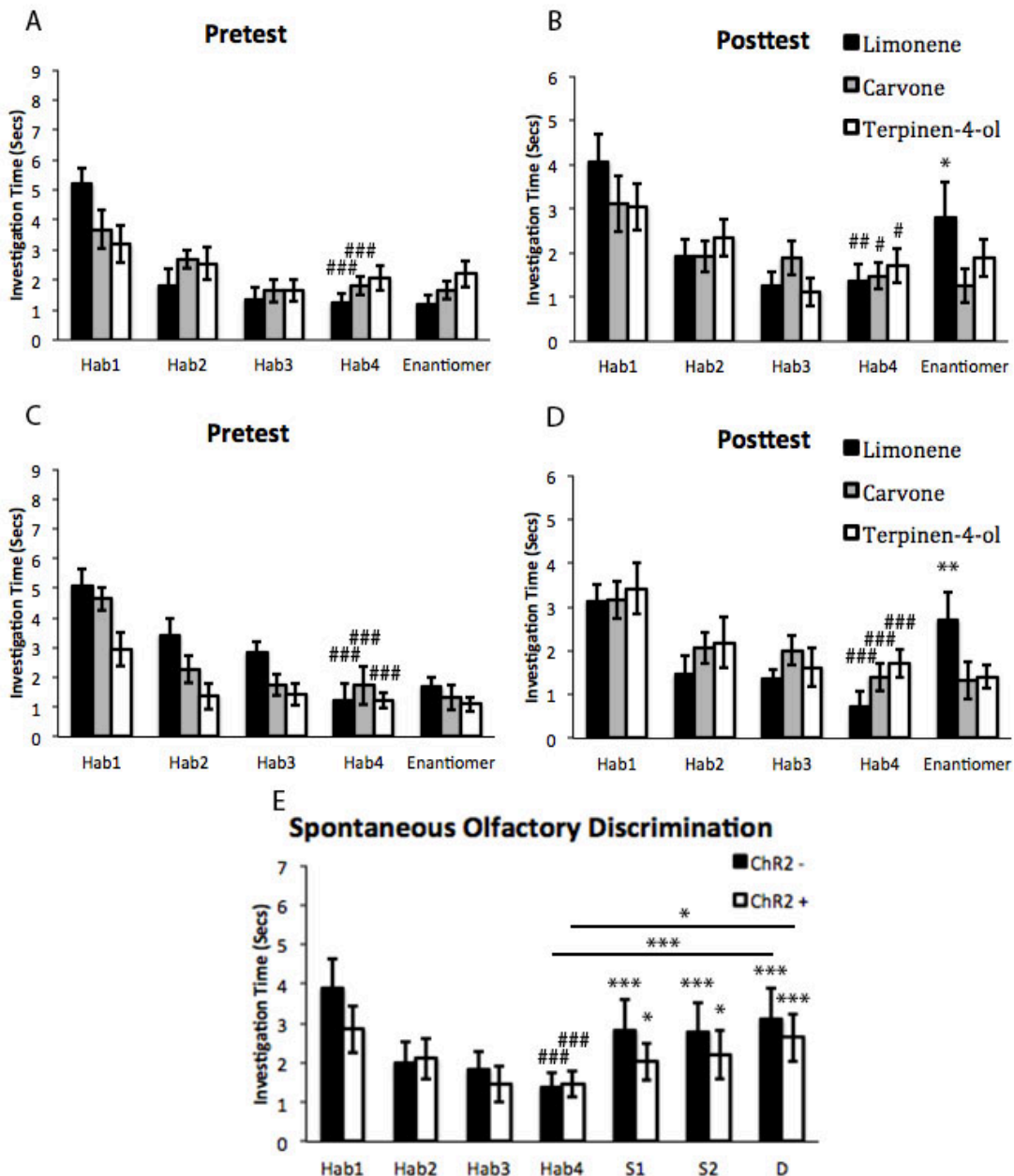


Figure 5.3. The ChAT ChR2 EFYP transgenic mouse line does not affect olfactory behaviors. A) ChAT ChR2 EFYP (ChR2) positive mice display habituation to + limonene and + carvone odorant and no discrimination to any of the enantiomer pairs before a 10-day +/- limonene enrichment period. B) ChR2 positive mice exposed to a 10-day +/- limonene enrichment period display habituation to all three odorant pairs and discrimination to only the limonene enantiomer pair ($F(1,13)=4.724$, $p=0.049^*$). C) ChR2 negative mice exhibit habituation behavior to the three odorant pairs and no enantiomer discrimination ability before a 10-day +/- limonene enrichment period. D) ChR2 negative mice were exposed to a 10-day +/- limonene enrichment period. These mice habituate to all three tested odorant pairs, but only discriminate +/- limonene ($F(1,10)=12.393$, $p=0.006^{**}$). E) ChR2 positive and negative mice exhibit normal habituation and general

discrimination behaviors. Hab4 was significantly lower than S1, S2 and D in both groups after Bonferroni corrections. Data are represented as mean $\pm$ SEM. Hab1 – 4 = habituation trials 1 – 4, S1 (moderately similar odorant), S2 (slightly similar odorant), and D (structurally and perceptually different odorant); [#]p $\leq$ 0.05, ^{##}p $\leq$ 0.01, ^{###}p $\leq$ 0.001 for statistically significant habituation between Hab1 and Hab4; *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 for statistically significant discrimination between Hab4 and Enantiomer, S1, S2 or D. See Table 5.2 for all F-statistics.

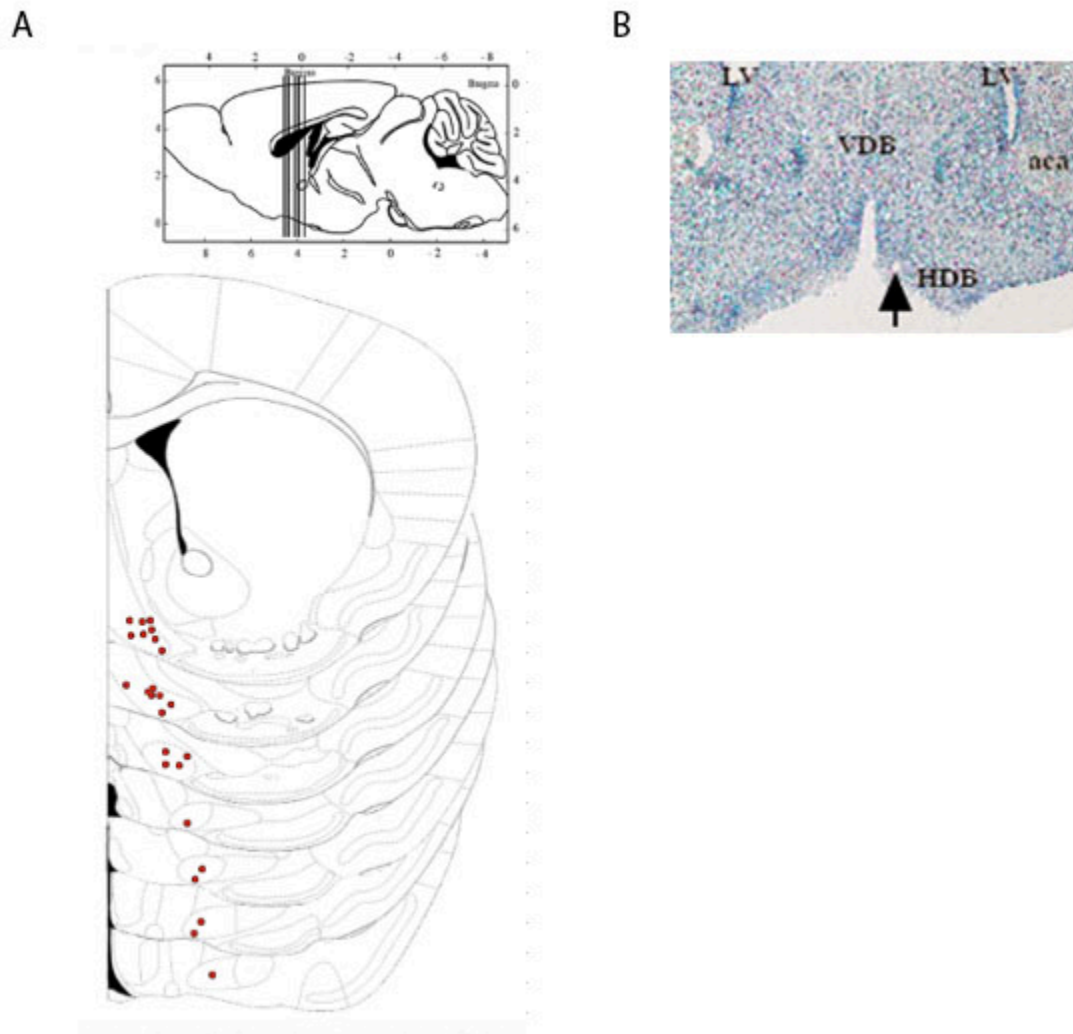


Figure 5.4. Optical fibers were implanted in the left nucleus of the horizontal limb of the diagonal band of Broca of male mice during the optogenetics experiment surgeries. A) Thirteen out of 14 ChAT ChR2 EYFP (ChR2) positive mice and 12 out of 13 ChR2 negative mice surgically implanted with optical fibers had the fiber correctly placed in the HDB. B) Hema 3 Fixative and Solutions was used on brain tissue to properly analyze fiber placement; aca = anterior commissure (anterior), HDB = nucleus of the horizontal limb of the diagonal band of Broca, LV = lateral ventricle, VDB = nucleus of the vertical limb of the diagonal band of Broca

Table 5.1. Optogenetics experiment statistics.

Group	Task	Behavior	Odorant Pair	Statistics
ChR2+ Laser	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,12)=26.524, p≤0.001 ^{###}
			Carvone	F(1,11)=83.102, p≤0.001 ^{###}
			Terpinen-4-ol	F(1,10)=20.751, p=0.001 ^{###}
		Discrimination	Limonene	F(1,11)= 1.419, p=0.259
			Carvone	F(1,11)=0.006, p=0.940
			Terpinen-4-ol	F(1,10)=0.088, p=0.772
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,12)=28.625, p≤0.001 ^{###}
			Carvone	F(1,11)=16.425, p=0.002 ^{##}
			Terpinen-4-ol	F(1,11)=31.174, p≤0.001 ^{###}
	Spontaneous Olfactory Discrimination	Discrimination	Limonene	F(1,12)=14.380, p=0.003 ^{**}
			Carvone	F(1,11)=5.187, p=0.044 [*]
			Terpinen-4-ol	F(1,11)=18.879, p=0.001 ^{***}
		Habituation		F(1,12)=72.139, p≤0.001 ^{###}
		Discrimination		F(3,10)=7.200, p=0.007 ^{**}
		Hab4 – S1		F(1,12)=25.408, p≤0.001 ^{***}
		Hab4 – S2		F(1,12)=17.508, p=0.001 ^{***}
		Hab4 – D		F(1,12)=11.550, p=0.005 ^{**}
ChR2- Laser	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,10)=63.194, p≤0.001 ^{###}
			Carvone	F(1,11)=28.886, p≤0.001 ^{###}
			Terpinen-4-ol	F(1,11)=9.379, p=0.011 [#]
		Discrimination	Limonene	F(1,9)=2.360, p=0.159
			Carvone	F(1,11)=0.896, p=0.364
			Terpinen-4-ol	F(1,11)=0.009, p=0.925
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,11)=39.098, p≤0.001 ^{###}
			Carvone	F(1,11)=11.969, p=0.005 ^{##}
			Terpinen-4-ol	F(1,11)=15.229, p=0.002 ^{##}
	Spontaneous Olfactory Discrimination	Discrimination	Limonene	F(1,11)=1.220, p=0.293
			Carvone	F(1,11)=3.163, p=0.103
			Terpinen-4-ol	F(1,11)=1.146, p=0.307
		Habituation		F(1,11)=116.408, p≤0.001 ^{###}
		Discrimination		F(3,9)=5.624, p=0.019 [*]
		Hab4 – S1		F(1,12)=7.873, p=0.008 ^{**}
		Hab4 – S2		F(1,11)=12.190, p=0.002 ^{**}
		Hab4 – D		F(1,11)=15.673, p=0.001 ^{***}
Both Laser	BrdU			F(1,16)=10.989, p=0.005 ^{**}
	BrdU NeuN			F(1,12)=0.029, p=0.867

ChR2 = ChAT ChR2 EYFP; #p≤0.05, ##p≤0.01, ###p≤0.001 for statistically significant habituation between Hab1 and Hab4 (habituation trials 1 and 4); *p≤0.05, **p≤0.01, ***p≤0.001 for statistically significant discrimination between Hab4 and Enantiomer, S1 (moderately similar), S2 (slightly similar), or D (structurally and perceptually different) odorants. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen

Table 5.2. ChAT ChR2 EFYP control experiment statistics.

Group	Task	Behavior	Odorant Pair	Statistics
ChR2+ Enrichment	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,12)=98.015, p<0.001 ^{###}
			Carvone	F(1,12)=19.113, p=0.001 ^{###}
			Terpinen-4-ol	F(1,12)=2.452, p=0.143
		Discrimination	Limonene	F(1,13)=0.025, p=0.876
			Carvone	F(1,13)=0.114, p=0.741
			Terpinen-4-ol	F(1,12)=0.009, p=0.927
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,13)=14.116, p=0.002 ^{##}
			Carvone	F(1,12)=6.778, p=0.023 [#]
			Terpinen-4-ol	F(1,13)=7.124, p=0.019 [#]
		Discrimination	Limonene	F(1,13)=4.724, p=0.049 [*]
			Carvone	F(1,12)=0.074, p=0.791
			Terpinen-4-ol	F(1,13)=0.105, p=0.751
ChR2- Enrichment	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,13)=15.417, p=0.002 ^{##}
			Discrimination	F(3,11)=5.987, p=0.011 [*]
			Hab4 – S1	F(1,13)=5.723, p=0.016 [*]
	Olfactory Perceptual Learning Posttest	Discrimination	Hab4 – S2	F(1,13)=5.633, p=0.017 [*]
			Hab4 – D	F(1,13)=18.828, p<0.001 ^{***}
	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,9)=35.292, p<0.001 ^{###}
			Carvone	F(1,10)=50.026, p<0.001 ^{###}
			Terpinen-4-ol	F(1,10)=25.547, p<0.001 ^{###}
		Discrimination	Limonene	F(1,10)=0.589, p=0.460
			Carvone	F(1,9)=0.921, p=0.362
			Terpinen-4-ol	F(1,11)=0.277, p=0.609
ChR2- Enrichment	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,10)=23.076, p=0.001 ^{###}
			Carvone	F(1,10)=19.181, p=0.001 ^{###}
			Terpinen-4-ol	F(1,11)=10.814, p=0.007 ^{##}
		Discrimination	Limonene	F(1,10)=12.393, p=0.006 ^{**}
			Carvone	F(1,10)=1.480, p=0.252
			Terpinen-4-ol	F(1,11)=0.526, p=0.484
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,11)=48.492, p<0.001 ^{###}
			Discrimination	F(3,9)=16.214, p=0.001 ^{***}
			Hab4 – S1	F(1,11)=22.975, p<0.001 ^{***}
	Olfactory Perceptual Learning Posttest	Discrimination	Hab4 – S2	F(1,11)=29.353, p<0.001 ^{***}
			Hab4 – D	F(1,11)=34.458, p<0.001 ^{***}

ChR2 = ChAT ChR2 EYFP; [#]p<0.05, ^{##}p<0.01, ^{###}p<0.001 for statistically significant habituation between Hab1 and Hab4 (habituation trials 1 and 4); ^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001 for statistically significant discrimination between Hab4 and Enantiomer, S1 (moderately similar), S2 (slightly similar), or D (structurally and perceptually different) odorants.

CHAPTER 6: ADULT NEUROGENESIS IS REQUIRED TO SUPPORT OLFACTORY DISCRIMINATION FOLLOWING OPTOGENETIC STIMULATION OF OLFACTORY BULB CHOLINERGIC FIBERS

Introduction

In the past two chapters, we determined that cholinergic activity is necessary and sufficient to support olfactory perceptual learning. In addition, the density of newly born cells in the granule cell layer (GCL) depends on cholinergic modulation. In this chapter, we will determine if the relationship observed between newly born cells and olfactory perceptual learning from Chapters 4 and 5 is due to correlation or causation by examining whether olfactory perceptual learning is dependent upon the presence of newly born cells while using an optogenetic stimulation protocol to stimulate nucleus of the horizontal limb of the diagonal band of Broca (HDB) cholinergic fibers.

Although several studies have shown that olfactory exposure decreases olfactory bulb (OB) interneuron cell death, promoting the survival of adult born cells (Woo et al., 2006; Bovetti et al., 2009), Moreno et al. (2009) first showed that newly born cells in the OB are necessary for olfactory perceptual learning. A follow-up study found that noradrenaline is important for olfactory perceptual learning by using the pharmacological blockade of noradrenergic receptors during an olfactory enrichment period. Newly born cell density increased in the OB regions responsive to enriched odorants, where pharmacologically-antagonized mice lacked this selective cell survival. Enhanced

noradrenaline release showed the opposite effect, with increased non-specific cell survival and olfactory discrimination abilities. Treatment with the mitotic blocker, cytosine arabinoside (AraC) blocked this noradrenergic effect, demonstrating that the noradrenergic system must act through newly born cells to impact olfactory perceptual learning (Moreno et al., 2012). We believe acetylcholine may also be important in this process, functioning similarly to noradrenaline.

Methods

For more information on the methods used in Chapter 6 experiments, see “Olfactory Cross-Habituation Tasks” and “Statistical Analysis” in Chapter 7.

AraC

In 1951, Bergmann and Feeney isolated the nucleosides spongothymidine (AraT) and spongouridine (AraU) from *Tethya crypta*, a sponge species native to the Caribbean Sea. These nucleosides, later identified as D-arabinosyl nucleosides, have an arabinose sugar covalently linked to a nitrogenous base in lieu of the ribose or deoxyribose sugar typically found in nucleosides (Bergmann and Burke, 1955). In 1959, Walwick et al. first synthesized AraC, a drug with powerful anti-tumor and anti-viral properties. Its sister drug, vidarabine (AraA) was synthesized just one year later (Lee et. al, 1960).

AraC is now known as one of the most effective drugs in treating acute myeloid leukemia (Löwenberg et al., 1999). It is catalyzed to its active form, cytosine arabinoside triphosphate (araCTP), via three phosphorylation enzymes: deoxycytidine kinase (dCK), nucleoside monophosphate (NMP) kinase and nucleoside diphosphate (NDP) kinase (Verschuur et al., 2002). Since araCTP closely resembles deoxycytidine triphosphate (dCTP), it acts as a deoxynucleotide analogue, inhibiting normal DNA replication (Harris and Grahame-Smith 1980; Kufe et al., 1980; Wills et al., 1996). As expected, cells in the DNA synthesis (S) phase of the cell cycle are most affected, allowing AraC to function as a mitotic blocker. Since AraC can be enzymatically deaminated by cytidine deaminase to

non-toxic AraU (Eliolopoulous et al., 1998; Lachmann et al., 2013), we tonically infused AraC in our experiment.

In addition to the substitution of araCTP for dCTP into DNA, AraC acts as a competitive inhibitor of DNA polymerase (Furth and Cohen, 1968; Kimball and Wilson, 1968; Momparler, 1969; Graham and Whitmore, 1970; Benhura, 1995). In contrast, although there is some data that supports an effect of AraC on RNA polymerase, these claims suggest only a weak RNA polymerase-araCTP interaction (Chuang and Chuang, 1975, 1976; Dhiman and Schleif, 2000).

AraC has been shown to kill postmitotic neurons when used in high concentrations (Wallace and Johnson, 1989; Martin et al., 1990). Although the pharmacokinetics of mouse intraventricular (IVT) AraC administration have not been examined, AraC has a half-life of 5.3 hours in the rat following IVT infusion and an LD₅₀ of approximately 3000-4000mg/kg following oral or intraperitoneal (IP) administration in the mouse (Groothuis et al., 2000; Lewis, 2004). To avoid possible cytotoxic effects, we used a low dose (4% AraC in saline) for our experiment.

Subjects

See “Subject Overview” in Chapter 5 methods. For detailed methods, see “Subjects” in Chapter 7.

Surgery Overview

In order to test if improvements in discrimination require the incorporation of newly born cells, we silenced cell proliferation in the subventricular zone (SVZ) using continuous micro osmotic pump administration of the mitotic blocker, AraC into the lateral ventricles. This was done in ChR2 positive mice, allowing for optogenetic drive of HDB fibers. The micro osmotic pump and optical fiber were implanted during the same surgery. For detailed methods, see “Surgery” in Chapter 7.

LED Stimulation

In the AraC experiment, we chose to utilize an LED system (PlexBright Optogenetic Stimulation System, Dallas, TX) for our stimulation protocol to maximize the number of mice we could stimulate during each 10 consecutive day period. Using an Arduino (Arduino UNO R3 board with DIP ATmega328P, Arduino S.R.L., Italy) and Matlab 8.5 (The Mathworks, Inc., Natick, MA), we recreated our stimulation protocol from the laser stimulation experiment (Chapter 5). Mice still received an hour of stimulation every day for 10 days, with eight second randomized 5-50 Hz trains of light pulses and 23 second breaks between each train (adapted from Ma and Luo, 2012). For an overview of all manipulations, see “Olfactory Manipulation” in Chapter 7.

Olfactory Perceptual Learning

See “Olfactory Perceptual Learning Overview” in Chapter 2 methods. For detailed methods, see “Olfactory Perceptual Learning” in Chapter 7.

Spontaneous Olfactory Discrimination

See “Spontaneous Olfactory Discrimination Overview” in Chapter 2 methods. For detailed methods, see “Spontaneous Olfactory Discrimination” in Chapter 7.

Bromodeoxyuridine

See “Bromodeoxyuridine Overview” in Chapter 2 methods. For detailed methods, see “Bromodeoxyuridine Injections,” “Perfusion and Histology Preparation” and “Immunohistochemistry” in Chapter 7.

Timeline

To minimize the experiment length after micro osmotic pump implantation, mice were subjected to a pretest prior to surgery. On surgery day, mice were implanted with an optical fiber aimed at the HDB, and a micro osmotic pump was subcutaneously implanted under the nape of the neck. The osmotic pump was attached to a cannulae inserted into the SVZ via polyvinyl tubing. Using the osmotic pump, AraC was infused to inhibit cell proliferation in the SVZ, at a rate of 0.25 μ L per hour (protocol adapted from Enwere et al., 2004; Moreno et al., 2009; Moreno et al., 2012). Saline pump implants were used as a control. Six days after surgery, the mice were injected with bromodeoxyuridine (BrdU) to confirm the successful inhibition of neurogenesis. In lieu of enrichment, mice were stimulated for one hour a day for 10 consecutive days using an LED one week after surgery. Three days after LED stimulation was completed, we conducted a posttest and spontaneous olfactory discrimination. Mice were perfused 39 days after BrdU administration. Since the osmotic pumps only hold 100 μ L, the pumps became empty about 17 days after implantation. Therefore, the pretest, posttest and spontaneous

olfactory discrimination tasks were all conducted in the absence of AraC (Figure 6.1).

For an overview of all timelines, see “Experiment Timelines” in Chapter 7.

Results

Acetylcholine has been shown to influence olfactory tuning and odorant discrimination (Linster et al., 2001; Mandairon et al., 2006; Chaudhury, et al., 2009; Ma and Luo, 2012). It also influences the process of OB neurogenesis (Cooper-Kuhn et al., 2004; Kaneko et al., 2006). However, no studies have examined whether acetylcholine's effects on olfactory perceptual learning depend upon newly born cell survival.

In Chapter 5, we found that optogenetic stimulation of cholinergic projections is sufficient to drive discrimination ability within all three enantiomer pairs. In order to determine if improvements in olfactory discrimination rely on newly born cells, we inhibited SVZ cell proliferation using AraC in ChR2 positive mice. At pretest, we found no effect of AraC administration or surgery on habituation or cross-habituation [AraC (Habituation: Limonene - $F(1,8)=27.117$, $p=0.001^{###}$; Carvone - $F(1,8)=22.002$, $p=0.002^{##}$; Terpinen-4-ol - $F(1,7)=9.453$, $p=0.018^{\#}$; Discrimination: Limonene - $F(1,8)=3.241$, $p=0.109$; Carvone - $F(1,8)=0.076$, $p=0.789$; Terpinen-4-ol - $F(1,7)=0.633$, $p=0.452$); Saline (Habituation: Limonene - $F(1,8)=19.462$, $p=0.002^{##}$; Carvone - $F(1,7)=55.682$, $p\leq 0.001^{###}$; Terpinen-4-ol - $F(1,8)=14.581$, $p=0.005^{##}$; Discrimination: Limonene - $F(1,9)=0.453$, $p=0.518$; Carvone - $F(1,8)=1.088$, $p=0.327$; Terpinen-4-ol - $F(1,8)=0.842$, $p=0.380$); Figure 6.2A and 6.2C]. Mice then underwent 10 days of optogenetic stimulation of HDB fibers, as described above. At posttest, mice that received the AraC infusion continued to fail to discriminate within all enantiomer pairs tested, while control mice that received saline via the micro osmotic pumps were able to discriminate the odorants within all three odorant pairs (AraC: Limonene - $F(1,8)=5.498$,

$p=0.047^*$ (significant decrease); Carvone - $F(1,8)=4.432$, $p=0.068$; Terpinen-4-ol - $F(1,8)=2.176$, $p=0.178$; Saline: Limonene - $F(1,9)=8.636$, $p=0.017^*$; $F(1,8)=5.713$, $p=0.044^*$; $F(1,8)=14.729$, $p=0.005^{**}$; Figure 6.2B and 6.2D). To determine if impairments in enhanced olfactory discrimination were the result of gross impairments in olfactory discrimination ability, we tested these mice on the spontaneous olfactory discrimination task. Both AraC- and saline-treated mice habituated to the control odorants (AraC - $F(1,8)=41.864$, $p\leq 0.001^{###}$; Saline - $F(1,9)=68.754$, $p\leq 0.001^{###}$; Figure 6.2E). Mice receiving saline showed no impairments in general discrimination abilities, while AraC-treated mice had a strong trend toward normal general discrimination (AraC - $F(3,4)=4.327$, $p=0.095$; Saline - $F(3,4)=6.847$, $p=0.047^*$; Figure 6.2E). In posthoc analyses, pairwise comparison between Hab4 and S1, S2 and D were statistically significant for both AraC and saline mice, suggesting only slight deficits in general olfactory abilities as a result of AraC infusion (AraC: S1 - $F(1,6)=7.708$, $p=0.016^*$; S2 - $F(1,6)=6.704$, $p=0.020^*$; D - $F(1,6)=10.677$, $p=0.009^{**}$; Saline: S1 - $F(1,6)=12.159$, $p=0.006^{**}$; S2 - $F(1,6)=8.896$, $p=0.013^*$; D - $F(1,6)=18.039$, $p=0.002^{**}$; Figure 6.2E). Surgical placements were confirmed with sectioning and Hema-3 staining (Figure 6.3). Using BrdU labeling of OBs from these mice, we confirmed that AraC led to a significantly reduction in the density of BrdU positive cells in AraC-treated animals compared to saline controls without impacting the percentage of BrdU positive cells exhibiting a neuronal phenotype (BrdU - $F(1,5)=29.770$, $p=0.003^{**}$; BrdU NeuN - $F(1,5)=0.204$, $p=0.670$; Figure 6.2F). All F-statistics are represented in Table 6.1.

Discussion

In this chapter, we provide support for our hypothesis that acetylcholine regulates olfactory perceptual learning through adult born cells. When we analyzed newly born cell density, we determined that manipulating the cholinergic system affects OB granule cell density, an effect that alters discrimination ability. In addition, the mitotic blocker, AraC, potentially causes mild deficits in general olfactory function, as indicated by the spontaneous olfactory discrimination data. From the pretest and posttest data, it appears that AraC does not influence olfactory sensitivity or habituation, only discrimination. However, overall investigation time is lower in AraC- than saline-treated mice during the spontaneous olfactory discrimination task.

Although AraC is associated with numerous side effects such as gastrointestinal issues, confusion, fatigue and respiratory problems, our posttest was conducted after a washout period to mitigate these effects (PubMed Health, 2016). In addition, previous studies have shown that AraC does not impact spontaneous olfactory discrimination, as well as numerous other abilities, such as associative learning, exploratory behaviors or locomotor activity (Imayoshi et al., 2008; Breton-Provencher et al., 2009). Therefore, it is possible our ANOVA failed because of a main effect of AraC on overall sniffing, diminishing our ability to observe intact general discrimination.

Although we did not analyze the effect of AraC on hippocampal neurogenesis, it has been previously suggested that AraC treatment into the lateral ventricles only significantly affects OB neurogenesis (Moreno et al., 2009). However, as AraC had a nonsignificant

trend towards reducing hippocampal neurogenesis in that study, the olfactory perceptual learning task is clearly a better choice than associative learning tasks, which may be impacted by changes in hippocampal neurogenesis instead of the OB. Additionally, olfactory perceptual learning requires newly born cells during task acquisition, while olfactory associative learning tasks require neurogenesis for memory retention (Moreno et al., 2009; Sultan et al. 2010). Overall, we have shown that cholinergic activity during the period of newly born neuron development and integration is imperative for olfactory perceptual learning, although future studies are needed to explore this effect in associative learning tasks.

While our experiments give a well-defined role for acetylcholine influencing learning through adult neurogenesis, and it is known that acetylcholine acts on both OB nicotinic and muscarinic receptors, we still do not understand the separate influences of these receptors when it comes to modulation via adult neurogenesis (Castillo et al., 1999; Pressler et al., 2007). Newly born neurons inhibit mitral cells through center-surround lateral inhibition. These cells enhance the contrast between neighboring mitral cell receptive fields, which allows animals to discriminate chemically similar odorants. Nicotinic receptors, positioned in the glomerular and mitral cell layers, influence the responsiveness of periglomerular cells, sharpening mitral cell olfactory tuning by minimizing mitral cell receptive field size (Castillo et al., 1999; Chaudhury et al., 2009). In contrast, muscarinic acetylcholine receptors located in the plexiform and granule cell layers influence granule cell excitability, enhancing network synchrony and gamma oscillations (Li and Cleland, 2013). Future work will tease apart the roles of cholinergic

modulation on adult neurogenesis via muscarinic and nicotinic receptors, to understand the spatial and temporal dynamics of this system.

In the last three chapters, we discussed several mechanisms through which acetylcholine could impact olfactory perceptual learning. However, due to the similarities between our results and the noradrenergic story published by Moreno et al. (2012), we would be remiss to dismiss a potential convergence of these two neuromodulatory systems as a mechanism through which acetylcholine has its effects. In many regions of the brain, including the neurogenic hippocampus and olfactory bulb, the activation of acetylcholine receptors (specifically nicotinic receptors) can induce noradrenaline release, either through direct activation of presynaptic nicotinic acetylcholine receptors on noradrenergic terminals, or through indirect mechanisms, such as altering GABA or glutamate levels in the GCL (El-Etri et al., 1999; Fu et al., 1999; Barik and Wonnacott, 2006; Beiranvand et al., 2014). If cholinergic modulation is piggybacking noradrenaline's effects, long-term potentiation, increased granule cell responsiveness and contrast enhancement among many other mechanisms could contribute to noradrenaline, and therefore acetylcholine's effects (Mouly et al., 1995; Kermen et al., 2010; Moreno et al., 2012).

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Figures and Table

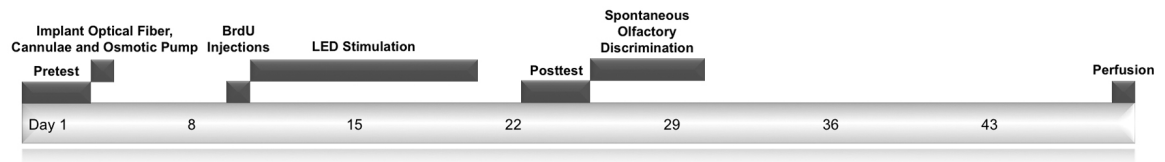


Figure 6.1. AraC experiment timeline.

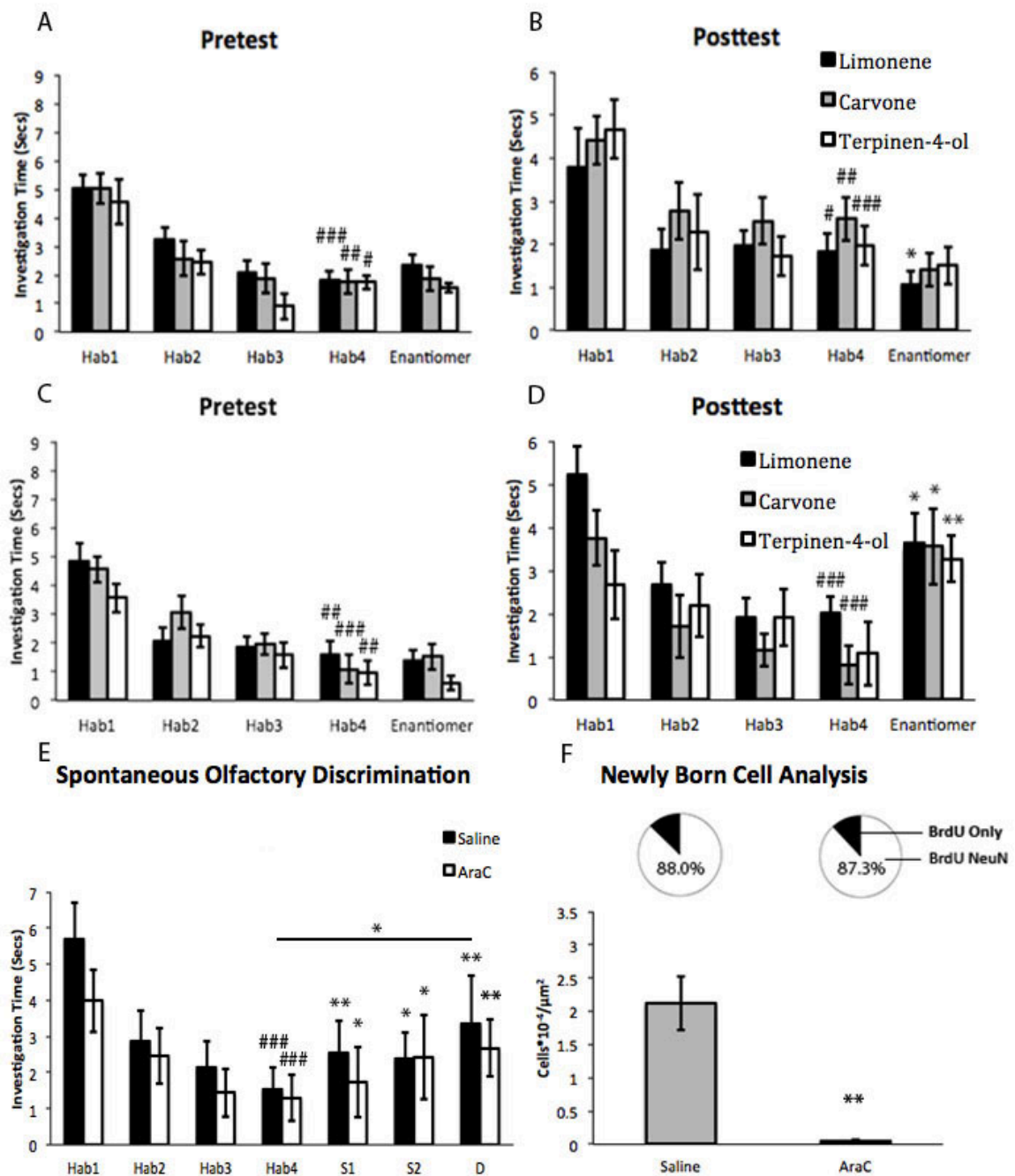


Figure 6.2. Acetylcholine regulates olfactory perceptual learning through adult neurogenesis. A) AraC mice showed normal habituation and discrimination behavior post-surgery and pre-stimulation. B) AraC mice did not learn to distinguish any of the odorants within the three enantiomer pairs post-stimulation. C) Control mice implanted with saline-filled pumps showed normal habituation and cross habituation behavior post-surgery but pre-optogenetic stimulation. D) Control mice implanted with a saline-filled pump showed habituation behavior after optogenetic stimulation as well as discrimination

within all three tested enantiomer pairs (Limonene - $F(1,9)=8.636$, $p=0.017^*$, Carvone - $F(1,8)=5.713$, $p=0.044^*$, Terpinen-4-ol - $F(1,8)=14.729$, $p=0.005^{**}$). E) Saline and AraC mice were tested on their general olfactory habituation and discrimination abilities. Hab1 – 4 = habituation trials 1 – 4, S1 (moderately similar odorant), S2 (slightly similar odorant), and D (structurally and perceptually different odorant); $^{\#}p\leq 0.05$, $^{##}p\leq 0.01$, $^{###}p\leq 0.001$ statistically significant habituation between Hab1 and Hab4; $^*p\leq 0.05$, $^{**}p\leq 0.01$, $^{***}p\leq 0.001$ statistically significant discrimination between Hab4 and Enantiomer, S1, S2 or D. F) AraC significantly reduces the number of BrdU positive cells ($F(1,5)=29.770$, $p=0.003^{**}$). The percent of neurons in BrdU positive cells is unchanged in mice implanted with a saline-filled versus AraC-filled pumps. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen. Data are represented as mean $\pm$ SEM. See Table 6.1 for all F-statistics.

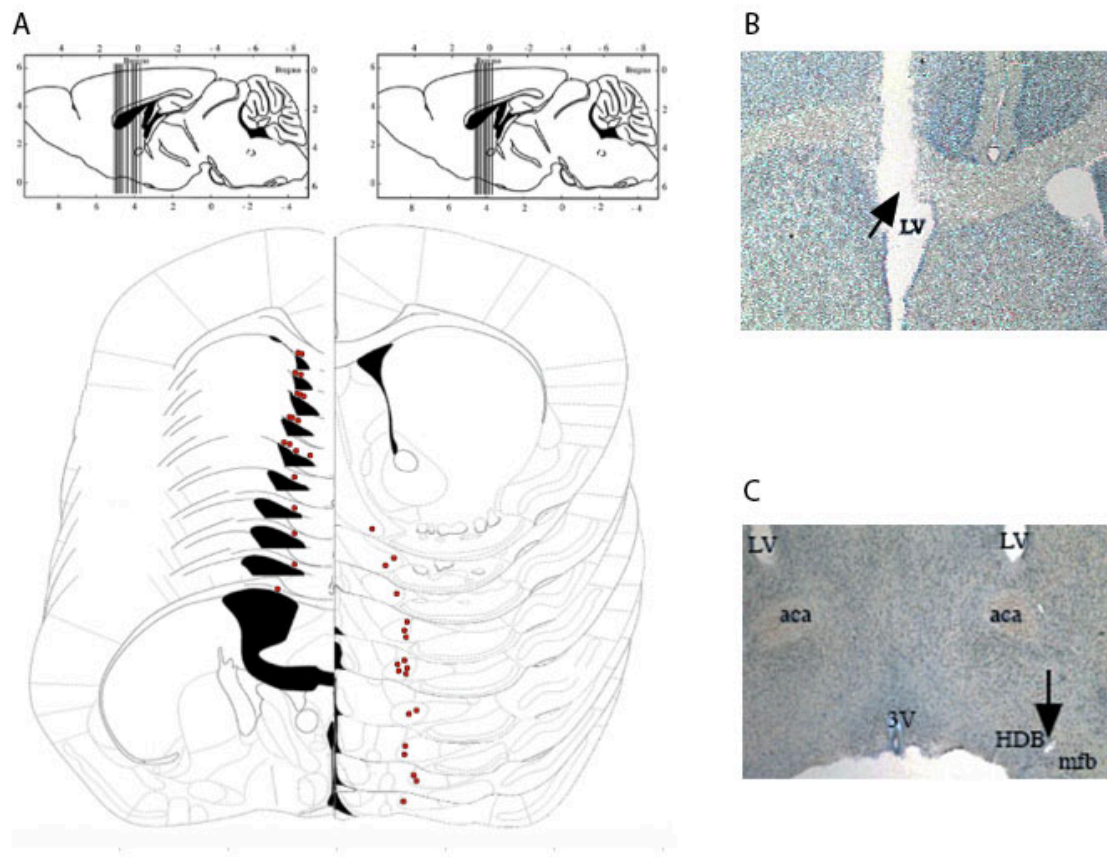


Figure 6.3. Optical fibers and cannula were implanted in the left nucleus of the horizontal limb of the diagonal band of Broca and right lateral ventricle, respectively of male mice during the implantation surgeries. A) Out of 27 surgeries, 19 total mice had the correct placement for both the LV and HDB coordinates (Saline: 14 surgeries - 10 hits, three miss, one could not confirm placement; AraC: 13 surgeries - 9 hits, two miss, two could not confirm placement). Hema 3 Fixative and Solutions was used on brain tissue to properly analyze B) cannulae and C) fiber placement. 3V = third ventricle, aca = anterior commissure (anterior), HDB = nucleus of the horizontal limb of the diagonal band of Broca, LV = lateral ventricle, mfb = medial forebrain bundle

Table 6.1. AraC experiment statistics.

Group	Task	Behavior	Odorant Pair	Statistics
AraC	Olfactory Perceptual Learning Pretest	Habituation	Limonene	F(1,8)=27.117, p=0.001 ^{###}
			Carvone	F(1,8)=22.002, p=0.002 ^{##}
			Terpinen-4-ol	F(1,7)=9.453, p=0.018 [#]
		Discrimination	Limonene	F(1,8)=3.241, p=0.109
			Carvone	F(1,8)=0.076, p=0.789
			Terpinen-4-ol	F(1,7)=0.633, p=0.452
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,8)=7.403, p=0.026 [#]
			Carvone	F(1,7)=14.349, p=0.007 ^{##}
			Terpinen-4-ol	F(1,8)=40.818, p≤0.001 ^{###}
		Discrimination	Limonene	F(1,8)=5.498, p=0.047 [*]
			Carvone	F(1,8)=4.432, p=0.068
			Terpinen-4-ol	F(1,8)=2.176, p=0.178
Saline	Olfactory Perceptual Learning Pretest	Habituation		F(1,8)=41.864, p≤0.001 ^{###}
				F(3,4)=4.327, p=0.095
				F(1,6)=7.708, p=0.016 [*]
		Discrimination		F(1,6)=6.704, p=0.020 [*]
				F(1,6)=10.677, p=0.009 ^{**}
	Olfactory Perceptual Learning Posttest	Habituation	Limonene	F(1,8)=19.462, p=0.002 ^{##}
			Carvone	F(1,7)=55.682, p≤0.001 ^{###}
			Terpinen-4-ol	F(1,8)=14.581, p=0.005 ^{##}
		Discrimination	Limonene	F(1,9)= 0.453, p=0.518
			Carvone	F(1,8)=1.088, p=0.327
			Terpinen-4-ol	F(1,8)=0.842, p=0.380
	Spontaneous Olfactory Discrimination	Habituation	Limonene	F(1,9)=22.856, p=0.001 ^{###}
			Carvone	F(1,8)=36.634, p≤0.001 ^{###}
			Terpinen-4-ol	F(1,8)=4.560, p=0.065
		Discrimination	Limonene	F(1,9)=8.636, p=0.017 [*]
			Carvone	F(1,8)=5.713, p=0.044 [*]
			Terpinen-4-ol	F(1,8)=14.729, p=0.005 ^{**}
Both	BrdU BrdU NeuN	Habituation		F(1,9)=68.754, p≤0.001 ^{###}
				F(3,4)=6.847, p=0.047 [*]
				F(1,6)=12.159, p=0.006 ^{**}
		Discrimination		F(1,6)=8.896, p=0.013 [*]
				F(1,6)=18.039, p=0.002 ^{**}
				F(1,5)=29.770, p=0.003 ^{**}
				F(1,5)=0.204, p=0.670

[#]p≤0.05, ^{##}p≤0.01, ^{###}p≤0.001 statistically significant habituation between Hab1 and Hab4 (habituation trials 1 and 4); ^{*}p≤0.05, ^{**}p≤0.01, ^{***}p≤0.001 statistically significant discrimination between Hab4 and Enantiomer, S1 (moderately similar), S2 (slightly similar), or D (structurally and perceptually different) odorants. BrdU = bromodeoxyuridine, NeuN = neuronal nuclear antigen

CHAPTER 7: OVERARCHING METHODS

All experiments were done in accordance with Brown University's IACUC approved protocols and carried out in accordance with the guide for the care and use of laboratory animals.

Subjects

Eight to 12 week old male C57Bl6/N WT (Chapters 2 – 4) and ChAT ChR2 EYFP mice (Chapters 5 – 6; B6.Cg-Tg (Chat-COP4*H134R/EYFP,Slc18a3)6Gfng/J; RRID: IMSR_JAX:014546) on a C57Bl6/N background (Charles River Laboratories, Wilmington, MA) were bred in-house. All mice were housed on a 12-hour light/ 12-hour dark cycle with water and food provided ad libitum. Testing occurred during the first half of the light phase of the light/dark cycle.

ChAT ChR2 EYFP Mice

Created from a bacterial artificial chromosome (BAC) technique, ChAT ChR2 EYFP mice express channelrhodopsin2 (ChR2) under the promoter for choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine. These mice express the mhChR2::YFP fusion protein derived from the green alga *Chlamydomonas reinhardtii*. When blue light (450 – 490 nm) is shone on cholinergic neurons, these cells depolarize and action potentials are produced. We chose to use hemizygous mice in our experiments

as they are viable and fertile. ChAT ChR2 EYFP mice express channelrhodopsin2 in cholinergic neurons all around the brain, including but not limited to the basal forebrain, striatum, cortex, hippocampus, trochlear nucleus, medial habenula, interpeduncular nucleus and brainstem (Zhao et al., 2011). Although these mice may have some spatial and working memory deficits, they have improved motor endurance and comparable anxiety- and depressive-like behaviors, metabolism and sensorimotor function to wild type littermates (Kolisnyk et al., 2013).

Experiment Timelines

In order to determine acetylcholine's role in olfactory perceptual learning, we first confirmed that mice treated with saline show olfactory perceptual learning to an enriched pair of odorants after ten days of olfactory enrichment (Figure 2.4). This learning is long-term, lasting between three and six months (Figure 3.1). We then used a pharmacological approach to block central nervous system (CNS) muscarinic acetylcholine receptors while mice received olfactory enrichment to determine if mice lose their potential to learn (Figure 4.1). We utilized optogenetics to stimulate the nucleus of the horizontal limb of the diagonal band of Broca (HDB) cholinergic fibers, increasing acetylcholine release into the bulb, and showed that enhanced acetylcholine promotes olfactory discrimination in lieu of odorant enrichment (Figure 5.1). Finally, we conducted a second optogenetics experiment (AraC experiment), where we inhibited adult neurogenesis to determine if acetylcholine impacts learning through newly born cell survival (Figure 6.1).

Olfactory Cross-Habituation Tasks

Olfactory cross-habituation tasks are used to assess the degree to which mice spontaneously discriminate odorants (Cleland et al., 2002; Mandaïron et al., 2006; Bath et al., 2008; Moreno et al., 2009). We purposefully used spontaneous discrimination tasks, as they rely on a mouse's innate curiosity to investigate novelty, allowing us to explore discrimination abilities without engaging other systems involved in learning, memory or reward.

Mice are intrinsically motivated to investigate novel odorants more than familiar ones (Cleland et al., 2002). Therefore, we can measure investigation time (calculated by the amount of time sniffing within one inch of an odorant) as an indicator of odorant novelty. If we introduce a mouse to an odorant over multiple trials, we expect attenuated investigation time with each subsequent trial, indicating successful habituation. However, if we introduce a new odorant into the cage and investigation time increases, this is indicative of the mouse's ability to discriminate the original habituation and novel odorants. If investigation time does not increase when a novel odorant is presented, the animal is not able to perceive differences between the habituation and novel odorants, and therefore generalizes, or cross-habituates to the novel odorant.

In our study, we used two olfactory cross-habituation tasks, olfactory perceptual learning and spontaneous olfactory discrimination. Stimulus odorants were diluted in the odorless carrier medium, mineral oil (MO) to 0.1 or 0.01 Pascal (Pa) vapor pressure, respectively. Volume/volume dilutions varied by odorant, and vapor pressures were estimated using

ACD ChemSketch software (Advanced Chemistry Development, Toronto, Ontario, Canada). All trials were videotaped using Ethovision XT 8.5 tracking software (Noldus), after which investigation time was manually scored with the rater blind to drug group or genotype and odorant identity.

Olfactory Perceptual Learning

Mice were individually placed in polycarbonate cages before testing. For each trial, odorants were introduced by placing 100 μ l of an odorant onto a piece of filter paper (Whatman, Florham Park, NJ) and suspending the filter paper in each mouse's cage by enclosing the paper in a tea ball hanging in the cage's upper corner. During the first four trials, mice were exposed to 0.1 Pa of a habituation odorant. For the fifth trial, the enantiomer to the habituation odorant was presented in lieu of the habituation odorant. Each trial was one minute in length followed by a five-minute inter-trial interval. Fresh odorants and filter paper were used in each trial. Investigation time was subsequently hand-scored and analyzed.

Enantiomer pairs were chosen for their perceptual similarity and the inability of our mice to behaviorally distinguish them in our pretest data (Linster et al., 2001; Figure 2.6A). Three enantiomer pairs (one habituation odorant and its enantiomer) were used in each behavioral experiment: +/- limonene, +/- carvone and +/- terpinen-4-ol (Table 7.1). For consistency, the + form of the enantiomer pair was always used in the habituation trial and the – form was always used in the enantiomer trial. Only one enantiomer pair was tested per day, so the pretest and posttest were each three days.

Spontaneous Olfactory Discrimination

Unlike the linear topographic organization in other sensory systems such as audition and vision, the chemical senses are much more complex. Odorants differ in a non-linear, multidimensional fashion, so it is difficult to discern what aspects of these molecules get interpreted in the brain, and how the olfactory bulb (OB) organizes this information.

Carbon chain length has been used as an axis for graded odorant similarity in terms of glomerular activation patterns and olfactory perception (Linster and Hasselmo, 1999; Rubin and Katz, 1999; Kaluza and Breer, 2000; Belluscio and Katz, 2001; Meister and Bonhoeffer, 2001; Cleland et al., 2002). Specifically, longer odorants activate glomeruli that are progressively more ventral (Johnson et al., 1999; Johnson and Leon, 2000; Ho et al., 2006) (Figure 7.1). In our spontaneous olfactory discrimination task, we chose straight-chain aliphatic odorant molecules as our habituation odorants. Since carbon chain length correlates with perception, each odorant set consisted of three unbranched aliphatic odorants differing by a single carbon, followed by a structurally and perceptual different molecule (Cleland et al., 2002; Bath et al., 2008).

Each mouse was tested individually into polycarbonate cages. For each trial, the odorants were presented by introducing 100 μ L of 0.01 Pa diluted odorant onto a piece of filter paper (Whatman, Florham Park, NJ), and placing the filter paper inside a tea ball (Table 7.2). The tea ball was subsequently draped inside the upper corner of the cage. The mice were presented with a single aliphatic acid odorant on four consecutive trials (habituation). Three novel odorant trials followed, where three odorants were introduced

in a counter-balanced randomized order: a moderately similar (S1 – differing by a single unbranched hydrocarbon from the habituation odorant), slightly similar (S2 – differing by two unbranched hydrocarbons from the habituation odorant) and dissimilar odorant (D – structurally and perceptually unique odorant). All trials were one minute long with a five-minute inter-trial interval. This procedure was repeated with five different odorant sets over the course of five days. Following manual scoring, the five days of testing were averaged, and the data was pooled by trial type and drug group or genotype to assess the effects of drug group or genotype on odorant detection, habituation and discrimination.

Olfactory Manipulation

Before any olfactory manipulation (enrichment, laser, LED) took place, mice were tested on their ability to distinguish three sets of enantiomers on three consecutive days: + and – limonene, + and – carvone, and + and – terpinen-4-ol. After this pretest, mice were put through 10 consecutive days of olfactory manipulation for one hour a day, followed by a three-day break and a three-day posttest.

Bromodeoxyuridine Injections

Since the peak arrival of newly born cells to the OB takes place by 10 days after 5-bromo-2-deoxyuridine (BrdU) injection (Winner et al., 2002), we intraperitoneally injected mice with BrdU (Sigma; 50 mg/kg in saline three times a day, two hours between each injection) eight days before the beginning of the olfactory manipulation period. Therefore, olfactory manipulation began as most BrdU labeled cells were arriving in the OB. BrdU is a thymidine analog that incorporates itself into DNA (by substituting bromouracil for thymidine) during the S phase of the cell cycle. Thus, we can label adult born neurons and analyze their density in olfactory tissue.

Surgery

For the optogenetics experiments, we used eight to 12 week old ChAT ChR2 EYFP male mice. Transgenic and control (C57Bl6/N) mice were injected intraperitoneally (IP) with the analgesic buprenorphine hydrochloride 20 minutes before surgery [0.1 mg/kg, Buprenex Injectable, Reckitt Benckiser Healthcare (UK) Ltd., Hull, England]. Mice were anesthetized using an isoflurane-oxygen mixture (induction = 2-3%, maintenance = 1-2.5, O₂ flow rate=75 ppm), shaved and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). A craniotomy was made on the skull above the coordinates for the left HDB (AP = +0.3 mm, ML = -2.0 mm, 13° lateral to medial), and a glass optical fiber was implanted (DV = 5.0 mm; fiber made in-house, core diameter = 200 µm, NA = 0.22). The fiber was fixed in place using C&B Metabond Quick Adhesive Cement System (Parkell Inc., Edgewood, NY) and dental acrylic (Repair Resin Quick Set Lab Pack, All 4 Dentist by Interguide Dental & Medical Supply, Burlingame, CA).

AraC Experiment Surgery

For the AraC experiment, mice were additionally implanted subcutaneously with a micro osmotic pump (Model 1002, ALZET Osmotic Pumps, Cupertino, CA). The pump was connected via polyvinyl tubing to a cannulae aimed at the right subventricular zone (SVZ; AP = +1.2 mm, ML = +0.9 mm, DV = 3.0 mm; brain infusion kit 3, ALZET Osmotic Pumps, Cupertino, CA). The pump was filled with 100 µL cytosine-β-d-arabinofuranoside (AraC; 4% in 0.9% saline solution, Sigma Aldrich, St. Louis, MO) with a flow rate of 0.25 µL per hour (Moreno et al., 2012). Post-surgery observations were maintained for three days.

Perfusion and Histology Preparation

Thirty-nine days after the BrdU injections, mice were sacrificed for histology. Each mouse was placed individually into a clean polycarbonate cage for one hour, followed by a one hour exposure to 100 µl undiluted + limonene in a tea ball. Mice were then injected IP with a lethal dose of a pentobarbital and phenytoin solution [300 µl; Beuthanasia-D Special, Intervet Inc (d/b/a Merck Animal Health), Madison, NJ] and intracardially perfused using PBS with 0.1% sodium nitrite, a vasodilator, followed by 4% PFA in PBS. Brains were removed and postfixed in 4% PFA for one hour at room temperature. The PFA was subsequently replaced by 30% sucrose. At least 48 hours after bathing in 30% sucrose, the brains were coronally sectioned to 30 µm using a freezing microtome (American Optical Company, Buffalo, NY). Sections were stored in a cryoprotectant solution at -20°C.

Immunohistochemistry

BrdU

In order to gain access to the BrdU antibody, cellular DNA was denatured by treating sections with 0.1 M citric acid and 2 N HCl. Sections were subsequently incubated overnight at 4°C with a mouse monoclonal anti-BrdU antibody (1:100; BD Biosciences Cat# 556028, RRID: AB_396304). After three PBS washes, sections were incubated in a biotinylated goat anti-mouse antibody (1:200; Vector Laboratories Cat# BA-9200, RRID: AB_2336171) for one hour at room temperature, followed by an avidin-biotin-peroxidase complex (VECTASTAIN Elite ABC-Peroxidase Kit antibody, Vector Laboratories Cat# PK-7100, RRID: AB_2336827) and diaminobenzidine/peroxide (DAB; Vector Laboratories Cat# SK-4100, RRID: AB_2336382) treatment. Sections were dehydrated in a graded ethanol series, cleared with Histo-Clear II (National Diagnostics, Atlanta, Georgia) and coverslipped with xylene-based Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI) (Figure 7.2A).

BrdU NeuN Double Labeling

In order to determine the percent of BrdU positive cells that differentiated into mature neurons, we performed a double labeling protocol with BrdU and NeuN, a neuronal nuclei marker. Free floating sections were incubated in a preheated 50% formamide/ 50% 2X SSC solution at 50°C for 20 minutes, 1 N HCl for 40 minutes (20 minutes at room temperature followed by 20 minutes at 37°C), and neutralized with borate buffer. Sections were then submerged in a blocking solution containing rat anti-BrdU (1:1000; Creative Diagnostics Cat# DMAB3062, RRID: AB_2343839) and mouse anti-NeuN

(1:10,000; LifeSpan Cat# LS-C73393-500, RRID: AB_10588506) at 4°C. After 72 hours, sections were washed, treated with the appropriate secondary antibodies (BrdU: goat anti-rat, 1:1000, 594 – red, Molecular Probes Cat# A-11007, RRID: AB_141374; NeuN: donkey anti-mouse, 1:1000, 488 – green, Molecular Probes Cat# A-21202, RRID: AB_141607), mounted on Superfrost Plus Microscope Slides (Fisher Scientific, Pittsburgh, PA), and coverslipped with ProLong Gold antifade reagent (Life Technologies Corporation, Eugene, OR) and clear nail polish (Figure 7.2B).

Fiber Placement

Free floating sections were washed in PBS, mounted and air dried. Slides were then brought through a Hema 3 Fixative and Solutions (Fisher HealthCare, Pittsburgh, PA) protocol in order to visualize the HDB. After washing slides in distilled water, the slides were dehydrated, cleared with Histo-Clear II (National Diagnostics, Atlanta, Georgia) and coverslipped with xylene-based Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI). After drying, sections were analyzed using an Eclipse E600 microscope (Nikon, Melville, NY) to determine if the HDB (and SVZ in the cannulated mice) had been successfully targeted.

Cell Counting

We coronally sectioned each OB to 30 μ m and stained every fifth section using DAB (BrdU) or fluorescence (BrdU NeuN). Using Neurolucida (Microbrightfield, Inc., Burlington, VT), the perimeter of each section's granule cell layer was traced at 4x. The contour was then analyzed at 40x, where each BrdU or BrdU NeuN cell was hand-counted.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics 20, excluding outlier trials that deviated by ± 2 or more standard deviations from the mean. Alpha was set to 0.05.

Behavior

We used repeated measures general linear model ANOVAs between Hab1 and Hab4 to determine if the mice were habituating to each odorant. In order to determine if mice discriminated odorants within enantiomer pairs in the olfactory perceptual learning tasks, repeated measures ANOVAs were conducted between Hab4 and the enantiomer trials. In the spontaneous olfactory discrimination tasks, discrimination was assessed with general linear model repeated measures ANOVA between Hab4, S1, S2 and D. We further analyzed the relationship between Hab4 and each novel odorant individually by running repeated measures ANOVAs (with Bonferroni corrections for multiple tests) between Hab4 and S1, S2 and D.

BrdU Cell Count

We determined the BrdU positive cell density for each mouse by taking the average number of BrdU positive cells from each section and dividing it by the average section area. To assess differences between BrdU positive cell densities, we carried out one-way ANOVAs.

BrdU NeuN

Using Neurolucida (Microbrightfield, Inc., Burlington, VT), we randomly sampled 50 BrdU positive cells per brain and determined if they were positive for BrdU only or BrdU NeuN. We calculated the percent neurons in BrdU positive cells by dividing the number of BrdU NeuN cells randomly sampled in each brain by 50. We compared these percents using a one-way ANOVA.

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Figures and Tables

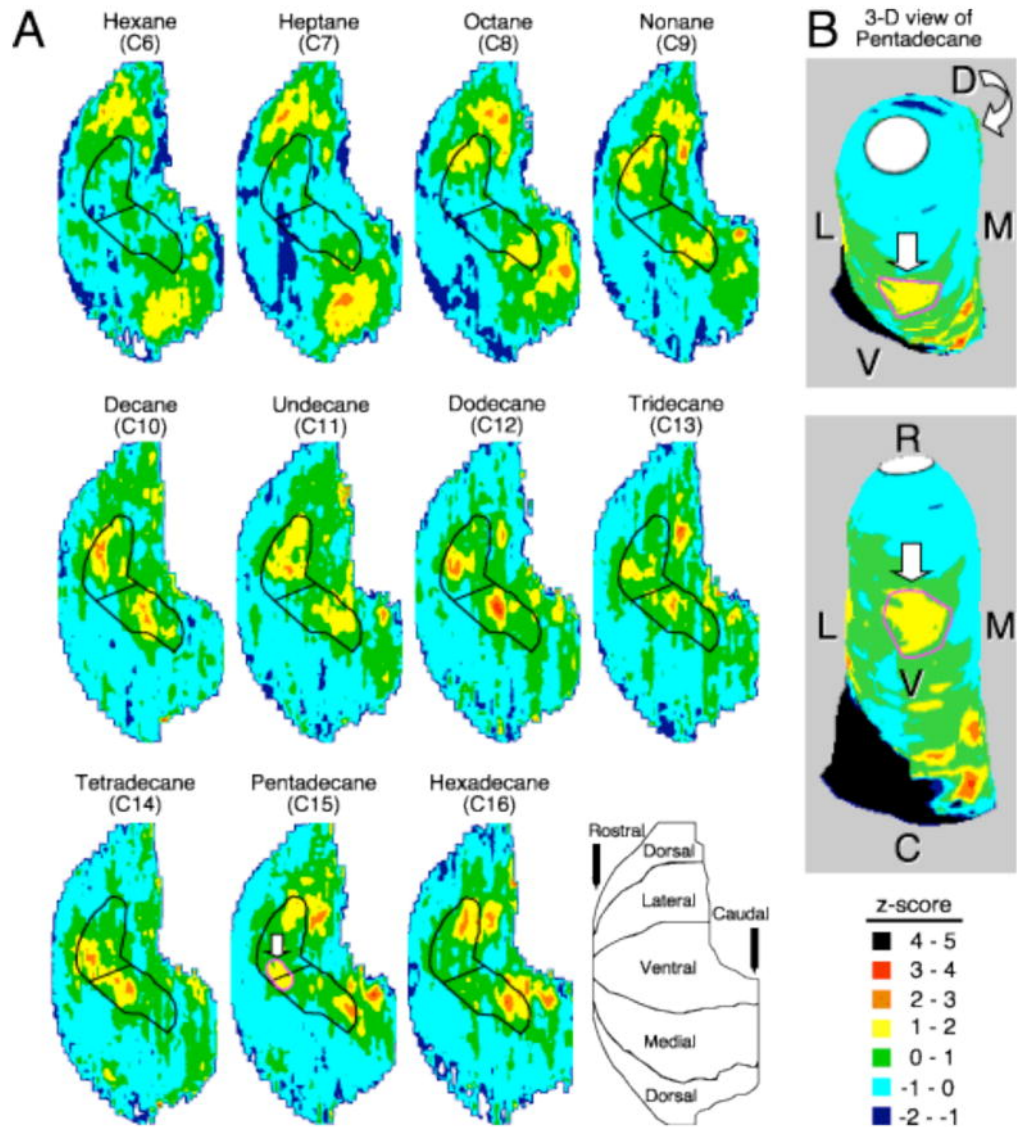


Figure 7.1. Longer saturated aliphatic hydrocarbons activate progressively more ventralized glomeruli. A) Using [^{14}C]2-deoxyglucose (2-DG) uptake, glomeruli activation was assessed in straight-chained alkanes from six carbons (hexane) to sixteen carbons (hexadecane). After deriving z-scores from averaging four individuals per odorant and subtracting out control activation, a heat map was created, where glomerular responses can be assessed from lowest (dark blue) to highest (black) activity. B) Example 3D view (from pentadecane). C = caudal, D = dorsal, L = lateral, M = medial, R = rostral, V = ventral. *Figure from Ho et al., 2006.*

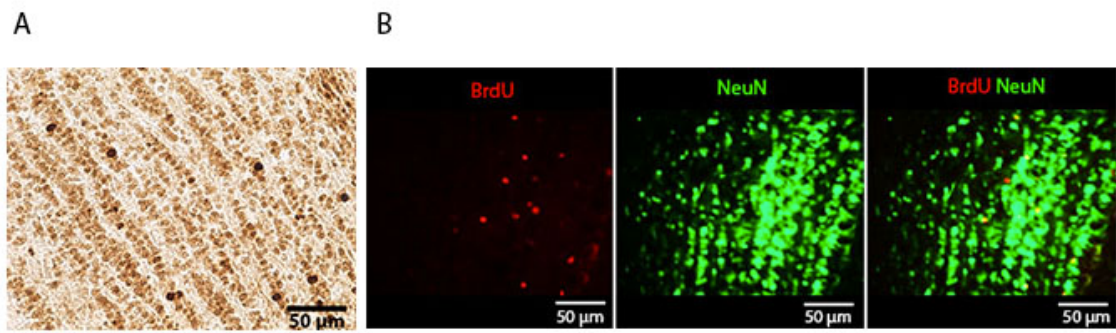


Figure 7.2. Immunohistochemistry example images. A) Bromodeoxyuridine (BrdU) and B) BrdU NeuN (Neuronal nuclear antigen) antibody staining.

Table 7.1. Olfactory perceptual learning odorant sets.

Name	Trial	0.1 Pa
+ Limonene	Limonene Habituation	2.0×10^{-4}
- Limonene	Limonene Enantiomer	2.0×10^{-4}
+ Carvone	Carvone Habituation	4.7×10^{-3}
- Carvone	Carvone Enantiomer	4.7×10^{-3}
+ Terpinen-4-ol	Terpinen-4-ol Habituation	6.6×10^{-3}
- Terpinen-4-ol	Terpinen-4-ol Enantiomer	6.6×10^{-3}

During the olfactory perceptual learning pretest and posttest, mice were behaviorally tested to see if they could distinguish within three sets of similar odorant molecules. Odorants were diluted in a carrier media, mineral oil to a vapor pressure of 0.1 Pa. The mice were always exposed to the plus form of the molecule first, followed by the minus form.

Table 7.2. Spontaneous olfactory discrimination odorant sets.

Trial	Name	Synonym	0.01 Pa
Hab	N-hexyl acetate	Apple, cherry, pear, floral	2.3×10^{-5}
S1	N-amyl acetate	Banana	7.2×10^{-6}
S2	N-butyl acetate	Fruity, diffusive	2.2×10^{-6}
D	Anisole	Phenyl methyl ether	5.2×10^{-6}
Hab	Propanoic acid (C3)	Propionic acid	1.3×10^{-5}
S1	Butanoic acid (C4)	Butyric acid	1.2×10^{-5}
S2	Pentanoic acid (C5)	Valeric acid	4.5×10^{-5}
D	5-methylfurfural	Sweet, spicy, warm, caramel, almond	3.0×10^{-5}
Hab	Octanal	Octyl aldehyde	1.5×10^{-5}
S1	Heptanal	Heptylaldehyde	7.1×10^{-6}
S2	Hexanal	Hexyl aldehyde, hexaldehyde	2.2×10^{-6}
D	Anisole	Phenyl methyl ether	5.2×10^{-6}
Hab	Octanoic acid (C8)	N/A	1.3×10^{-3}
S1	Heptanoic acid (C7)	Enanthoic Acid	4.6×10^{-4}
S2	Hexanoic acid (C6)	Caproic acid	1.5×10^{-4}
D	(R)-(+)-limonene	(+)-limonene	2.0×10^{-5}
Hab	Pentanol	1-pentanol	7.6×10^{-6}
S1	Hexanol	1-hexanol	2.6×10^{-5}
S2	Heptanol	1-heptanol	8.4×10^{-5}
D	Citronellal	Spicy, floral	1.7×10^{-4}

Each testing day began with four habituation trials (Hab1 – 4), followed by three novel odorants [moderately similar (S1), slightly similar (S2), and dissimilar (D) odorants delivered in a counter-balanced, randomized fashion]. Each block represents a different odorant set with the corresponding volume/volume dilution to 0.01 Pa in mineral oil. One odorant set was used per testing day. *Adapted from Bath et al., 2008.*

CHAPTER 8: CONCLUSION

Summary

Disturbances in olfactory sensory function are highly co-morbid with several neurological and psychiatric disorders, including Anxiety Disorder, Major Depressive Disorder, Alzheimer's Disease and Parkinson's Disease. In addition, preliminary work in our lab has indicated that early life stress (ELS), a major risk factor for pathology, impairs the ability of mice to learn odorant discrimination.

Since olfactory dysfunction may represent a biomarker of neurobiological disturbances, understanding the root cause of olfactory impairment could contribute to our understanding of the neurobiological basis of disorder, as well as inform future research and clinical interventions. In my dissertation, we tested the novel hypothesis that cholinergic projections to the olfactory bulb (OB) support learned discrimination of perceptually similar odorants by regulating the survival of newly born cells.

We used pharmacological and optogenetic approaches to manipulate cholinergic activity and assessed changes in perceptual discrimination abilities, adult neurogenesis and general olfactory function. After confirming that olfactory enrichment induces long-term changes in odorant discrimination, we revealed that the transient attenuation of cholinergic activity in the murine OB via acetylcholine receptor antagonist, Cogentin

during olfactory enrichment inhibited olfactory perceptual learning. Conversely, optogenetic stimulation of the nucleus of the horizontal limb of the diagonal band of Broca (HDB) fibers allowed for odorant discrimination within previously undistinguishable odorant pairs, even in the absence of enrichment. When we labeled and quantified newly born neurons in the OB granule cell layer (GCL), changes in newborn cell density were consistent with observed behavioral results; in comparison to control mice, pharmacological inhibition of cholinergic receptors led to a decrease in bromodeoxyuridine (BrdU) positive cell density and optogenetic stimulation of HDB fibers led to an increase in BrdU positive cell density. To establish whether a relationship exists between changes in neurogenesis and improved perceptual discrimination, we used the mitotic blocker, cytosine arabinoside (AraC) to inhibit neurogenesis in the lateral ventricles throughout the period of HDB cholinergic fiber optogenetic stimulation. We found that improvements in olfactory discrimination following cholinergic stimulation are dependent upon the presence of newly born cells in the OB.

Future Directions

This work provides strong evidence supporting the notion that neuromodulatory systems may support changes in perceptual abilities through their impact on neurogenesis. More work will be required to determine the unique contribution of the various cholinergic receptors to this process. In addition, future experiments will examine how acetylcholine impacts newly born cells. We will use Ki67 to see if our optogenetic manipulation affects cell proliferation, as well as caspase staining to examine cell apoptosis. Additional studies could include BrdU/cfos double labeling to determine if newly born cell survival is selectively enhanced in regions of the OB activated by odorant enrichment, OB cholinergic fiber density analysis to see if our manipulations impact the cholinergic fiber bundle and electrophysiological recordings of mitral and granule cells to determine if cholinergic modulation during an olfactory manipulation period alters OB neuron responsiveness.

Table

Table 8.1. Dissertation summary.

Chapter	Manipulation	Comparison	Cholinergic Activity	Olfactory Discrimination	Newly Born Cell Density
2	Saline Enrichment	Saline No Enrichment	N/A	Increase	Increase
3	Enrichment	No Enrichment	N/A	Increase: 0-, 1-, 3-month posttests No difference: 6-month posttest	N/A
4	Cogentin Enrichment	Saline Enrichment	Decrease	Decrease	Decrease
5	ChR2 + Stimulation	ChR2 – Stimulation	Increase	Increase	Increase
6	AraC Pump Stimulation	Saline Pump Stimulation	Equivalently Increase	Decrease	Decrease
<u>Conclusions</u> ↑Cholinergic Activity → ↑Newly Born Cell Density → ↑Discrimination					

STRESS EFFECTS ON BDNF EXPRESSION: EFFECTS OF AGE, SEX, AND FORM OF STRESS

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Abstract—Stress has long been associated with the development of neuropsychiatric and neurological disorders. The effects of stress vary depending upon the age during which the stress is incurred, the duration and severity of the stressor, and can further be influenced by levels of circulating gonadal hormones. To date, the majority of research investigating the link between stress and pathology development has focused on stress hormone secretion, receptor activity, and their impact on neuronal development and functioning in developing and adult male and female rodents. In recent years, work has begun to focus on additional neuromodulatory systems that may be significantly impacted by stress that may explain changes in developmental and sex-based susceptibility to stress. New research targets include molecules that play a role in neuronal development and plasticity. Specifically, stress-induced alterations in growth factors such as neurotrophins, in particular brain-derived neurotrophic factor (BDNF), have been identified as a strong candidate modulating stress-associated pathology. Furthermore, changing expression of BDNF and its receptors over development and in response to circulating gonadal hormones extend the attractiveness of this candidate signaling pathway for understanding differences in susceptibility to stress. This review focuses on what is known with regard to the effects of stress on neurotrophin expression in rodents, and the varied effects of stress on BDNF levels as a function of developmental status and sex.

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Key words: depression, anxiety, neurotrophins, plasticity, development, translational models.

INTRODUCTION

Stress has long been associated with the development of a variety of neuropsychiatric conditions and significantly influences the development and course of neurological disorders (Sapolsky et al., 1984; Sapolsky, 1999, 2000; Karatsoreos and McEwen, 2011). The effects of stress vary depending upon the age during which the stress is incurred and levels of circulating gonadal hormones (Luine, 2002; Luine et al., 2007). The primary targets for many researchers investigating stress-associated pathology have been the stress hormone corticosterone, factors mediating its release (corticotropin-releasing factor (CRF), adrenocorticotrophic hormone (ACTH)), and the activation of its receptors (mineralocorticoid and glucocorticoid receptors). However, in recent years work has begun to focus on additional neuromodulatory systems that may be significantly impacted by stress. New research targets include molecules and that play a role in neuronal development, plasticity, and have been implicated in stress-associated pathology development. Specifically, stress-induced alterations in growth factors such as neurotrophins, in particular brain-derived neurotrophic factor (BDNF), have been identified as a strong candidate pathway underlying stress-associated pathology. BDNF represents a particularly attractive target, as its expression is significantly influenced by stress, and altered neurotrophin expression has been associated with the development of affective pathology. Furthermore, BDNF levels change dramatically over development and in response to changes in circulating gonadal hormones, providing a potential additional mechanism through which the effects of stress vary as a function of age and sex. Here, we review recent work assessing the consequences of stress on neurotrophin expression throughout the brains of rodents. We highlight the complex relationship between stress and its impact on gene expression as a function of sex and age.

BDNF AND AFFECTIVE PATHOLOGY

In the clinic and in animal models, BDNF has been reliably linked with the development of affective disorders, including major depressive disorder (MDD), anxiety disorders, and post-traumatic stress disorder (PTSD) (Karege et al., 2002; Duman and Monteggia, 2006; Castren and Rantamaki, 2010; Dwivedi, 2010). Plasma BDNF levels are significantly lower in MDD patients compared with matched controls, and antidepressant treatment normalizes peripheral BDNF levels (Aydemir et al., 2005; Castren and Rantamaki, 2010; Molendijk

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Abbreviations: AMG, amygdala; BDNF, brain-derived neurotrophic factor; HC, Hippocampus; HYP, hypothalamus; MDD, major depressive disorder; PFC, prefrontal cortex; PirCTX, piriform cortex; VTA, ventral tegmental area.

et al., 2010). The degree of BDNF normalization is significantly correlated with symptom abatement (Gervasoni et al., 2005; Yoshimura et al., 2007; Tadic et al., 2010). In mouse models, genetically reducing BDNF levels, leads to increased anxiety and depressive-like behaviors (Chen et al., 2006; Duman and Monteggia, 2006). The direct administration or overexpression of BDNF has anxiolytic and antidepressant effects (Gourley et al., 2008; Deltheil et al., 2009). Exercise as well as antidepressant regimens augment BDNF in the hippocampus and are associated with decreased anxiety and positive outcomes (Nibuya et al., 1996; Russo-Neustadt et al., 2001; Garza et al., 2004; Koponen et al., 2004; Chen and Russo-Neustadt, 2007; Schmidt and Duman, 2010). Blocking the treatment induced augmentation of BDNF blocks the behavioral effects of antidepressants (Saarelainen et al., 2003; Chen et al., 2006; Ibarguen-Vargas et al., 2009). The culmination of these results have led to the development of a neurotrophic hypothesis of depression (Duman et al., 1997; Duman and Monteggia, 2006). The model suggests that diminished BDNF (through stress, genetic mutations, or pharmacological insult) leads to decreased trophic support, neuronal atrophy and death, and diminished neurogenesis which may underlie pathology development.

THE CHANGING ROLE OF BDNF OVER DEVELOPMENT

The expression of BDNF and its two primary receptors (TrkB and p75^{NTR}) are strictly controlled across development to guide the survival, differentiation, and plasticity of cells of the central nervous system (Labelle and Leclerc, 2000; Kuma et al., 2004). In rodents, throughout prenatal and early postnatal development, BDNF is expressed at very low levels. BDNF then undergoes a rapid increase in expression and peaks around 4 weeks of age followed by a decline over the lifespan (Kolbeck et al., 1999; Teng et al., 2005; Yang et al., 2009). BDNF exists in two forms, the preprocessed form (proBDNF) and the mature form which lacks the prodomain. These two isoforms of BDNF have differing affinities for the neurotrophin receptors TrkB and p75^{NTR}. Prototypically, mature BDNF binds to TrkB to promote neuronal survival, cellular differentiation, long-term potentiation (LTP), and dendritic outgrowth (Chao, 2003). proBDNF preferentially binds p75^{NTR} to mediate cell cycle arrest (Lopez-Sanchez and Frade, 2002; Cragnolini et al., 2009), synaptic long-term depression (LTD) (Woo et al., 2005), or apoptosis (Teng et al., 2005). In the embryonic and early postnatal brain, p75^{NTR} is highly expressed and then rapidly suppressed prior to elevations in BDNF expression, while levels of the full length form of TrkB remain relatively stable throughout the life of the animal (Yang et al., 2009). Interestingly, the expression or release of BDNF is significantly increased in response to estrogen (Sohrabji and Lewis, 2006; Begliuomini et al., 2007; Sato et al., 2007) with progesterone serving to counteract the effects of

estrogen on BDNF expression (Bimonte-Nelson et al., 2004). The modulation of BDNF by estrogen and progesterone may contribute to alterations in the expression of emotional pathology across sexual maturation and the estrous cycle and during the transitional period of peri-menopause (Forty et al., 2006; Cubeddu et al., 2010; Spencer et al., 2010). Environmental perturbations, such as stress or genetic mutation, which disrupt the fine balance in neurotrophin expression during sensitive periods in development can have lasting and deleterious effects on neural development and functioning, and may either contribute to the emergence of pathology (Bath et al., 2012) or diminish the effects of stress. An understanding of how these internal factors and environmental manipulations interact to potentially buffer or increase the likelihood of pathology, and the possible critical role played by BDNF, may provide important insights into the mechanisms underlying these debilitating and highly prevalent disorders.

IMPACT OF EARLY LIFE STRESS ON NEUROTROPHIN EXPRESSION

Prenatal stress exposure

Exposure to stress hormones *in utero* are known to have significant and detrimental effects on brain development. A number of lines of evidence have linked prenatal stress with increased risk for later development of anxiety and depressive disorders in rodent models (Weinstock, 2001, 2007; Abe et al., 2007). Recent work has begun to assess the impact of *in utero* stress exposure on neurotrophin expression. Interestingly, many of the studies only report the effects of prenatal stress on basal and stress-induced changes in neurotrophin gene expression when animals reach adulthood.

In rats, stress incurred during the last 7–10 days of gestation leads to alterations in BDNF expression that are strain and brain region dependent. The use of a variable stress (paradigm) in pregnant dams led to increased BDNF mRNA and decreased BDNF protein levels (proBDNF and mature BDNF) in the hippocampus of male Sprague Dawley and Lewis rats when pups reached adulthood, but had no effect on Fischer 344 rats (Neeley et al., 2011). Interestingly, prenatal stress impacted the way that BDNF changes in response to stress exposure in adulthood. In those same rats, Lewis and Sprague Dawley strains exposed to acute restraint showed a suppression in BDNF expression if the animal was previously stressed, whereas Fischer 344 rats that were previously stressed showed no change in BDNF expression following an acute stressor in adulthood (Neeley et al., 2011). Work by other groups have found that restraint stress of the pregnant dam leads to a significant decrease in BDNF protein levels in the prefrontal cortex and striatum of males when they reach adulthood (Fumagalli et al., 2004). However, these studies are contradictory to work by Zuena et al. (2008) who found that restraint of the pregnant rat led instead

to an increase in BDNF expression in male rat, and no change in BDNF expression in the female.

These studies are important, and indicate that early life stress can have long lasting effects on the expression of genes associated with neural development and ongoing plasticity. In addition, such findings suggest that early life stress can program later life stress-responsiveness of key genes involved in emotional regulation and cognitive functioning. These results also indicate that significant sex and strain differences in stress sensitivity with regard to neurotrophin expression, which would allow for the unique ability to compare and contrast the effects of these forms of stress on behavioral outcomes and relate them the effects on neurotrophin expression. However, more work is needed to assess the direct impact of prenatal stress on developmental expression of BDNF, and the impact of such changes on neurodevelopmental processes such as cell proliferation, migration, synaptogenesis, and apoptosis during early time points. In addition, more studies will be valuable in clarifying discrepancies in the literature at adult time points.

Stress as a consequence of diminished maternal care

Manipulations of maternal care are commonly used to induce stress in young rodents. In rats and mice, these manipulations often occur during the first 14 days of postnatal life, which approximate the third trimester of fetal development and early postnatal development in humans. The most commonly employed methods include the use of maternal separation (removing the mother daily for short bouts of time across several days), maternal deprivation (removing the mother for an extended time on a single occasion), and finally investigating the effects of natural variations in maternal care (high vs low care). Many of these paradigms have been associated with disruptions in later cognitive and emotional functioning of the animal (Romeo et al., 2003; Millstein and Holmes, 2007) and have potent and long lasting effects on neurotrophin expression.

In rats, maternal separation stress leads to decreased BDNF protein and mRNA levels in hippocampus (HC) of stressed relative to control rats as they reached adulthood (Lippmann et al., 2007; Maniam and Morris, 2011; Lee et al., 2012). These effects were largely restricted to the mature form of BDNF, with no changes being reported for proBDNF levels (Lippmann et al., 2007). The effects of maternal separation appear to be region specific, as this same manipulation led to increased BDNF expression in the ventral tegmental area (VTA) (Lippmann et al., 2007). In addition, levels of BDNF vary over development, as do the effects of stress on BDNF expression relative to control animals. For example, in the cerebral cortex, maternal separation stress led to a transient increase in BDNF mRNA expression from P15–P30, followed by reduced BDNF levels compared with control rats at P60 (Lee et al., 2012). The impact of this form of stress on neurotrophin receptor expression has also been studied. Maternal

separation led to a transient increase in TrkB expression in the cortex during the preadolescent and adolescence period (P16–P30) and a significant reduction in TrkB expression in early adulthood (P60) (Lee et al., 2012). Using similar paradigms, others have found that maternal separation leads to elevations in the expression of the pan-neurotrophin receptor, p75^{NTR}, in the medial septum (mS) that appears to persist into adulthood (Aisa et al., 2009).

Investigators using the more acute maternal deprivation stress paradigm, have found similar effects on BDNF levels to those reported following maternal separation. Exposing rat pups to maternal deprivation from P9 to P10 led to decreased expression of BDNF mRNA and protein in the adult HC compared with typically reared animals. However, other groups, using virtually identical procedures, have failed to replicate that effect (Choy et al., 2008), but found that maternal deprivation led to a more robust decrease in BDNF when rats were later exposed to corticosterone. The effects of maternal deprivation stress also depended upon the time at which BDNF levels are assayed and the sex of the animal being tested. Viveros et al. (2010) found that maternal deprivation led to a blunting of circadian changes in BDNF expression in the hypothalamus (HYP) of male but not female rat pups. Interestingly, in that same study, following the return of rats to the dam, females showed a significant augmentation in BDNF expression compared with levels observed at the same time in control rats.

The use of more naturalistic manipulations, in which investigators track the quality of maternal behavior, or expose animals to poor maternal care during the first week of life, have also provided interesting results. Rat pups that received low levels of maternal care showed significant reductions in BDNF expression throughout the HC as adults (Liu et al., 2000). These results appear to mirror what is seen at younger ages, where other groups have found that at weaning, rat litters receiving low care have reduced BDNF levels in the HC compared with litters receiving high care (Macri et al., 2010). Studies by Roth et al., using a paradigm in which rat pups are exposed to abusive dams, found a similar decrease in BDNF expression in the prefrontal cortex (PFC, but no change in the HC), and identified epigenetic changes as a potential mechanism mediating this effect (Roth et al., 2009).

These results provide interesting parallels to findings in human population. Work by Grassi-Oliveira and colleagues found that women who were exposed to maternal neglect tend to have higher rates of MDD in adulthood, and that exposure to physical neglect leads to decreased levels of salivary BDNF, even below that of patients with MDD who did not experience early life physical neglect (Grassi-Oliveira et al., 2008). Ongoing work in this area has the potential to more clearly identify the consequences of decreased BDNF levels following early life stress on neural development and functioning to better understand the neural basis of stress-associated pathology.

IMPACT OF LATER LIFE STRESSORS ON NEUROTROPHIN EXPRESSION

Social stress and neurotrophin expression

Manipulating social interactions in rodents have become a powerful methodology for the induction of pathological behavior, including the development of anxiety and depressive-like behaviors (Berton et al., 2006; Vialou et al., 2010). In these model systems, the time of stress induction can vary from prepubescent animals to aged models. Paradigms in which stress is induced through modulation of social interaction include the use of repeated social defeat, social isolation, or the implementation of a high degree of social variability (which often involves increased levels of social agonism as a function of repeated development of social hierarchies when new cage mates are introduced).

The majority of studies investigating effects on neurotrophin expression have used the social defeat paradigm. For this form of stress, effects have been reported across species, with repeated social defeat leading to an acute increase in BDNF expression in the PFC, nucleus accumbens (nAcc), amygdala (AMG), and VTA of rats (Nikulina et al., 2012), while defeat stress leads to a decreased BDNF levels in HC and piriform cortex (PirCTX) of golden hamster (Arendt et al., 2012), and decreased BDNF mRNA in the HC, PirCTX, and AMG of male mice (Pizarro et al., 2004).

Studies in which high degrees of social variability have been used as a means to induce stress have found significant decreases in BDNF levels in the HC of these animals as they reach old age (12 months), suggesting a potential impact of this form of stress on age related changes in markers of plasticity (Sterlemann et al., 2010). The use of social isolation as a means of inducing stress, leads to a similar decrease in BDNF expression in nearly all brain regions studied, with similar effects being observed in mice and rats. Specifically, social isolation of mice leads to decreased BDNF protein levels in the midbrain, HYP, PFC, and HC (Berry et al., 2012), and social isolation in rats leading to decreased BDNF levels in the HC and parietal cortex (ParCTX) (Barrientos et al., 2003).

The results of studies using social defeat in many models are difficult to interpret as the level and duration of agonistic behavior is often difficult to accurately quantify or control. In addition, agonism can lead to significant physical pain as a consequence of bites, and the contribution of chronic pain are similarly difficult to disentangle. However, the presence of reliable effects, despite a lack of clear control, suggest the potency of this form of stress to induce significant and lasting changes in gene expression. Furthermore, many of these studies are restricted to male rodents, as eliciting agonism in female rodents over the course of multiple days can be significantly affected by changes in circulating gonadal hormones and changes in sexual receptivity, limiting our ability to generalize these effects across sexes.

Restraint/immobilization (chronic and acute)

Restraint stress is one of the most commonly used means to induce stress in rodent models. Unlike social stressors, it is highly controllable, and can be employed to assess acute stress or repeated stress events. In addition, restraint stress can be combined with other stressors, such as predator odor to enhance the effectiveness of this form of stress. A variety of studies have employed this methodology to assess its effect on neuronal plasticity, including neurogenesis and dendritic remodeling, as well as its impact on long-term behavioral outcomes (including enhanced anxiety and depressive-like behaviors).

Many studies have focused on the use of the acute form of this stress which include the restraint of the animal in wire mesh restrainer, plastic cone, or strapping an animal to a board in the supine position on a single occasion for between 30 min and 6 h. Using these forms of stress, multiple groups have shown dynamic changes in BDNF expression as a function of the duration and type of stressor and time at which BDNF levels were assayed.

In rats, acute restraint led to rapid increase in HC BDNF mRNA (as soon as 60 min following the beginning of stress. By 180 min, mRNA levels in stressed rats had decreased rapidly to levels significantly below that of controls. This is effect was also evident at the level of BDNF protein level, which were elevated in HC compared with controls at 180 min, and then fell to levels that were indistinguishable from controls by 300 min (Marmigere et al., 2003). Other groups using slightly different stress paradigms, often with shorter duration, found that acute stress led to decrease in HC BDNF protein expression (Franklin and Perrot-Sinal, 2006; Roth and Sweatt, 2011), while mRNA levels were found by some to decrease (Ueyama et al., 1997; Lee et al., 2008; Roth and Sweatt, 2011) and other to increase (Nair et al., 2007; Alboni et al., 2011) relative to unstressed controls. The effects of this form of stress were not uniform across all brain regions, with some groups demonstrating a transient upregulation of BDNF in the HYP (Rage et al., 2002), and other demonstrating no effect of this form of stress on BDNF levels in the basolateral amygdala (BLA) or PFC (Roth and Sweatt, 2011).

Several groups have employed the use of more prolonged restraint stress paradigms to assess its impact on neurotrophin expression, with varied results. Repeated restraint led to a significant decrease in BDNF levels in the HC (Naert et al., 2011; Lakshminarasimhan and Chattarji, 2012), an effect that also occurs in females (Takuma et al., 2007) and is exacerbated by ovariectomy (Takuma et al., 2007). However, some groups have failed to find any changes in BDNF levels following repeated restraint stress (Kuroda and McEwen, 1998; Rosenbrock et al., 2005; Magarinos et al., 2011), and others have suggested that the effects on BDNF levels in the HC are present but transient (Lakshminarasimhan and Chattarji, 2012). The same groups that find transient effects of chronic restraint stress in HC have found that this same stressor leads to

Table 1. Summary of the effects of various forms of stress on BDNF levels in the hippocampus, amygdala, and cortex of rodents. These results highlight the varied effects of stress on BDNF as a function of age, brain region, and timing between cessation of stress and assay of BDNF levels. Given the very small number of studies assessing BDNF in females, outcomes were not further broken down by sex, and thus results are largely indicative of effects in male rodents

Stressor	Stress period	Assay period	Species	Region Assayed		
				Hippocampus	Amygdala	Cortex
<i>Maternal stress</i>						
Maternal deprivation	P9–P10	Adult	Rat	Mixed		
Maternal separation	P2–P14	Adolescent	Rat	Increase		Increase
Maternal separation	P2–P14	Adult	Rat	Decrease		Decrease
<i>Social stress</i>						
Social defeat	Early adult	Adult	Rat		Increase	Increase
Social defeat	Early adult	Adult	Mouse	Decrease	Decrease	Decrease
Social isolation	Adult	Adult	Rat/mouse	Decrease		Decreased
Social variability	Adult	Aged	Mouse	Decrease		
<i>Other stressors</i>						
Acute restraint	Adult	Immediate	Rat	Increase		
Acute restraint	Adult	Delayed	Rat	Decrease		
chronic Restraint	Adult	Adult	Rat	Decrease and N/C	Increase	
Unpredictable stressor	Adult	Adult	Rat/mouse	Decrease		

a significant and lasting increase in BDNF levels in the AMG, which is associated with hypertrophy of this region and increased anxiety-like behavior (Lakshminarasimhan and Chattarji, 2012).

These results taken together suggest a complex relationship between the dose and duration of stress on neurotrophin levels, that appears to depend significantly on the region being studied as well as the duration between the cessation of stress and the time at which you measure BDNF levels. Such effects could explain transient changes in affect and cognition following either acute or chronic stress, however, more studies will be needed to bear out the direction relationship between changing BDNF levels and these measures.

Footshock/unpredictable stress

The use of repeated variable footshock has been used to induce a form of learned helplessness in animals, and milder forms of this paradigm can contribute to the development of anxiety and depressive-like phenotypes in rodents. A number of groups have indicated that males and females respond quite differently to this form of stress (Shors et al., 2007; Dalla et al., 2008). Several studies have begun to assess the effects of this form of stress on neurotrophin levels in the brain of rats and mice, with interesting results across sexes being observed. In adult male rats, repeated footshock leads to decrease in the expression of BDNF in the dentate gyrus of these animals (Rasmusson et al., 2002). Using a similar paradigm in rats, the acute application of footshock led to an increase in BDNF expression in the hippocampus of female but not male rats (Lin et al., 2009). When this paradigm was extended to multiple sessions of footshock over several days, again females were solely affected and were found to have a significant decrease in BDNF levels in both the hippocampus and PFC compared with control animals (Lin et al., 2009). These results are similar to those

obtained in mice using a variable stress paradigm that included the use of footshock. However, in mice chronic stress led to decreased BDNF expression in the hippocampus of both males and females (Autry et al., 2009). Based upon these findings, BDNF levels in female appear to be particularly susceptible to this form of physical stress.

CONCLUSIONS

Throughout this review we have highlighted the effects of various forms of stress on BDNF expression in males and females rodents across development (summarized in Table 1). In reviewing this literature, it has become clear that stress can have potent effects on the on expression of neurotrophins, in particular BDNF, and that these effects are highly sensitive to the form, duration, and timing of stress as well as the sex of the subject receiving the stress and the brain region being studied. Based upon the previously established linkage between neurotrophin expression and affective pathology development and the emerging linkage between stress and neurotrophin expression, more work will be required in this area to understand the potential direct contributions of stress associated changes in BDNF expression with pathology development. The emergence of new genetic and molecular tools which will allow for timed elimination or expression of neurotrophins or direct modulation of their receptors in the context of stress have the potential to more clearly delineate the relationship between gene expression and stress-associated outcomes. Should this relationship bear out, targeting of neurotrophins and their receptors may provide a novel target for the treatment of stress associated illness, including affective pathology.

CONFLICTS OF INTEREST

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Original Article

Fragile X Mental Retardation Protein Regulates Olfactory Sensitivity But Not Odorant Discrimination

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Abstract

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and is characterized by cognitive impairments and altered sensory function. It is caused by absence of fragile X mental retardation protein (FMRP), an RNA-binding protein essential for normal synaptic plasticity and function. Animal models have provided important insights into mechanisms through which loss of FMRP impacts cognitive and sensory development and function. While FMRP is highly enriched in the developing and adult olfactory bulb (OB), its role in olfactory sensory function remains poorly understood. Here, we used a mouse model of FXS, the *fmr1*^{−/y} mouse, to test whether loss of FMRP impacts olfactory discrimination, habituation, or sensitivity using a spontaneous olfactory cross-habituation task at a range of odorant concentrations. We demonstrated that *fmr1*^{−/y} mice have a significant decrease in olfactory sensitivity compared with wild type controls. When we controlled for differences in sensitivity, we found no effect of loss of FMRP on the ability to habituate to or spontaneously discriminate between odorants. These data indicate that loss of FMRP significantly alters olfactory sensitivity, but not other facets of basal olfactory function. These findings have important implications for future studies aimed at understanding the role of FMRP on sensory functioning.

Key words: behavior, discrimination, FMRP, mouse, olfaction, sensitivity

Introduction

Fragile X syndrome (FXS) is an X-linked genetic disease and is the most common form of inherited intellectual disability (Hagerman et al. 2009). It is caused by the expansion of a trinucleotide (CGG) repeat at the 5′ untranslated region of the fragile X mental retardation 1 gene (*FMR1*) (O'Donnell and Warren 2002; Santoro et al. 2012). This expansion leads to DNA methylation in the CGG repeats

and *FMR1* promoter, preventing *FMR1* transcription and its protein product, fragile X mental retardation protein (FMRP) (Pieretti et al. 1991; Verheij et al. 1993; Coffee et al. 2002). More rare forms of FXS arise from deletions of *FMR1* or point mutations in the promoter, protein-coding sequence, introns, or untranslated regions (Gedeon et al. 1992; De Boulle et al. 1993; Collins et al. 2010).

FXS has been associated with alterations in sensory development and function, including tactile and auditory hypersensitivity

(Rogers et al. 2003; Tomchek and Dunn 2007; Baranek et al. 2008; Rotschafer and Razak 2014). FXS has also been associated with enhanced sympathetic arousal in response to stimulation across multiple other sensory domains, including olfaction, an effect that does not habituate to repeated stimulus presentation (Miller et al. 1999). Based upon work in the mouse model of FXS (*fmr1*^{-/-}), enhanced tactile and auditory sensitivity have been attributed to alterations in cellular excitability and channel sensitivity in primary somatosensory cortices (Rotschafer and Razak 2014; Zhang et al. 2014). Studying the effects of loss of FMRP on sensory development holds great potential for understanding the contribution of FMRP to neural development and function, as well as the identification of potential targets for treatment of FXS.

There is reason to believe that the effects of FMRP on sensory development extend beyond the auditory and tactile domain. FMRP is expressed at high levels in the developing and adult olfactory system (Christie et al. 2009; Akins et al. 2012). It is localized in the somatodendritic compartment in olfactory glomerular, granule cell, and nerve layers. FMRP is also localized in the axons of mature olfactory sensory neurons, where it exists in distinctive fragile X granules (FXGs) (Christie et al. 2009; Akins et al. 2012). Notably, in many brain regions, FXGs are expressed only during times of synaptic integration (approximately P15–P30 in the mouse). However, in the olfactory system, these axonal FMRP-containing structures persist into adulthood (Christie et al. 2009; Akins et al. 2012). This distinctive developmental profile suggests that FMRP may play a role in both olfactory development and ongoing olfactory sensory function. While impairments in olfactory identification have been described in a related disorder, fragile X tremor ataxia syndrome (FXTAS) (Juncos et al. 2012), to our knowledge, olfactory perceptual abilities have not been well characterized in FXS patient populations. In *Drosophila*, FMRP (dFMR1) has been shown to be necessary for long-term, but not short-term olfactory habituation, as indicated by an olfactory avoidance task (Sudhakaran et al. 2014). In *fmr1*^{-/-} mice, there are indications that learning to discriminate olfactory stimuli may be impaired (Larson et al. 2008). However, that study used a reinforced responding paradigm, which may recruit additional neuromodulatory systems that could significantly alter attention and sensory processes, masking alterations in basal functioning of the system. To date, no studies have examined basal olfactory function in the absence of a learning paradigm. The identification and characterization of alterations in olfactory sensory function have the potential to provide additional insights into the role of this protein on neural development and function, with implications for understanding system disturbance in FXS.

In the current study, we examined the role of FMRP on basal olfactory sensory processing by using the mouse model of fragile X (*fmr1*^{-/-} mice) and a spontaneous discrimination paradigm. We compared the performance of wild type (WT) and *fmr1*^{-/-} mice on a spontaneous olfactory cross-habituation task, in which we tested for effects of gene deletion on olfactory sensitivity, habituation, and discrimination ability. We found that *fmr1*^{-/-} mice had a significant decrease in olfactory sensitivity. When we controlled for differences in sensitivity, WT and *fmr1*^{-/-} mice did not differ in their ability to habituate to or spontaneously discriminate between even similar olfactory stimuli. Taken together, our results demonstrate that FMRP is important for olfactory sensitivity but not olfactory habituation nor spontaneous discrimination.

Materials and methods

Animals

Nine- to sixteen-week old male WT ($n = 10$) and *fmr1*^{-/-} mice ($n = 12$) (Bakker et al. 1994) on a C57Bl6/N background (Charles River Laboratories, Wilmington, MA) were bred in-house. Prior to weaning, mice were tail tipped and genotyped (Transnetyx, Cordova, TN). Following weaning, mice were group housed on a 12 h light/12 h dark cycle with water and food provided ad libitum. Testing occurred during the first half of the light phase of the light/dark cycle. All experiments were approved by the Brown University's IACUC, and carried out in accordance with the guide for the care and use of laboratory animals.

Methods

To assess olfactory sensory function in WT and *fmr1*^{-/-} mice, we employed a spontaneous olfactory cross-habituation task (Cleland et al. 2002; Bath et al. 2008). Each mouse was placed individually into a polycarbonate cage. For each trial, the odorants were presented by placing 100 μ L of the diluted odorant onto a piece of filter paper (Whatman, Florham Park, NJ), and placing the filter paper inside a tea ball. The tea ball was subsequently draped inside the upper corner of the cage. Odorants were diluted in the odorless carrier media, mineral oil (MO). The mice first habituated to MO as well as the tea ball carrier for 2, 1-min trials. Mice were then presented with a single aliphatic acid odorant on 4 consecutive trials (habituation). The final 3 trials consisted of 3 novel odorants: a highly similar (S1—differing by a single unbranched hydrocarbon from the habituation odorant), moderately similar (S2—differing by 2 unbranched hydrocarbons from the habituation odorant), and dissimilar odorant (D—structurally and perceptually unique odorant), presented in a counter-balanced randomized order. All trials were 1 min in duration with a 5-min inter-trial interval. This procedure was repeated with 5 different odorant sets (Supplementary Table 1) over the course of 5 days. To assess differences in olfactory sensitivity, the above procedure was carried out using 4 different odorant concentrations across 4 weeks of testing (with all 5 odorant sets repeated at each concentration). Stimulus odorants were diluted in MO to 0.001, 0.01, 0.1, or 1.0 Pascal (Pa) vapor pressure prior to testing. Volume/volume dilutions varied by odorant (Supplementary Table 2) and vapor pressures were estimated using ACD/ChemSketch software (Advanced Chemistry Development, Toronto, Ontario, Canada). The order of odorant concentrations was counterbalanced across weeks, but concentration was held constant within each given week (5-day period). During testing, mice were video recorded with Ethovision XT 8.5 tracking software (Noldus). Following testing, time spent sniffing the tea ball (measured by sniffing within 1 inch of the tea ball) was measured with the rater blind to genotype, odorant identity, and concentration. Following scoring, the 5 days of testing within each odorant concentration were averaged, and the data was pooled by trial type, genotype, and concentration to assess the effects of genotype on odor detection, habituation, and cross-habituation.

Statistical analysis

To determine the effects of gene deletion on olfactory sensitivity and habituation at each concentration, we conducted repeated measures ANOVAs with trial as our within subjects factor and genotype as our between subjects factor. For each individual genotype, we used repeated measures ANOVAs with trial as our within subjects factor to test for effects of odorant concentration on cross-habituation between the MO and the first habituation (Hab1) trials. To assess

ability of mice to discriminate odorants, we carried out a 1-way ANOVA followed by paired *t*-tests (with Bonferroni corrections for multiple tests) between Hab4 and S1, S2 and D. Statistical analyses were conducted using IBM SPSS Statistics 20, excluding outlier trials that deviated by ± 2 or more standard deviations from the mean [71 of 1800 control trials ($\sim 3\%$ of trials), 96 of 2160 FMRP trials ($\sim 4\%$ of trials)]. Alpha was set to 0.05.

Results

Olfactory sensitivity

To test the effect of *fmr1* gene deletion on olfactory sensitivity (the ability to detect the presence of an odorant), we looked at the first portion of the spontaneous olfactory cross-habituation task, the transition from MO to the first habituation (Hab1) trial. Briefly, mice received 2 habituation trials where they were exposed to a tea ball containing only the carrier media (MO) and were then presented with a tea ball containing an odorant diluted in MO to a vapor pressure of 0.001, 0.01, 0.1, or 1.0 Pa. Within each odorant concentration, 5 different habituation odorants were used on 5 consecutive days. The investigation times from the 5 trials within each odorant concentration were averaged. Failure to show increased investigation on the Hab1 trial compared with the MO trial indicated cross-habituation, or an inability to distinguish the vehicle from the odorant. When comparing the second MO trial and Hab1 across 4 different odorant concentrations, WT mice showed cross-habituation at the 0.001 Pa concentration ($F_{1,9} = 1.669$, $P >$

0.05; Table 1). WT mice significantly discriminated Hab1 odorants from MO at 0.01 Pa ($F_{1,9} = 20.704$, $P = 0.001$) and 0.1 Pa ($F_{1,9} = 48.375$, $P < 0.001$; Table 1). WT mice did not show significant discrimination at 1.0 Pa ($F_{1,9} = 1.986$, $P > 0.05$, Table 1). For *fmr1*^{-/-} mice, we found cross-habituation between MO and Hab1 at concentrations of both 0.001 Pa ($F_{1,11} = 1.198$, $P > 0.05$) and 0.01 Pa ($F_{1,11} = 0.227$, $P > 0.05$; Table 1). *Fmr1*^{-/-} mice showed statistically significant discrimination only at 0.1 Pa ($F_{1,11} = 26.065$, $P < 0.001$) and 1.0 Pa ($F_{1,11} = 11.465$, $P = 0.006$; Table 1). We found a significant interaction for investigation time between WT and *fmr1*^{-/-} mice at 0.01 Pa ($F_{1,20} = 20.977$, $P < 0.001$; Table 1), indicating differences in odorant investigation by genotype at this concentration. Since neither genotype displayed an increase in investigation at the 0.001 Pa level, we omitted this concentration from further analysis.

Habituation

To test if loss of *fmr1* impacts the ability of mice to habituate to repeated odorant presentation, we tested for differences in investigation times across odorant habituation trials (Hab1–Hab4) at the lowest odorant concentration successfully detected (0.01 Pa WT, 0.1 Pa *fmr1*^{-/-}) as well as highest concentration discriminated (0.1 Pa WT and 1.0 Pa *fmr1*^{-/-}).

If mice successfully habituate to the odorants, they should demonstrate a significant decrease in investigation time across the 4 habituation trials. At the lowest concentration detected, we found a statistically significant reduction in investigation time over the 4 habituation trials for both WT 0.01 Pa ($F_{3,7} = 8.963$, $P = 0.009$) and *fmr1*^{-/-} 0.1 Pa ($F_{3,9} = 8.834$, $P = 0.005$) mice (Figure 1a). At the highest concentration discriminated, we again observed a significant reduction in investigation time over the 4 habituation trials for WT 0.1 Pa ($F_{3,7} = 22.935$, $P = 0.001$) and *fmr1*^{-/-} 1.0 Pa ($F_{3,9} = 8.212$, $P = 0.006$) mice (Figure 1b). Direct comparison of habituation at each concentration was also carried out within group (Supplementary Figure 1).

Cross-habituation (discrimination)

In order to test the ability of WT and *fmr1*^{-/-} mice to discriminate between odorants, we utilized the later portion of the spontaneous olfactory cross-habituation task. Following habituation (Hab1–Hab4), animals were presented with the S1, S2, and D odorants in a counterbalanced and randomized order. We used 5 different S1, S2, and D odorants and 5 different habituation odorants over a 5-day period; the resulting investigation times were averaged. If mice failed to detect the odorant as new, they should cross-habituate

Table 1 Olfactory sensitivity differs between WT and *fmr1*^{-/-} mice

Concentration	WT, s ($\pm$ SEM)		<i>Fmr1</i> ^{-/-} , s ($\pm$ SEM)	
	MO2	Hab1	MO2	Hab1
0.001 Pa	3.0 (0.7)	2.6 (0.5)	2.8 (0.6)	2.7 (0.6)
0.01 Pa ^{5,***}	2.6 (0.5)	5.7 (1.1)***	2.4 (0.4)	2.2 (0.5)
0.1 Pa ⁵	2.0 (0.3)	3.9 (0.5)***	2.6 (0.5)	5.9 (1.1)***
1.0 Pa	2.7 (0.6)	3.5 (0.5)	3.0 (0.6)	5.0 (1.0)**

Mean investigation time for WT and *fmr1*^{-/-} mice ($\pm$ SEM) on the final acclimation trial, mineral oil 2 (MO2) and initial odorant presentation trial (Hab1) at 4 different odorant concentrations (0.001–1.0 Pa). Five aliphatic acid odorants were tested at each odorant concentration and the resulting investigation times were averaged. To test for effects of trial and/or genotype on investigation time, we used repeated measures ANOVA analyses. We tested for main effect of trial ⁵ $P < 0.01$, trial $\times$ genotype interaction *** $P \leq 0.001$, and follow-up comparisons (MO2 vs. Hab1) ** $P < 0.01$, *** $P \leq 0.001$.

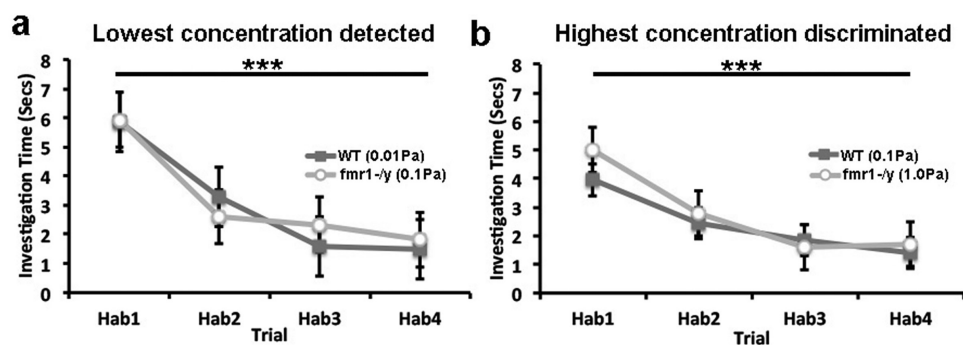


Figure 1. Loss of *fmr1* does not impact olfactory habituation. Five odorants were averaged to create the investigation time presented. Investigation time ($\pm$ SEM) is plotted across the 4 habituation trials (Hab1–4) for (a) the lowest detected concentration and (b) highest discriminated concentration of odorants tested for WT and *fmr1*^{-/-} mice. Statistical comparison were carried out using a 1-way ANOVA, main effect of trial, *** $P \leq 0.001$.

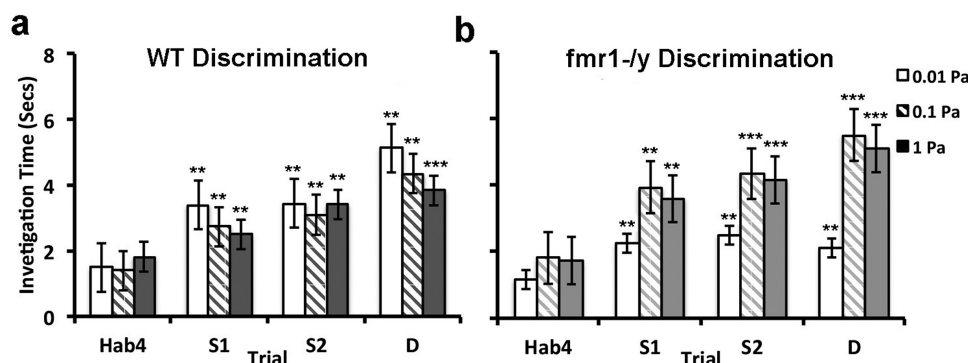


Figure 2. Olfactory discrimination ability is concentration- and genotype-dependent. Bar graphs plotting the average investigation time ($\pm$ SEM) for the Hab4 = final habituation, S1 = strongly similar, S2 = moderately similar, D = dissimilar odorant trials. Data is presented for (a) WT and (b) *fmr1*^{-/-} mice. Five unique odorants were used for each novel trial. Statistical comparisons were carried out with GLM repeated measures ANOVA, with post hoc comparisons and Bonferroni corrections for multiple tests, ** $P < 0.01$, *** $P \leq 0.001$.

(show no increase in investigation time relative to Hab4). If the mice successfully discriminate the odorants, they should increase their investigation time to the new odorant, relative to Hab4. We tested for differences in sniffing time on Hab4 relative to S1, S2, and D trials using GLM repeated measures ANOVA with post hoc comparisons. At the lowest concentration detected by each genotype, we found a significant main effect of trial for both WT 0.01 Pa ($F_{3,7} = 5.061$, $P = 0.036$) and *fmr1*^{-/-} 0.1 Pa ($F_{3,9} = 12.178$, $P = 0.002$) mice (Figure 2a). We also found a significant main effect of trial at the highest concentration discriminated (WT 0.1 Pa, $F_{3,7} = 4.602$, $P = 0.044$; *fmr1*^{-/-} 1.0 Pa, $F_{3,9} = 9.722$, $P = 0.003$). Post hoc comparisons showed that both WT and *fmr1*^{-/-} mice significantly discriminated S1, S2, and D trial odorants compared with Hab4 (Bonferroni corrections for multiple tests; $P < 0.05$; Figure 2b). Further analysis revealed that WT mice show a significant difference between S1 and D at all concentrations and S2 and D at 0.01 and 0.1 Pa. However, in *fmr1*^{-/-} mice, we only observed a significant effect on odorant type at the 1.0 Pa concentration with a significant difference in investigation time between S1 and D, and a marginal effect between S2 and D (Supplementary Table 3).

Discussion

In the current study, we used a spontaneous olfactory discrimination task at 4 different odorant concentrations to test for changes in odorant sensitivity and the ability of *fmr1*^{-/-} mice to habituate to or discriminate between odorants compared with WT controls. We found that *fmr1*^{-/-} mice have a lower sensitivity to detect low concentration odorants than control mice. However, after controlling for differences in sensitivity, we observed no differences in the ability of *fmr1*^{-/-} mice to habituate to repeated presentation of an odorant or discriminate between very similar or dissimilar odorants using a spontaneous olfactory discrimination task (olfactory cross-habituation).

When assessing olfactory sensitivity in WT mice, we observed a trend where WT mice showed increased investigation time as the odorant concentration increased, but then failed to investigate the odorant when the concentration reached 1.0 Pa. This failure could be the consequence of the high concentration of odorant permeating the cage, obviating the need for mice to approach and directly smell the source of the odorant, or possibly an aversion to the high odorant concentration. Importantly, in *fmr1*^{-/-} mice, we observed what appears to be a decrease in sensitivity, with decreased investigation

at lower odorant concentrations, but intact discrimination even at the highest odorant concentration (1.0 Pa). Based upon the cross-habituation between MO2 and Hab1 trials in *fmr1*^{-/-} mice at low concentrations, we conclude that *fmr1*^{-/-} mice require higher concentrations of odorants than WT mice in order to detect the stimulus.

When we controlled for differences in sensitivity and tested the ability of mice to discriminate between odorants at the lowest concentration of odorant successfully detected, we observed no differences in habituation or discrimination ability between WT and *fmr1*^{-/-} mice. Our failure to detect effects of gene knockout on discrimination ability was particularly surprising, as other reports have indicated that *fmr1*^{-/-} mice have impairments in the ability to learn to discriminate olfactory signals (Larson et al. 2008). However, that study used an active learning paradigm with reinforced responding. Previous work has indicated that reinforced responding could significantly influence discrimination abilities, possibly through the recruitment of feedforward neuromodulatory systems (Cleland et al. 2002). It is possible that the results from Larson et al. (2008) were the consequence of impaired learning and may not have been due to differences in basal discrimination abilities across genotypes. Alternatively, the recruitment of these feedforward systems may enhance sensitivity or attention to the stimulus, providing a measure of optimal performance of the system. However, such methods may fail to detect differences in basal detection and processing of odorants, which may have greater relevance for detecting and processing ambient signals in the environment. The current work strongly suggests that loss of FMRP impacts basal sensory function, and possibly the development of the olfactory system. Future studies will help to arbitrate between the current findings and previous work using alternative methods to assess olfactory sensory function (Larson et al. 2008).

Our observation that loss of FMRP leads to decreased olfactory sensitivity could be the result of altered development of the olfactory system or more acute effects on neuronal function. FMRP is expressed in olfactory sensory neurons as well as in the glomerular, granule cell, and olfactory nerve layers of the olfactory bulb (OB) (Christie et al. 2009; Akins et al. 2012). However, few studies have assessed the impact of loss of FMRP on the development and organization of the OB. Loss of FMRP could impact receptor density, lead to mistargeting of olfactory receptor axons, or alter ion channel distribution in the OB or olfactory cortex. However, in the current study, we did not carry out anatomical analyses of the OB to test any of these hypotheses.

There is also significant reason to believe that the effect of FMRP may extend beyond the development of the OB and continue to play

a role in olfactory function during adulthood. While axonal FMRP is highly expressed in the OB early in life and associated with FXGs in virtually all brain regions, FXG expression diminishes over early development. However, in the OB, FXG levels remain high into adulthood (Christie et al. 2009). The continued expression of FXGs in the adult structure could indicate an ongoing role for FMRP in the regulation of olfactory sensory function or plasticity. As an example, loss of *fmr1* leads to altered structure and function of adult born granule cells, resulting in an increase in granule cell spine density and length, and diminished spinogenesis (Scotto-Lomassese et al. 2011). The phenotype of *fmr1*^{-/-} dendritic spines mimics the look of immature dendritic spines (Scotto-Lomassese et al. 2011). FMRP also has a role in activity-dependent synapse pruning and dendritic remodeling (Pfeiffer et al. 2010; Scotto-Lomassese et al. 2011). The morphological and functional changes in granule cells could lead to an over exuberance of inhibition and ultimately a dampening of sensitivity. More work will be required to understand the direct relationship between these morphological changes and olfactory abilities.

In addition to potential effects of FMRP on olfactory granule cell dendritic development, FMRP may also play a role in axonal development and functioning. FMRP has been classically defined by its postsynaptic functions in somata and dendrites. However, the protein and its homologs, FXR1P and FXR2P, are also localized axonally and presynaptically into FXGs in the OB (Christie et al. 2009; Akins et al. 2012). While the functional role of FMRP in the presynaptic compartment is not yet known, it is possible that loss of FMRP could impact axonal targeting, synaptic development and stabilization, and ultimately synaptic transmission, with consequences on olfactory sensory function.

Conclusions

In the current study, we found that olfactory sensitivity was diminished in *fmr1*^{-/-} mice compared with WT mice. When we controlled for this shift in sensitivity, we found no differences between genotypes in the ability to habituate to or spontaneously discriminate between odors. These data suggest that loss of FMRP has a significant impact on either the development or functioning of the olfactory system, with implications for olfactory sensitivity. Such effects could have far reaching consequences for the regulation and expression of behaviors that are largely guided by olfactory signals in mice, such as socialization and navigation. In addition, these studies identify the olfactory system as a potentially fertile testing ground to investigate the role of FMRP on sensory development and function, with implications for understanding disturbance in neural development and behavior in FXS.

Supplementary material

Supplementary material can be found at <http://www.chemse.oxford-journals.org/>

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