Drew University
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Sniffing out the effects of nicotine on olfactory cortical neuron activity and odor preference

A Thesis in Neuroscience

by

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To all those who endeavor in the relentless pursuit of scientific inquiry.

To my family, friends, and professors without whom I would not be where I am now and by which these contributions to the field are as much theirs as they are my own.

Thank you.

ABSTRACT

The olfactory tubercle (OT) is a lesser-studied cortical area that lies at the intersection of the brain's olfactory and reward systems, effectively mediating processes of the two. As a component of the ventral striatum, the OT receives dense innervation from the ventral tegmental area) and is associated with reward pathways. As part of the olfactory system, it receives information from the main olfactory bulb and piriform cortex. Although there appears to be a possibility for an obvious association between processes of drug addiction and olfaction to take place in the OT, there is little empirical research that has aimed to delineate the interplay between the two. In this study, two experiments were conducted to understanding the effects of acute and chronic nicotine exposure on the neural systems of olfaction and reward that intersect within the OT. In experiment 1, electrophysiological recordings were conducted in the OTs of adult Sprague-Dawley rats while a sequence of 12 monomolecular odorants were repeatedly presented. Additionally, a dose of intracerebroventricular 5 μg/0.5 μl nicotine ditartrate was administered halfway through the session. Results show that acute nicotine infusion can potentially have excitatory or inhibitory effects on neuronal firing rate. Furthermore, nicotine may selectively alter the rate of odor-elicited activity. These findings provide new information on the role of the OT in processes of both nicotine addiction and olfaction. Experiment 2 aimed to assess the effect of a conditioned odorant paired with mecamylamine-precipitated nicotine withdrawal on odor preference as assayed on a custom-built olfactory hole-board apparatus. Although these results were inconclusive, there still remains an opportunity for drug-withdrawal associated cues to perpetuate drug-seeking and drug-taking behavior.

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INTRODUCTION

I. Public Health and Epidemiology of Tobacco Use

Cigarette smoking is the most pervasive form of tobacco use and is the leading cause of preventable death in the United States, accounting for over 480,000 deaths annually (Center of Disease Control). Smoking results in a wide variety of health issues and diseases such as cancer, stroke, cardiovascular disease, and respiratory disease, damaging organs throughout the entire body and not exclusively the lungs, bronchus, and trachea. In 2014, over 40 million Americans were estimated to be smokers, 16 million of which were suffering from at least one smoking-related disease. Smoking is not only a hazard to smokers, but to those around them as well. Resultantly, frequent exposure to secondhand smoke can have effects on health similar to those of smoking itself, as it accounts for nearly 34,000 deaths among nonsmokers annually.

There are many chemical constituents and additives in cigarettes that contribute to the toxic effects of smoking. In fact, a typical cigarette contains carcinogens and several hundred hazardous substances including acetone, arsenic, lead, and mercury (Novotny et al., 2011; Talhout et al., 2011), and the potential for these substances to cause bodily damage increases with the rate of smoking. Tobacco companies also add several ingredients to cigarettes that serve to improve their palatability and attractiveness, thus making cigarettes more likely to be smoked. However, of all the chemical compounds in cigarettes, nicotine is the primary ingredient that reinforces smoking behavior and instates dependency (Balfour et al., 2000; Caggiula et al., 2001).

Electronic cigarettes, or e-cigarettes, have been popularized in recent years and are marketed as a safer alternative to traditional tobacco cigarettes since they work by vaporizing a liquid solution rather than burning tobacco leaves. Like regular cigarettes, e-cigarettes also come infused with various flavors to enhance their taste and likeability. However, while e-cigarettes may possess exponentially fewer toxicants compared to traditional cigarettes (Goniewicz et al., 2013), they nonetheless still contain the highly addictive and toxic substance nicotine. For reference, the median lethal dose (LD_{50}) of nicotine is 50 mg/kg for rats and 3 mg/kg for mice (Mayer, 2017). Adult humans can succumb to 30-60 mg of nicotine, or a dose of 0.5-1.0 mg/kg, potentially making nicotine more toxic than cyanide, which has an LD_{50} of 1.52 mg/kg (Agency for Toxic Substances and Disease Registry). Nicotine overdose is extremely rare, however, since the average cigarette only contains 2-3 mg nicotine and the amount that is actually absorbed by the body tissue is far less.

II. The Nicotinic Acetylcholine Receptor

In the nervous system, the pentameric nicotinic acetylcholine receptor (nAChR) typically exclusively binds the endogenous excitatory neurotransmitter acetylcholine (ACh). There exist as many as 12 subtypes of nAChRs, which can vary in location and number across the neuronal membrane and throughout the nervous system (Dani, Ji & Fu-Ming Zhou, 2001). This transmembrane receptor protein can be heteromeric, consisting of α- and β-subunits in a ratio of two to three, or homomeric, with five α-subunits (Markou, 2008). There are nine isoforms of the α-subunit (α2-α10) and three of

the β-subunit (β2-β4). Each nAChR subtype, comprised of different combinations of α- and β-subunits, thus varies in structure and function.

ACh normally binds to nAChRs and causes them to undergo conformational changes that cause receptor channels to open and allow positively charged ions such as Na^+ and K^+ to flow into and depolarize the neuron, ultimately resulting in the firing of an action potential and/or activation of second messenger pathways. After ACh has diffused from the binding site, the receptor channel closes and returns to a resting state in which reopening requires further binding by ACh. Upon frequent activation, however, the receptor may become desensitized and subsequently enter into an inactive state wherein no other ligand can react with the binding site for a few milliseconds. In this way, a given nAChR can be in one of any three conformational states: closed (resting), open (activated), or desensitized (inactivated).

III. The Reward System

The striatum, comprised of the ventral tegmental area (VTA), nucleus accumbens (NAc), olfactory tubercle (OT), and other areas such as the putamen and caudate, is the locus of one of the brain's primary cholinergic and dopaminergic systems and is heavily involved in processes of motor coordination and reward (Zhou et al., 2002). Collectively, the NAc and portions of the OT are referred to as the ventral striatum, which is integrated and associated with a variety of areas other than just those involved in reward, such as the cortex, thalamus, amygdala, hippocampus, and midbrain (Gadziola & Wesson, 2016). From about 10-20% of striatal neurons are local cholinergic interneurons (CIN) while the

remainder consists primarily of medium spiny projection neurons (MSN; Zhou et al., 2002). Local cholinergic interneurons in the striatum have remarkably dense and widespread arborizations and receive considerable dopaminergic projections originating from midbrain structures such as the substantia nigra pars compacta (SNpc) and VTA, a prime area in the brain's reward pathways.

The interplay between cholinergic and dopaminergic signaling in the striatum is evidenced by the exceptionally high concentrations of acetylcholine (ACh), dopamine (DA), acetylcholinesterase (AChE; ACh degradation), and tyrosine hydroxylase (TH; DA synthesis) observed in striatal areas. Coinciding with the high levels of striatal ACh and DA is the fact that there is also a proportionally high number of receptors for these neurotransmitters (Zhou et al., 2002). As such, D_1 and D_2 dopamine receptors and nAChRs are found in great quantities on the membranes of neurons throughout the striatum. The relationship between ACh and DA is better defined when considering that nAChRs are widely distributed across dopaminergic cell bodies and axon terminals, suggesting that nAChR activation modulates DA release. In fact, administration of the competitive nAChR antagonist DHβE sharply attenuates DA transmission.

IV. The Neurobiology of Nicotine Addiction

Upon neurotransmission, a discreet amount of ACh is released by the presynaptic cholinergic neuron that is then allowed to bind to and activate postsynaptic nAChRs before diffusing away and being degraded by AChE (Dani, Ji & Fu-Ming Zhou, 2001). Nicotine, a nAChR agonist, however, is not hydrolyzed by AChE and thus is allowed to

have a greater postsynaptic effect. As previously mentioned, repeated activation of nAChRs can cause them to become desensitized and functionally inactivated. Prolonged desensitization of nAChRs by repeated nicotine exposure can trigger a cascade of neuroadaptive responses that serve to compensate for the deregulatory effects of the drug. The homeostatic response to persistent inactivation of nAChRs caused by elevated nicotine levels and maintained by smoking is increased expression and decreased degradation of the receptor protein, which ultimately lead to more nAChRs remaining in the cellular membrane.

However, the desensitization of dopaminergic nAChRs by the excitatory properties of nicotine does little to explain the finding that nicotine maintains a heightened extracellular level of DA (Imperato et al., 1986; Pidoplichko et al., 2004). As it would be, there are also nAChRs on presynaptic sites of glutamatergic neurons that synapse onto dopaminergic neurons in the VTA and do not readily desensitize, potentially allowing for the sustained levels of DA observed after nicotine exposure (Mansvelder & McGehee, 2000). Smoking can also affect neuronal activity by various other means as well. Another hallmark of tobacco use is inhibition of monoamine oxidase B (MAOB), which normally serves to degrade and remove DA from the synapse. With MAOB inhibited, the free DA can more readily bind to postsynaptic receptors and potentially further reinforce the rewarding effects of nicotine. Inhibition of MAOB in reward pathways is therefore another means by which smoking and tobacco use can drive mechanisms of nicotine addiction.

Experiment 1: The effect of acute nicotine infusion on olfactory tubercle activity

I. INTRODUCTION

I. The Olfactory System

Olfaction begins when molecular odorants bind to protein receptors on the surface of olfactory receptor neurons within the main olfactory epithelium (MOE) or the vomeronasal organ (VNO). Once activated by an odorant, olfactory receptor neurons transmit signals to olfactory cortical areas such as the main olfactory bulb (MOB) and accessory olfactory bulb (AOB). From there, projections branch out to synapse upon a variety of target areas such as olfactory tubercle, piriform cortex, the bed nucleus of the stria terminalis, amygdala, and hypothalamus, from which a vast range of behavioral and hormonal responses may result.

Second order neurons within the glomeruli of the MOB known as mitral and tufted cells fasciculate to form the lateral olfactory tract (LOT) and send axonal projections to associated olfactory areas. Mitral cell projections from the LOT predominately innervate the piriform cortex while those of tufted cells mainly extend direct inputs to the dense cell layer of medium spiny neurons within the OT (Wesson & Wilson, 2011). Similarly, the OT also receives direct input from piriform cortex, although it is thought that the accessory olfactory bulb projects indirectly to the OT via the posteromedial nucleus of the amygdala. In addition to those of the reward and olfactory systems, the OT also receives considerable auditory, visual, amygdalar, hippocampal, thalamic, hypothalamic, and brainstem projections as well. Thus, the OT is markedly a highly integrated region that sends and receives signals to and from a wide range of

functionally diverse brain areas and is therefore ideally situated for involvement in the relation between sensory stimuli processing and subsequent behavioral response, particularly in those elicited by olfactory stimuli.

II. The Olfactory Tubercle

The ventral striatum, comprised primarily of the nucleus accumbens (NAc) and olfactory tubercle (OT), receives a considerable portion of the dopaminergic projections from the VTA and has been found to be significantly involved in processes of drug reward and addiction (Ikemoto et al., 2006). The OT in particular has sparked an appreciably large amount of interest in recent scientific publications concerning drug reward and addiction (Bissonette et al., 2013; Fitzgerald et al., 2014; Gadziola & Wesson, 2016; Striano et al., 2014). However, and as the name suggests, the olfactory tubercle is also a component of the olfactory system, receiving input from the olfactory bulb and other olfactory cortical areas as well as encoding odor valences that guide odor-related behavior (Gadziola et al., 2015). Olfactory tubercle neurons have also been shown to respond selectively to certain odorants as opposed to others and are likely to participate in processes of odor discrimination (Wesson & Wilson, 2010). Consequently, considering a majority of the focus on the OT has been in terms of reward processes, and although the OT has been recognized as a component of the olfactory system for over 50 years, there is an unsurprising scarcity of research on the role of the OT in olfactory processing (Wesson & Wilson, 2011).

The cytoarchitecture of the OT is structured in ways that are both laminar and nuclear; the ventral portion is more akin to cortical areas and is organized into three layers while the dorsal aspect contains dense cell clusters collectively referred to as the islands of Calleja (Wesson & Wilson, 2011). The cells that comprise the outside of these clusters, or "islands," are innervated predominately by dopaminergic projection from the NAc and SNpc, and the neuropil that surround these islands receive extensive cholinergic projections. Indeed, the islands stain positive for AChE and choline acetyl transferase (ChAT; ACh synthesis). Layer II of the OT consists mainly of medium spiny neurons that project to the NAc and regions of the ventral pallidum such as the caudate and putamen, which together comprise the dorsal striatum. Interestingly, the olfactory tubercles of olfactory bulbectomized animals exhibit an upregulation in D_2 receptor expression and a modified responsivity to the rewarding properties of *d*-amphetamine. This suggests that incoming olfactory information to the OT may be able to effectively modulate reward processes (Wesson & Wilson, 2011). Furthermore, rats will self-administer cocaine into the OT, but not when a D_1 or D_2 antagonist is coadministered (Ikemoto, 2003). Together, these findings describe a functional significance of the OT in mediating dual functions of olfaction and reward.

III. Electrophysiology

One prominent experimental method for studying neural activity is electrophysiology, which is the field of research concerned with the electrical properties involved in neural functions. Electrophysiology can be conducted *in vitro* or *in vivo* and

is capable of measuring the electrical activity of neurons from a small group of individual cells known as units to larger groups known as neuronal ensembles. Single unit activity (SUA) can be recorded through insertion of a metal microelectrode detecting changes in electrical potential caused by the rapid movement of ions across the cell membrane that occurs during action potential firing. In this way, the rate of neuronal firing can be studied, in particular in response to the addition of some experimental manipulation, such as a sensory stimulus or a drug. Through SUA, however, not only can the firing rate of individual cells be gauged, but also other features of neuronal communication that are characterized by a change in firing rate, such as tuning, sensitization, habituation, and inhibition.

IV. Specific Aims & Experimental Design

Thus, the specific aim of this study was to assess the effects of nicotine exposure on the firing rate and tuning of neurons in the olfactory tubercle in response to a set of olfactory stimuli in order to better understand the functional role of the OT in olfactory processing as well as mechanisms by which nicotine can modify odor-elicited neuronal activity. This was accomplished comparing neuronal firing rates via single unit electrophysiological recording of OT neurons in urethane-anesthetized rats during presentation of a series of monomolecular odorants before and after an infusion of nicotine into the lateral ventricle.

The experimental hypotheses are based on a string of experimental findings which provides that nAChRs are widely distributed throughout the ventral striatum, that nicotine

binds to and activates nAChRs, that nAChR activation occasions striatal DA release, that DA release is involved in processes of drug reward and addiction, and that there is an interaction between processes of reward and olfaction within the OT. As such, a wide range of experimental outcomes may be plausible. First, nicotine injection may result in an increase or a decrease in neuronal firing, whether in response to an odorant or not, compared to baseline. Second, the tuning profile of OT neurons may widen or narrow following nicotine injection compared to baseline. Third, there may be an observable effect of time after nicotine injection on odor responsivity and/or tuning.

II. METHODS

Subjects. Adult female Sprague Dawley rats (Harlan, Indianapolis, IN; 300-500 g) were used in this study. Subjects were individually housed in clear plastic cages and maintained on a 12 hr light/dark cycle in a vivarium with a controlled temperature and humidity. Access to food and water was provided *ad libitum*.

Surgery. Surgical procedures were conducted to allow for lateral-to-medial advancement of a pair of recording electrode to the olfactory tubercle. Subjects were anesthetized with urethane (3.0 mg/kg, i.p.) and mounted in a standard stereotactic frame. Anesthesia depth was gauged by absence of the toe-pinch reflex. The skull surface was exposed and cleaned and an aluminum post was affixed to the posterior nasal bones with dental acrylic and steel skull screws. The subjects were turned onto the side with the left hemisphere up to allow for electrode penetration from above. The left masseter muscle was deflected

and a small section of the mandible and jugal bone removed. The temporalis muscle was deflected to expose the ventrolateral surface of the skull. A craniotomy was performed to reveal the cortex around the middle cerebral artery from approximately 1.0 mm anterior to 3.0 mm posterior to bregma. A small craniotomy was made in the left temporal bone to allow for the penetration of a reference electrode. Body temperature was maintained with an electric heating pad if needed.

Electrophysiological recording. A pair of tungsten recording electrodes were advanced medially through the cortex and aimed towards OT with stereotactic guidance. Recordings were conducted in a Faraday cage and were begun when sufficiently large spike waveforms were detected at depths > 2.0 mm in order to increase the chance of hitting tubercle neurons. A reference electrode was placed into the left parietal lobe to subtract electrical noise from neuronal activity. Signals were amplified, filtered, and digitized for storage and later offline analysis (A-M Systems, Carlsborg, WA). Data acquisition was carried out using Spike2 (Cambridge Electric Design, Cambridge, UK). Spike waveforms were extracted and isolated using OfflineSorter (Plexon, Inc., Dallas, TX). Cells were sorted based on interspike interval, waveform properties, and principle component analyses. All other analyses were conducted using NeuroExplorer (Nex Technologies, Madison, AL) and MATLAB (The Mathworks, Natick, MA).

Stimulus presentation. After a stable 2 min baseline recording period, a sequence of 12 monomolecular odorants (2 sec duration, 30 sec ISI) was presented using a custom flow-

dilution olfactometer positioned 3.0 cm from the nares (**Fig. 1**). Odorants included benzaldehyde, citral, cineole, cumene, ethyl acetate, heptanal, heptanone, isoamyl acetate, isophorone, limonene, 5-methyl-2-hexanone, and propyl butyrate. All odorants were diluted to 350 ppm in mineral oil and delivered in nitrogen gas at a flow rate of 1.0 l/m. The predator pheromones fox urine and cat urine were also used but were not considered in subsequent data analyses. At least 10 trials of each odorant presentation were made per recording session.

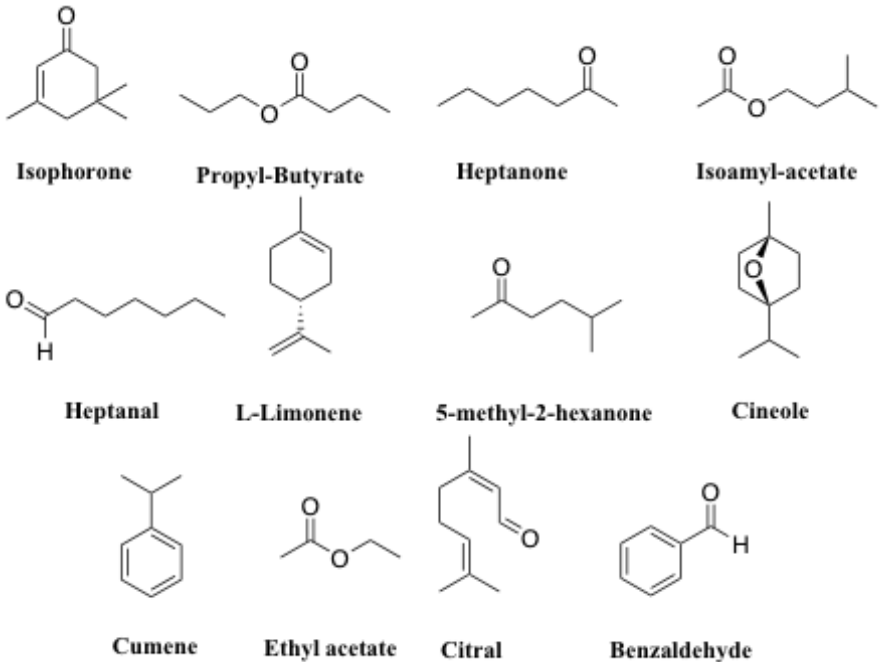


Figure 1: Molecular structures of experimental odorants. A sequence of twelve novel, monomolecular odorants were presented during electrophysiological recording sessions.

Intracerebroventricular nicotine infusion. After the unit was screened for odor responsivity during real-time data analysis in NeuroExplorer proceeding at least four presentations of each of the experimental odorants, a dose of 5 µg/0.5 µl nicotine ditartrate (Sigma-Aldrich, Saint Louis, MO; 3.12 mg free base) was infused into the left

lateral ventricle at the stereotactic coordinates AP = -1, ML = -1.7, DV = -4 relative to bregma (Paxinos & Watson, 1997) to achieve a biologically relevant cerebral nicotine concentration similar to that found in a human smoker (Matta et al., 2007).

Statistical analyses. Paired sample t-tests were run in SPSS (IBM Corporation, Armonk, NY) to compare mean within-subject neuronal firing rates (3 sec bins) before and after both odor onset and nicotine infusion. Cells were determined to be odor-responsive if they exhibited a significant change in firing rate pre- compared to post-onset to at least one of the monomolecular odorants. Likewise, cells were determined to be nicotine-responsive if they exhibited a significant change in firing rate pre-compared to post-infusion of nicotine. An alpha level of significance of .01 was used for all tests.

III. RESULTS

Now and hereafter, "onset" will refer to the instance in which a given odorant was presented to the subject while "infusion" will refer to the time at which nicotine was administered into the lateral ventricle. Paired samples t-tests were performed in order to detect statistically significant effects of odorant presentation and nicotine infusion by comparing mean neuronal firing rate (NFR) during 3 sec before and after onset of each odorant and before and after an acute i.c.v. infusion of 5 μg/0.5 μl nicotine over the course of the recording session. Corrections were made for multiple comparisons (α = .01). Pairwise comparisons described in **Fig. 2**.

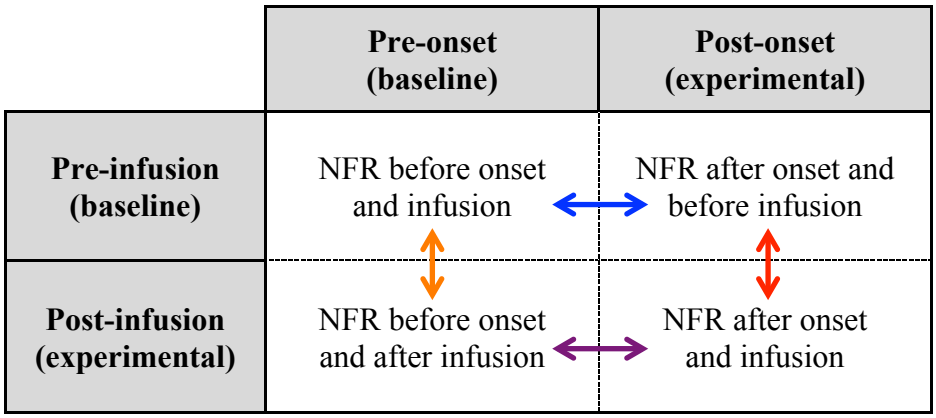


Figure 2: Pairwise comparisons of neuronal firing rate. Determination of odor responsivity before (blue arrows; Pair 1) and after (purple arrows; Pair 2) nicotine infusion found by comparing pre- and post-onset NFR values. Determination of nicotine responsivity before (orange arrows; Pair 3) and after (red arrows; Pair 4) odorant onset found by comparing pre- and post-infusion NFR values.

Fifteen units were isolated from three adult female Sprague Dawley rats in the current dataset. Of these fifteen cells, thirteen (86.7%) were found to be odor selective, demonstrating an increase NFR in response to odorant presentation. Furthermore, six of the eight (75%) cells exposed to nicotine a response to nicotine, which manifested as either an increase or a decrease in activity relative to the pre-infusion period. Given the small sample size, cells were conveniently named according to the following convention for data reporting and will hereafter be referred to as such: subject_electrode_cell, wherein, for example, S2_2_6 refers to cell 6 recorded on electrode 2 from subject 2 (**Table 1**). An infusion of nicotine was given at least halfway though the recording session for cells 1-8 while cells 9-15 were never exposed to nicotine. Cells recorded during the same session were always found to be all odor-responsive or not. However, not all odor responsive cells from the same session all showed nicotine responsivity. There was variance in the mean NFR between units (range: 0.11-5.71 imp/sec).

Interestingly, while cells recorded during the same session were likely to be odor-responsive, they did not necessarily have similar mean firing rates

ID			Spikes	Session Duration (sec)	Mean NFR (imp/sec)	Odor Responsive?	Nicotine Responsive?
Subject	Electrode	Cell					
Nicotine							
S1	**1**	**1**	1,966	4500	0.44	Yes	No
		2	1,346	4500	0.30	Yes	Yes
		3	1,697	4500	0.38	Yes	No
S2	**1**	**4**	20,178	4500	4.48	Yes	Yes
		5	8,031	4500	1.78	Yes	Yes
	2	**6**	1,602	4500	0.36	Yes	Yes
S3	**1**	**7**	459	4300	0.11	No	Yes
		8	1,731	4300	0.40	No	Yes
Control							
S1	**1**	**9**	5,556	4200	1.32	Yes	-
		10	12,946	4200	3.08	Yes	-
S2	**1**	**11**	15,870	4300	3.69	Yes	-
		12	22,853	4300	5.44	Yes	-
	2	**13**	9,616	4300	2.24	Yes	-
		14	4,310	4300	1.00	Yes	-
		15	1,779	2900	0.61	Yes	-

Table 1: Nomenclature and features of isolated units. Data referred to by subject, electrode, and cell number for nicotine and control conditions. The majority of units demonstrated odor and nicotine responsivity in nicotine-exposed cells.

I. Nicotine occasioned prolonged changes in the firing rate of olfactory neurons

In order to assess the effects of nicotine infusion on NFR in nicotine-exposed cells, 200 msec tailing averages of neuronal firing rate were related to the mean baseline neuronal firing rate (1000 msec before infusion) to determine the percent change (**Fig. 3**). A number of nicotine-exposed cells demonstrated a change in NFR greater than 50% of that of baseline NFR. Overall, the activity of nicotine-exposed cells appeared to decrease, especially within the first 300 msec after nicotine infusion.

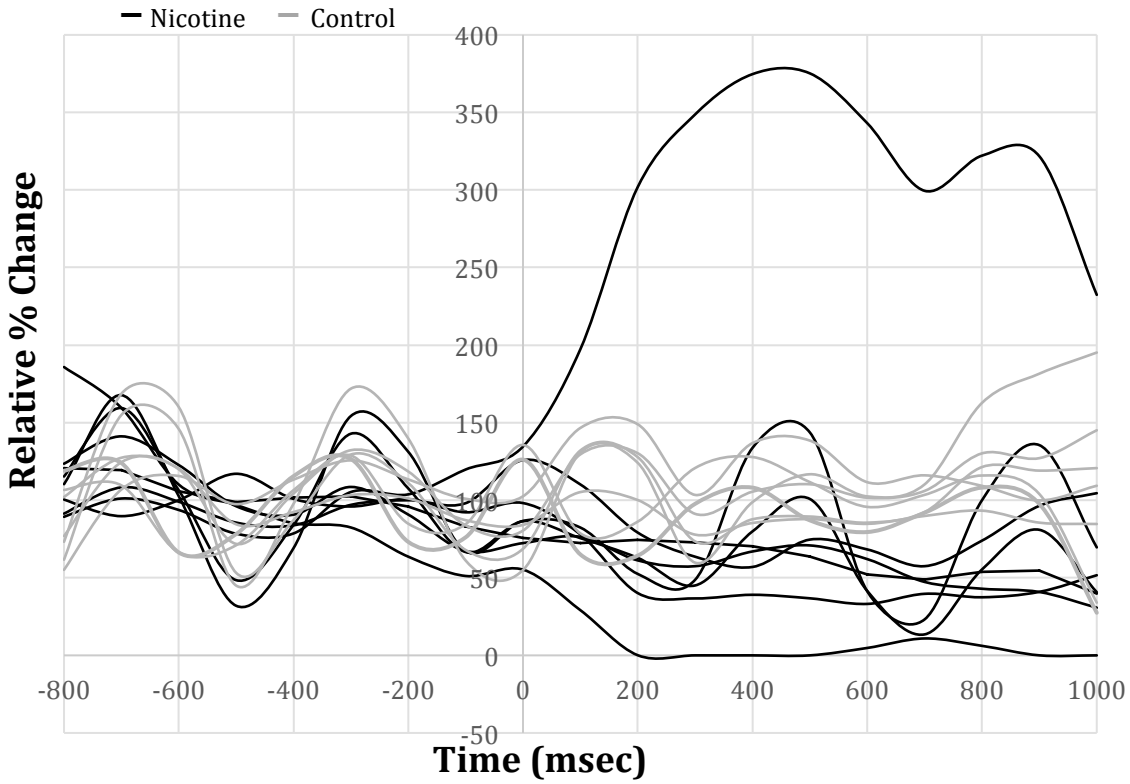


Figure 3: The activity of nicotine-infused cells was altered after infusion. Percent change in NFR during 100 msec bins relative to the mean pre-infusion NFR for each cell. For nicotine cells, nicotine infusion at t = 0 msec. For control cells, two consecutive 1000 sec periods were compared. A number of nicotine-exposed cells (black traces) demonstrate changes in NFR post-infusion of nicotine, particularly within the first 300 msec after infusion.

These drug-induced changes in NFR lasted for several hundred milliseconds in some cases, but there was variance in the magnitude and time to maximal effect between cells (**Fig. 4**; **Appendix**). In adjacent cells recorded from the same electrode, a similar pattern of activity was observed during sessions with monomolecular odorant presentation. While the rate of neuronal firing differed between individual cells, the occurrences of peaks and troughs were largely in synchrony among adjacent cells throughout the course of the recording session.

IV. The odor-related activity of olfactory neurons was altered by nicotine infusion

The effects of i.c.v. nicotine infusion on NFR as shown in **Fig. 3** were found to also apply to activity around odor onset (**Fig. 5**). Perievent rasters and histograms around all odorant presentations pre- (**Panel A**) and post-infusion (**Panel B**) demonstrate marked differences in NFR both before and after odor onset. In particular, nicotine infusion appeared to have different effects on two odor-responsive cells recorded simultaneously on the same electrode (S2_1_4 and S2_1_5). It is unclear if nicotine can selectively potentiate or attenuate the rate of odor-elicited activity or if it affects overall NFR of odor-responsive cells, and consequentially the rate of odor-elicited activity.

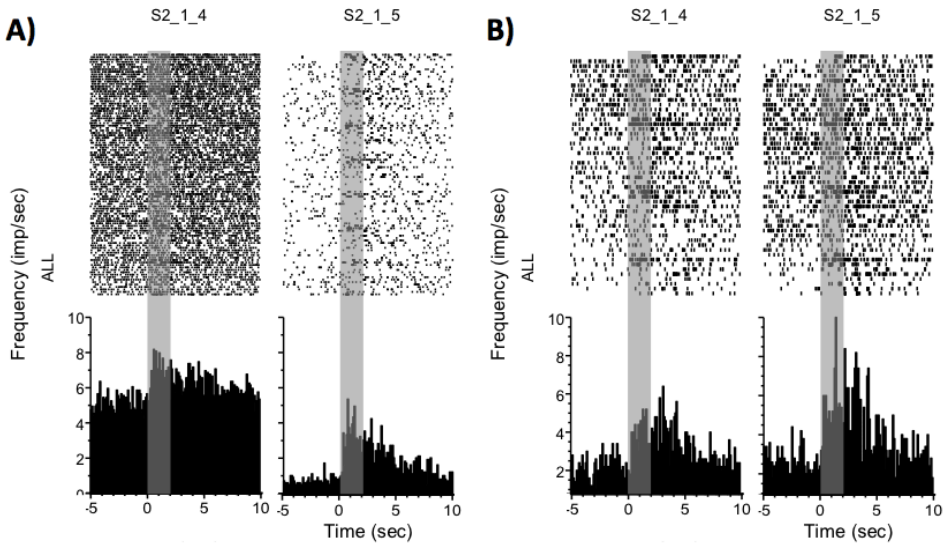


Figure 5: Odor-elicited activity was affected by nicotine infusion. Perievent rasters of frequency of neuronal firing in imp/sec around presentation of all experimental odorants (t = 0; grey bars) pre- (**A**) and post-infusion (**B)** of nicotine. The firing rates of S2_1_4 after odor onset seemed to decrease after infusion while that of S2_1_5 increased.

Pairwise comparisons of mean average neuronal firing rate factored by odor onset revealed significant differences as a function of nicotine infusion (**Table 2**, white boldface font). Cells of tables are colored for the relative minimal (reddest red) and

maximal (greenest green) NFR for each unit with red representing lower values and green, higher values. Nicotine infusion induced significant changes in NFR in certain cells. However, in some cases this effect was only seen after rather than before onset, as in S1_1_2 and S2_2_6, providing evidence that nicotine may selectively attenuate odor-elicited activity without significantly affecting baseline activity. S1_1_1 and S_1_3 did not exhibit nicotine responsivity while cells S3_1_7 and S1_1_8 failed to demonstrate odor-elicited activity. S2_1_5 was the only cell in which nicotine infusion occasioned a significant increase in neuronal firing rate, both pre- and post-onset of odorants. S3_1_7 showed a silencing of neuronal firing after infusion, although this cell was not determined to be odor-responsive.

Cell	Nicotine Responsivity			
	Pair 3		Pair 4	
	Before Onset		After Onset	
	Pre-Infusion	Post-Infusion	Pre-Infusion	Post-Infusion
S1_1_1	0.4	0.6	7.8	7.4
S1_1_2	0.7	0.3	**3.8**	**2.9**
S1_1_3	1.1	0.8	2.3	2.2
S2_1_4	**16.8**	**7.3**	**21.1**	**12.4**
S2_1_5	**2.2**	**7**	**10.4**	**15.5**
S2_2_6	0.6	0.4	**5.4**	**1.4**
S3_1_7	**0.7**	**0**	**0.9**	**0**
S3_1_8	**1.5**	**0.7**	**1.6**	**0.8**

Table 2: Comparisons of neuronal firing rate per cell in response to nicotine. Green-red color scale and values represent NFR in imp/sec. White boldface font denotes pairs with values that are statistically significantly different ($p < .01$). All cells except S1_1_1 and S1_1_3 demonstrated a post-infusion NFR change before and/or after onset of odorants. (Pairs 3 & 4).

Pairwise comparisons of mean average neuronal firing rate factored by nicotine infusion reveal significant differences (**Table 3**, white boldface font). Nicotine infusion did not significant affect the odor-responsive properties of the recorded cells to all odorants overall. That is, cells that were odor-responsive pre-infusion continued to be so post-infusion (1-6), as for cells that were not odor-responsive (7-8).

Cell	Odor Responsivity			
	Pair 1		Pair 2	
	Before Infusion		After Infusion	
	Pre-Onset	Post-Onset	Pre-Onset	Post-Onset
S1_1_1	0.4	7.8	0.6	7.4
S1_1_2	0.7	3.8	0.3	2.9
S1_1_3	1.1	2.3	0.8	2.2
S2_1_4	16.8	21.1	7.3	12.4
S2_1_5	2.2	10.4	7	15.5
S2_2_6	0.6	5.4	0.4	1.4
S3_1_7	0.7	0.9	0	0
S3_1_8	1.5	1.6	0.7	0.8

Table 3: Comparisons of neuronal firing rate per cell in response to odorant. Green-red color scale and values represent NFR in imp/sec. White boldface font denotes pairs with values that are statistically significantly different ($p < .01$).

As shown in **Fig. 6**, cells continued to be odor responsive following nicotine infusion, however the magnitude of response was altered. The activity of S2_1_4 around presentation of individual odorants is shown to have decreased pre- (**Panel A**) compared to post-infusion (**Panel B**) of nicotine while that of S2_1_5 increased.

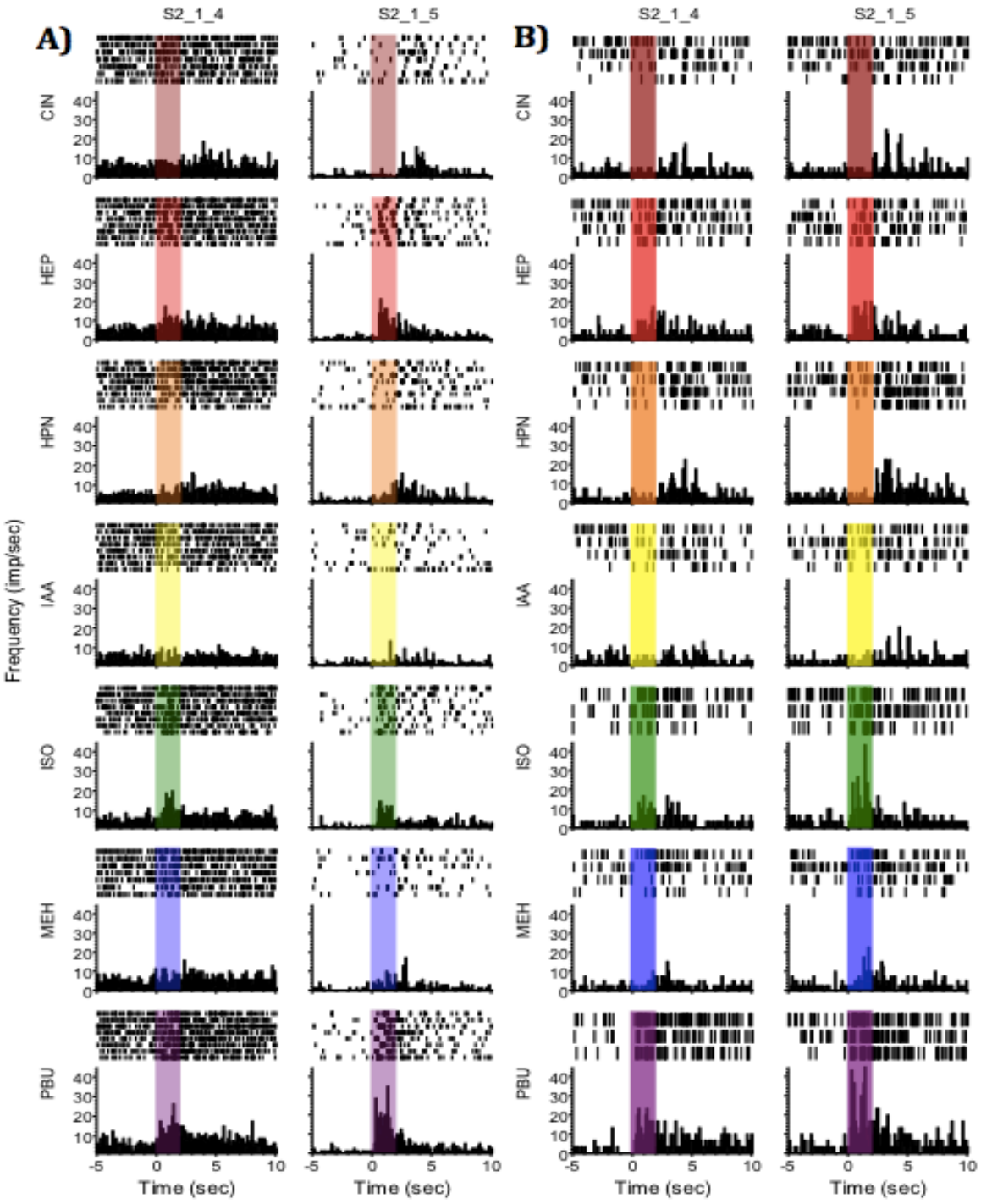


Figure 6: Nicotine differentially affects responses to odorants. Perievent rasters and histograms of firing rate of two cells on the same electrode in response to 2 sec presentations of monomolecular odorants at t = 0 (colored bars) during pre- (**A**) and post-infusion (**B**) periods.

The rate of an individual neuron's firing 2.5 sec after onset of each of the 12 monomolecular odorants (FR) was normalized to the firing rate to the odorant that produced the maximal level of activity for that unit (FR_m) in order to determine the odor ranking and tuning breadth of odor-responsive cells (**Fig. 7**). To assess how nicotine might affect tuning breadth, the firing rate after infusion (FR_a) was compared relative to the firing rate before infusion (FR_b) and normalized to the odorant that produced the maximal level of activity after infusion (FR_m):

$$\frac{FR}{FR_m} = normalized\ FR$$

$$\frac{FR_a/FR_b}{FR_m} = normalized\ relative\ FR$$

As shown in **Panel A** and **B**, under control conditions cells exhibited a moderately narrow tuning breadth, responding robustly to only a select few of the odorants. Following drug administration (**Panel C**), select cells exhibited a widening of tuning breadth wherein they responded robustly to an increased number of odorants, as seen in S1_1_1, S1_1_2, and S2_1_4. However, on average the population of nicotine-exposed units did not reliably demonstrate a change in tuning breadth.

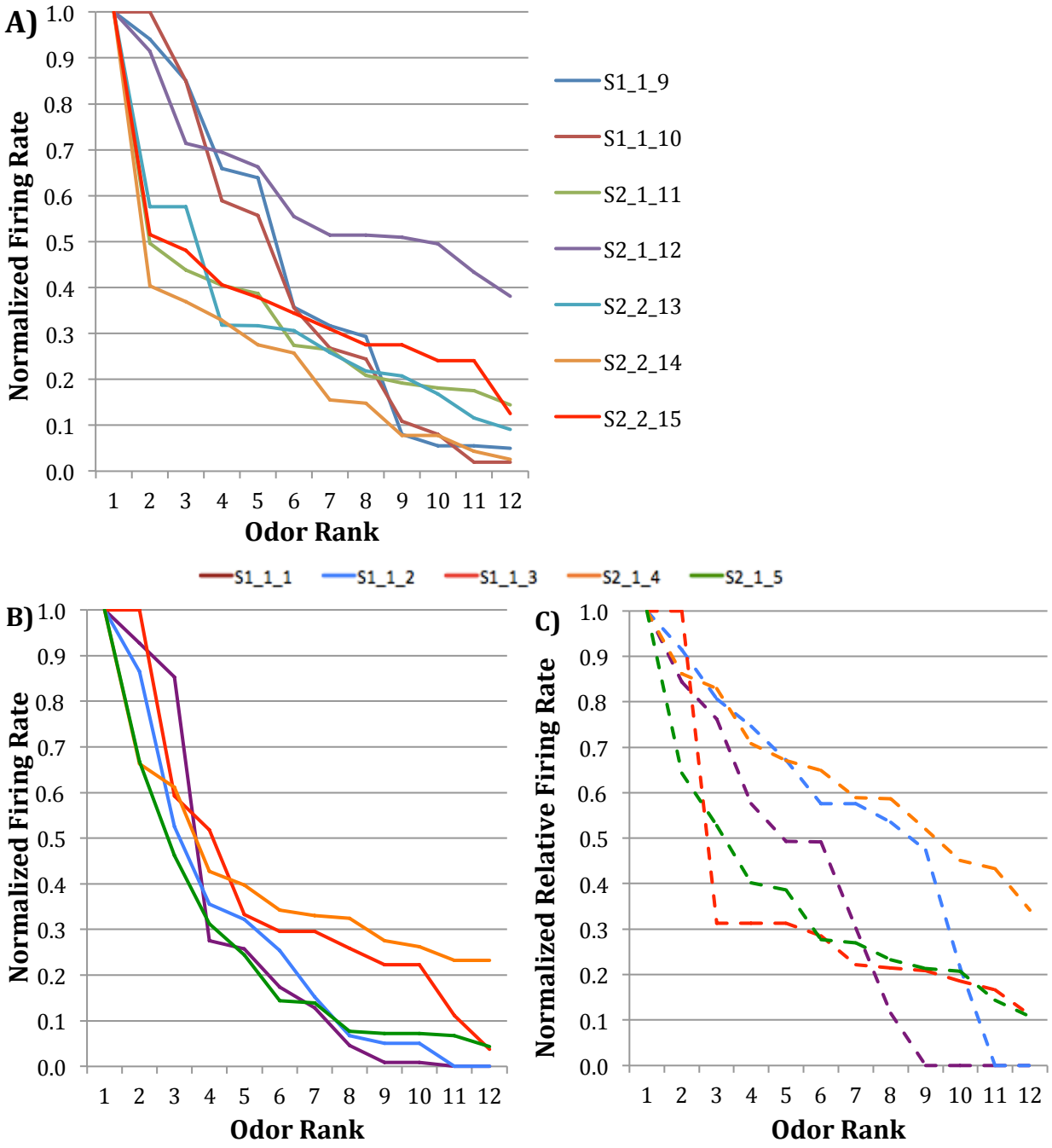


Figure 7: Nicotine did not widen the tuning breadth of odor-responsive cells. A) Tuning breadth of seven control odor-responsive cells. Units tended to be narrowly tuned. **B)** Five isolated odor-responsive cells pre-infusion**.** Cells demonstrated a moderately narrow tuning breadth. **C)** The five same cells post-infusion. Select cells demonstrated a wider tuning breadth compared to that during the pre- infusion period, however there was no effect overall.

III. Odor-responsive cells exhibited similar tuning profiles

In adjacent units recorded from the same electrode, there was a similar pattern of activity during sessions with monomolecular odorant presentation (**Fig. 8**; **Appendix**). While the rate of neuronal firing differed between individual cells, the occurrences of peaks and troughs were in synchrony among adjacent cells throughout the course of the recording session. These trends were also observed between nonadjacent cells recorded simultaneously from different electrodes within the same subject (**Panel B**). Frequency of peak firing was seen to decrease over the duration of the session in some cells, such as S1_1_10, S2_1_12, and S2_2_13. Spike data for S2_2_15 during $t < 1400$ unable to be sorted and was thus excluded from analysis.

Odor responsivity was determined via paired samples *t*-test of firing rate 3 sec before and after onset of each odorant ($\alpha = .01$). Adjustments were made for multiple comparisons. A majority of units (88.7%) were found to be odor responsive and demonstrated a significant increase in neuronal firing rate proceeding presentation of at least one of the experimental odorants (**Table 4**). Results for cells 1-8 based on data from pre-infusion period only. Cells recorded simultaneously, whether on the same or different electrodes, were found to all be either odor responsive or all not. Additionally, simultaneously recorded cells also responded selectively to similar odorants and demonstrated similar tuning profiles and breadths. The mean tuning breadth was approximately 4 odorants wide. Odorants elicited responses in the following order from greatest to least: heptanal (HPN; 76.9%), propyl butyrate (PBU; 61.5%), cumene (CUM; 46.2%), heptanone (HEP), isoamyl acetate (IAA), and cineole (CIN; 30.8%), limonene

(LIM; 23.1%), ethyl acetate (ETA), citral (CIT), and benzaldehyde (BEN; 15.4%), 5-methyl-3-hexanone (MET; 7.7%), and isophorone (ISO; 0%).

Cell	ISO	PBU	HEP	IAA	HPN	LIM	MET	CIN	CUM	ETA	CIT	BEN	Total/12
S1_1_1	✖	O	–	O	O	✖	–	✖	✖	✖	✖	–	3
S1_1_2	✖	–	–	O	O	O	O	✖	–	✖	✖	✖	4
S1_1_3	✖	–	✖	–	O	✖	✖	✖	–	✖	✖	✖	1
S2_1_4	✖	O	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	1
S2_1_5	–	O	O	✖	O	✖	✖	✖	✖	✖	✖	✖	3
S2_2_6	–	–	O	✖	O	✖	–	✖	–	✖	✖	✖	2
S3_1_7	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	0
S3_1_8	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	0
S1_1_9	✖	✖	✖	O	O	O	✖	O	O	O	O	O	8
S1_1_10	✖	✖	✖	O	O	O	✖	O	O	O	O	O	8
S2_1_11	✖	O	O	✖	O	✖	–	O	O	✖	✖	✖	5
S2_1_12	✖	O	✖	✖	✖	✖	✖	✖	O	✖	✖	✖	2
S2_2_13	✖	O	✖	✖	O	✖	–	O	O	✖	✖	✖	4
S2_2_14	✖	O	O	✖	O	✖	✖	✖	O	✖	✖	✖	4
S2_2_15	✖	O	✖	✖	✖	✖	✖	✖	✖	✖	✖	✖	1
Total/13	0	8	4	4	10	3	1	4	6	2	2	2	

Table 4: Tuning profiles and breadths of recorded units. The odorant producing a response in the greatest number of recorded cells was heptanal and in the least number was isophorone. Cells 7 and 8 were not odor responsive. Data from cells 1-8 from pre-infusion period only. Thick horizontal lines separate cells recorded on different electrodes while shading (white or light grey) separate cells recorded in different sessions. (O) = $p < .01$; (–) = $p < .05$; (✖) = $p > .05$.

Odor-responsive cells demonstrated an overall increase in activity following odor presentation (**Fig. 9**). In cells 9 and 10 there was a robust, consisted odor-elicited response that was concomitant with odor onset (t = 0). In cells 11-14, odor-elicited activity was associated with odor offset (t = 2). These responses to odorant presentation overall were similar between adjacent cells recorded on the same electrode and nonadjacent cells recorded on different electrodes within the same subject. Similarities in perievent rasters between cells recorded during the same session suggest similar tuning.

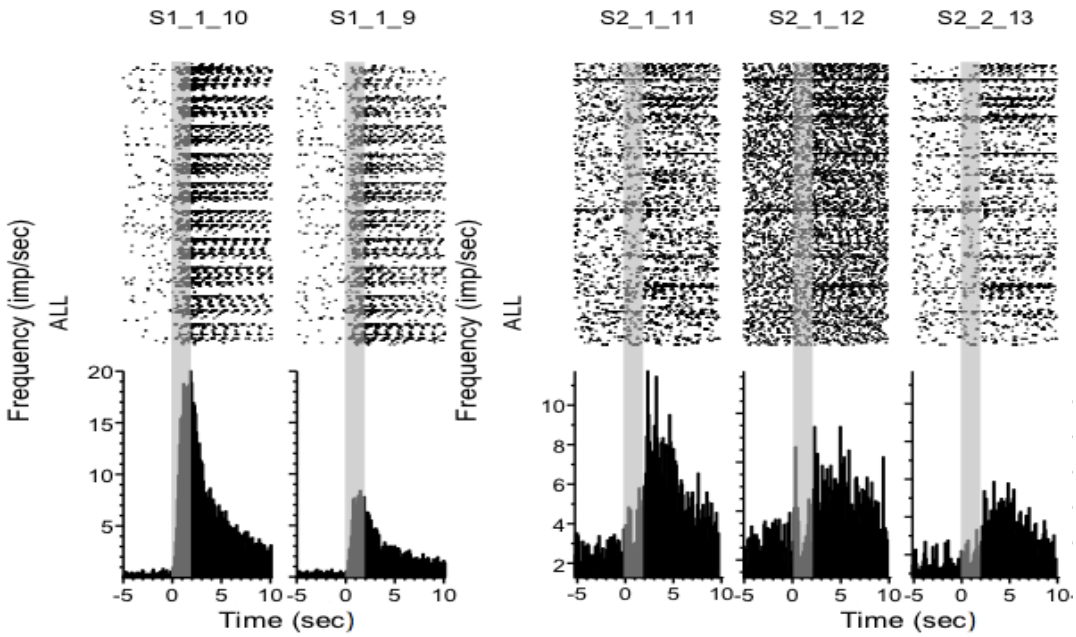


Figure 9: Odor-elicited activity proceeded odor onset or offset. Perievent rasters of frequency of neuronal firing in imp/sec around presentation of all experimental odorants. Cells 9 and 10 exhibited a reliable increase in firing rate following onset (t = 0; grey bars) while cells 11-13 expressed delayed-onset odor-elicited activity.

Cells 9 and 10 demonstrated identical tuning profiles and breadths, responding robustly and selectively to the same eight monomolecular odorants at onset (t = 0), five of which are shown in **Fig. 10**. Phasic activity was repeatedly observed following offset (t > 2) for odorants to which these cells were responsive that was robust and reliably reproduced between trials of that odorant. Cells 11-14 also demonstrated similar tuning profiles, but the latency to odor-elicited activity was longer (**Fig. 11**). These similarities in tuning were extended to nonadjacent cells recorded on different electrodes as well. Transient increases in NFR can be seen after offset of certain odorants that are phase-locked with the respiration cycle.

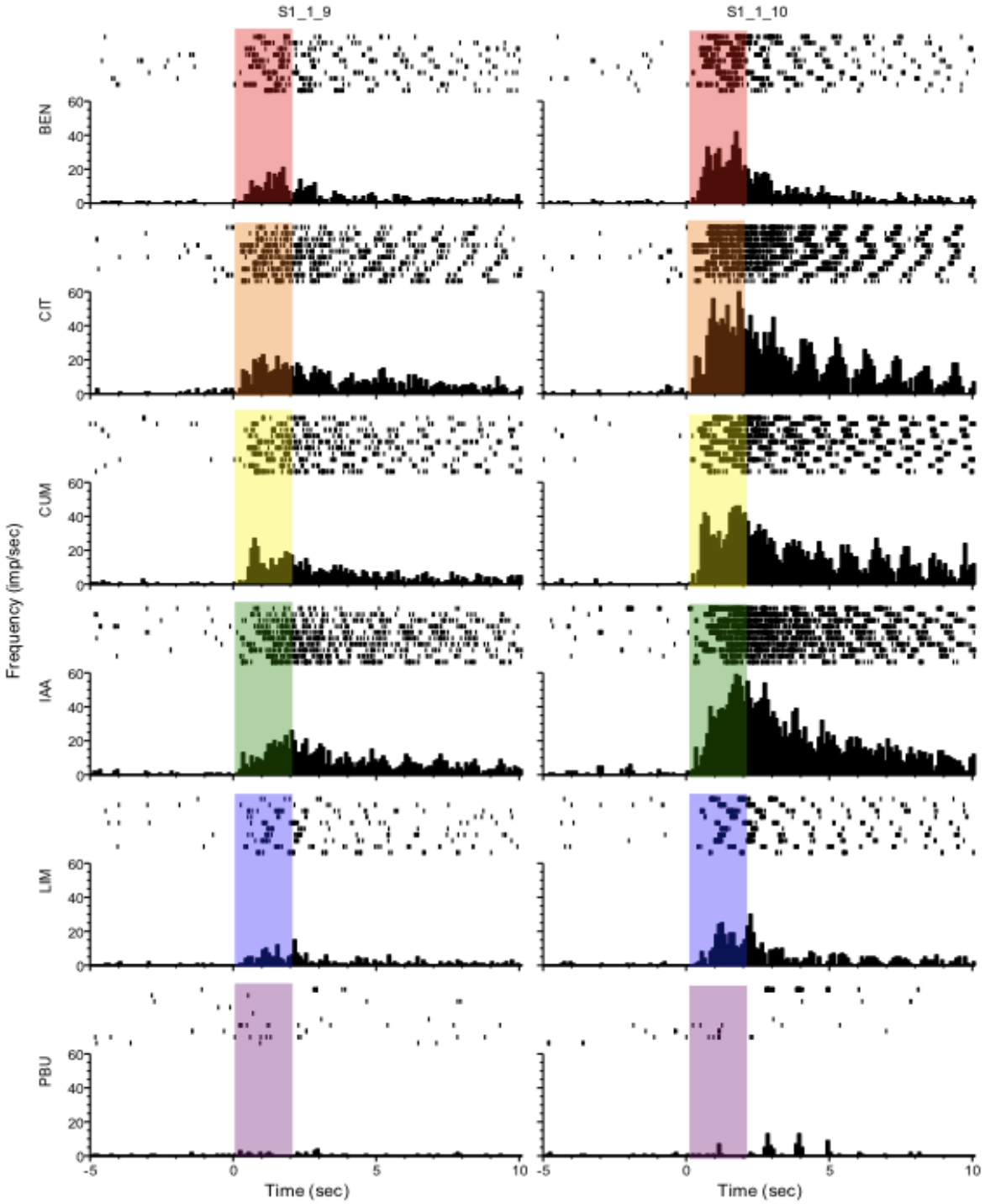


Figure 10: Adjacent neurons demonstrated robust odor-elicited activity and similar tuning. Perievent rasters and histograms of firing rate of two adjacent cells in response 2 sec presentations of monomolecular odorants at t = 0 (colored bars). Cells responded similarly yet selectively to the same odorants at onset. Entrainment of neuronal activity with the ongoing respiration cycle can be seen proceeding odor offset (t > 2).

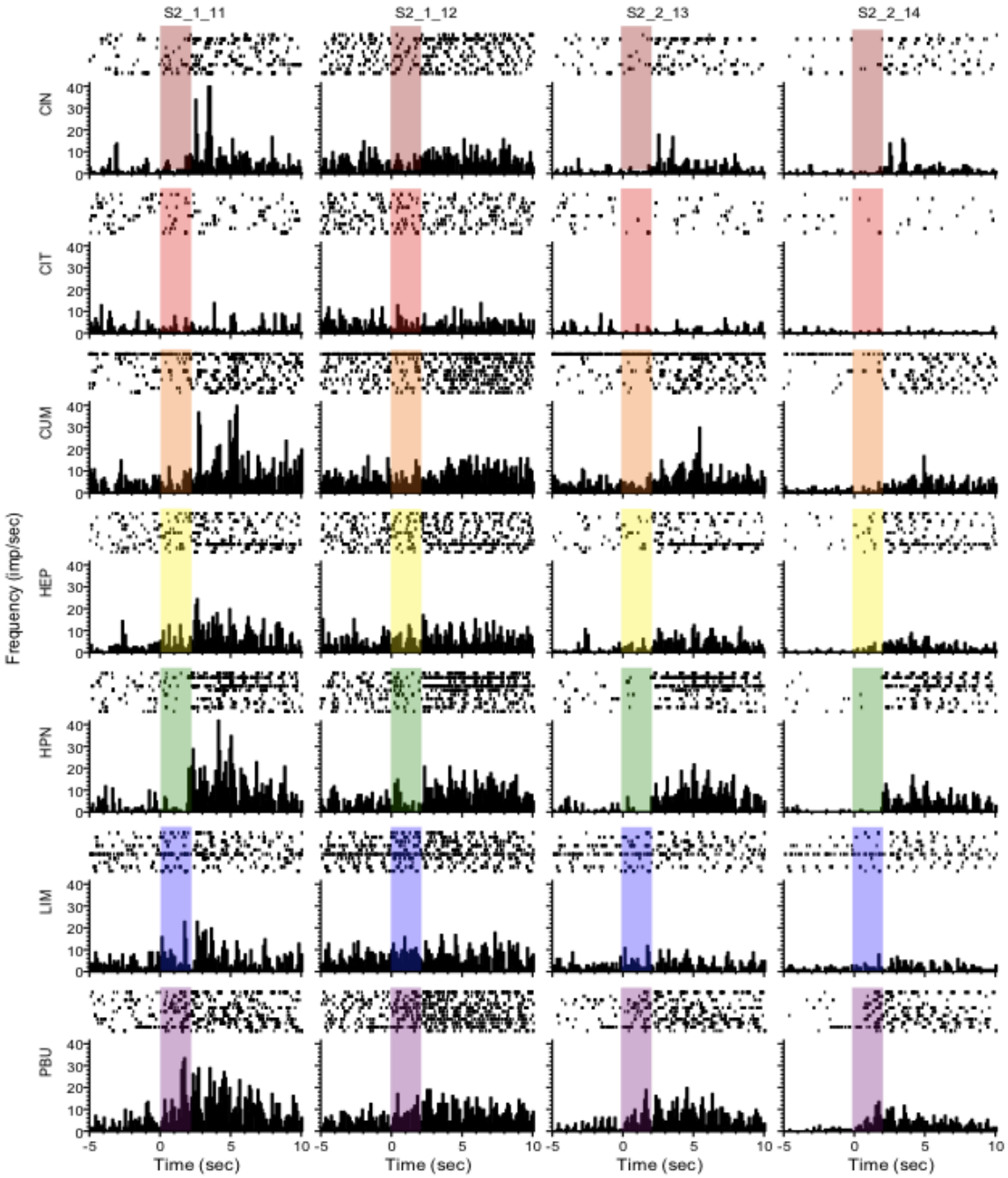


Figure 11: Non-adjacent neurons demonstrated robust odor-elicited activity and similar tuning. Perievent rasters and histograms of firing rate of four cells on two electrodes in response 2 sec presentations of monomolecular odorants at t = 0 (colored bars). Cells responded similarly yet selectively to the same odorants despite whether they were simultaneously recorded on the same or different electrodes. The latency to response was longer in these cells.

IV. DISCUSSION

I. Nicotine infusion occasions prolonged changes in firing rate of olfactory neurons

Based on statistical inference, seventy-five percent of units (n=8) exposed to an i.c.v. infusion of nicotine appeared to demonstrate a marked change in neuronal firing rate (**Fig. 3**). In S2_1_5, this change took form as a drastic increase in NFR compared to the pre-injection period. This observation is in line with the characteristic effects of nicotine exposure in which excitation would likely result from increased activation of nAChRs due to exogenous agonist binding on dopaminergic neurons that heavily innervate the OT (Voorn et al., 1986). However, the majority of these changes manifested as a decrease in NFR compared to the pre-injection period. Of particular interest, in one instance, nicotine infusion occasioned the total silencing of neuronal firing for a prolonged period of time (**Fig. 9**, **Panel C**). This finding was initially unanticipated, as nicotine is typically expected to have excitatory effects upon reward pathways. Therefore, a separate mechanism was needed to explain the prevalent inhibitory effects seen in the present dataset.

Although nicotine is traditionally thought to mediate the rewarding effects of drug use by having excitatory effects as a result of increased striatal dopamine and glutamate concentrations, there are in fact a variety of other neurotransmitter systems that are affected as well (Markou, 2008). Nicotinic acetylcholine receptors are also present on presynaptic sites of GABA-ergic neurons that inhibit dopaminergic neurons of the VTA and project to other striatal areas such as the OT. However, unlike the α4β2-containing and α7 homomeric nAChR subtypes found on dopaminergic and glutamatergic neurons

of the VTA, GABA-ergic nAChRs are particularly susceptible to desensitization and their inhibitory effect abates with higher concentration of nicotine. Thus, nicotine-induced release of striatal GABA via acute i.c.v. infusion is one mechanism through which the activity of OT neurons may be inhibited following acute nicotine infusion.

II. The odor-responsive properties of olfactory neurons are altered by nicotine infusion

Statistical analysis suggested that nicotine had a significant effect on neuronal activity surrounding events of odor presentation (**Table 4**). Specifically, nicotine infusion was found to occasion changes in NFR during both the pre- and post-onset periods. However, S1_1_2 and S2_1_5, which were both odor-responsive, demonstrated a significant change in activity exclusively during the post-onset period. The effect on post-onset (odor-elicited) activity was inhibitory in both units, providing evidence that nicotine may selectively affect odor-elicited activity without significantly altering baseline activity. While observed in only in two isolated cases, this effect on odor-responsive cells in the OT may in part underlie processes through which the rewarding effects of nicotine can be attributed to other external stimuli such as odorants and result in the attribution of affective valence to drug-associated cues (Markou, 2008).

III. Odor-responsive cells exhibit similar tuning profiles

Electrophysiological recordings were performed at depths > 2.0 mm from the brain surface as an approximate for the recording of olfactory tubercle neurons, although the exact recording site was not histologically confirmed. Units recorded simultaneously

whether on the same or different electrodes shared similar patterns of neuronal activity as indicated by the close timing of peaks and troughs between cells over the duration of the recording session, and peak timing was often found to closely coincide with that of odor onset (**Fig. 8**). Resultantly, 87% of the recorded units (n=15) were found to be odor-responsive as determined through statistical comparisons of mean firing rates 3 sec before and after onset of each of the 12 monomolecular odorants. These findings are similar to those reported by previous studies investigating properties of olfactory cortical neurons (Gadziola et al., 2015; Payton et al., 2012; Stettler & Axel, 2009; Wesson & Wilson, 2010).

Heptanal and propyl butyrate were the most likely to elicit a response in olfactory neurons (76.9% and 61.5%, respectively) while 5-methyl-3-hexanone and isophorone were the least likely (7.7% and 0%, respectively). By utilizing a set of 12 monomolecular odorant stimuli, this study has a greater possibility to detect differences in tuning breadth and profile between individual cells that are more difficult to be elucidated with smaller stimulus sets (Motokizawa, 1996; Lehmkuhle et al., 2003). However, more extensive, systematic studies that utilized more than double the number of monomolecular odorants used in the present study as well as hundreds of others in the form of odorant mixtures and in both anesthetized and awake animals have already been performed (Davison & Katz, 2007). Nevertheless, this study has been able to find a vast range of odor-elicited responses despite the relatively small stimulus set and sample size.

Also in line with the literature is the finding that recorded units were narrowly tuned and on average, demonstrated robust odor-elicited activity in response to only a

subset (~4/12; ~33.3%) of the experimental odorants (**Fig. 7**, **Panel A**; Wesson & Wilson, 2010). Some cells, such as S1_1_9 and S1_1_10, however, had a wide tuning breadth (8/12; 66.6%). Without a clearer sense of the site of electrophysiological recording, however, it is difficult explain any physiological observation of tuning breadth in terms of the local anatomy. It is possible that cells 9 and 10 were recorded from a functionally different olfactory cortical area, thus accounting for their unusually wide tuning breadth. An alternative explanation might be that the constrained stimulus set included a disproportional number of odorants to which these two particular cells were responsive, which would overrepresent the width of their tuning breadths

Odor-responsive cells were also found to share similar tuning profiles. S1_1_9 and S1_1_10 in particular demonstrated a marked increase in NFR in response to the same eight odorants (**Table 4**). This similar tuning was not peculiar to adjacent cells recorded simultaneously on the same electrode, but extended to nonadjacent cells recorded on different electrodes as well. This is a noteworthy finding that does not fit the current understanding of how odors are represented in olfactory cortical areas, such as in piriform cortex (Franks & Isaacson, 2006). Specifically, afferents from the main olfactory bulb are thought to be broadly distributed and innervate olfactory cortex nontopographically. As a result, in areas such as the piriform cortex, the olfactory neurons that respond to a given odorant are spatially distributed and have noncontiguous receptive fields while adjacent cells do not typically respond to the same odorants and thus have different tuning properties. However, in the present study not only were adjacent cells tuned similarly with each other, but also with nonadjacent cells recorded on

a different electrode. This may suggest a chemotopic organization of odor representation in the OT that is unseen in other olfactory cortical areas and instead is more akin to what is seen in primary cortical areas, such as auditory, visual, and somatosensory cortices.

Odor-responsive cells exhibited odor-elicited activity following odor onset, although the latency to response was longer in some cases and seemed to coincide more so with odor offset (**Fig. 11**). While an infrequent observation, odor-elicited activity related to offset might be indicative of ON/OFF olfactory cortical neurons that respond to the presentation and removal of a sensory stimulus. Such cells have been found in the main olfactory epithelium of cockroaches, which respond selectively to rises or drops in odorant concentration (Burgstaller & Tichy, 2010). Unrelatedly, a subset of odor-responsive cells exhibited phasic bursts of activity following odor offset that was entrained with the ongoing respiration cycle (**Fig. 10**). This is a characteristic expressed commonly among olfactory cortical neurons (Wesson & Wilson, 2011).

Experiment II: Valence attribution to conditioned cues paired with withdrawal

I. INTRODUCTION

I. Mechanisms of Withdrawal

The recovery of nAChRs from desensitization can also at least partially explain mechanisms of nicotine withdrawal and tolerance (Dani & Heinemann, 1998). As the desensitized nAChRs return to a functional state after cessation of nicotine exposure, pathways possessing these receptors may become hyperexcitable to synaptically released Ach and contribute to the uncomfortable and aversive psychological and somatic

symptoms of nicotine withdrawal that then drive further drug-taking behaviors through negative reinforcement. In this way, abstinence from smoking can cause stress to the smoker that is readily, yet temporarily, abated by reinstatement of smoking (Grunberg, 2007).

II. Drug- and Drug Withdrawal-Associated Cues

However, smoking is not driven solely by the euphoric and hedonic effects of nicotine nor entirely by the desire to avoid the agitating and aversive effects of nicotine withdrawal. Drug-associated cues, such as the sight of a cigarette or the smell of tobacco smoke, that are repeatedly present during drug use can gain positive affective valence and motivate further drug-taking behaviors (Caggiula et al., 2001). Similarly, contextual cues can also become associated with the unpleasant experience of drug withdrawal and increase drug craving, thus motivating drug-taking by instead gaining a negative affective valence (Kenny & Markou, 2005).

More specifically, nicotine withdrawal-associated cues have the potential to decrease activity of brain reward systems and produce reward deficit in a way that mimics the naturally occurring neurobiological and electrophysiological characteristics of spontaneous withdrawal where symptoms arise after cessation of smoking when nicotine is no longer readily available in the brain. Resultantly, drug-associated and drug withdrawal-associated cues may be just as, and if not more, important than nicotine itself in perpetuating drug-seeking and drug-taking behaviors.

In animal models of addiction, withdrawal can be precipitated in nicotine dependent animals by administration of antagonists, such as the non-competitive nAChR antagonist mecamylamine (Xiu et al., 2007). Mecamylamine precipitates somatic signs of withdrawal by blocking nicotine from binding to nAChRs, mimicking conditions akin to those of spontaneous withdrawal. In this way, mecamylamine is a very useful pharmacological tool for the experimental study of nicotine withdrawal, as the conditions under which the animal experiences withdrawal can be precisely manipulated and controlled.

III. Specific Aims & Experimental Design

In this experiment, adult male Sprague Dawley rats were divided into three experimental groups: control, paired, and unpaired. Control rats were not nicotine dependent and were given mecamylamine injections along with exposure to an odorant. Paired rats were nicotine dependent and were given mecamylamine injections paired with exposure to an odorant. Unpaired rats were also nicotine dependent but were not exposed to an odorant along with mecamylamine injection.

In order to determine the affective valence gained by exposure to an odorant paired with nicotine withdrawal, an odor preference assay on a custom-built olfactory hole-board was performed. Variations of the olfactory hole-board have been used to assess the effects of biologically relevant odors on the behavior of rats and mice (Wernecke & Fendt, 2015; File & Wardill, 1975). A circular six-hole custom-built olfactory hole-board was used to evaluate conditioned odor preference and aversion in

rats. Three monomolecular odorant, propyl butyrate, limonene, and octadiene, were placed under even numbered holes. Paired and control rats were exposed to propyl butyrate after mecamylamine injection which thus served as the conditioned withdrawal-associated odorant in paired rats. All rats were preexposed to limonene, the familiar unconditioned odorant, in the absence of mecamylamine, and octadiene, the novel odorant presented for the first time during the hole-board assay.

Nicotine-dependent rats injected with the nAChR antagonist mecamylamine exhibit somatic signs of withdrawal, such as cheek tremors, body shakes, genital licks, headshakes, teeth chatters, and yawns. Repeated pairings of propyl butyrate with nicotine withdrawal should result in the gaining of a negative affective valence and thus rats should demonstrate an aversion in the form of less head dips into and less time spent around the hole containing this odorant compared to controls. Alternatively, rats may show a preference for the unconditioned familiar odorant limonene such that they will make more head dips into and spend more time around the hole containing this odorant. Neither a preference nor an aversion is expected to be seen for the novel odorant octadiene, since there has been no experimentally manipulated valence attributed to it.

II. METHODS

Subjects. Twenty-one adult male Sprague Dawley rats (Harlan, Indianapolis, IN; 350-500 g) were used in this experiment. Subjects were singly- or co-housed. Subjects were handled 5 mins per day and preexposed to the withdrawal chamber for 30 mins per day for two days prior to experimentation. Paired rats were nicotine dependent and

experienced precipitated withdrawal in the presence of an odorant, propyl butyrate. Withdrawal rats were also nicotine dependent but were not exposed to a withdrawal-associated odorant. Control rats were not nicotine dependent but were exposed to the same odorant and in the same environment as paired rats.

Osmotic mini-pump implantation. Subjects were implanted with 2ML2 osmotic mini-pumps (Alzet, DURECT Corporation, Cupertino, CA) containing either 8.0 mg/kg/day nicotine tartrate (Sigma-Aldrich, Saint Louis, MO; 3.12 mg free base) or saline. Mini-pumps were prepared and implanted according to manufacturer's instructions. Subjects were anesthetized with 2-3 % isoflurane and an incision was made along the scapulae. A hemostat was used to create a subcutaneous pocket and the mini-pump was inserted parallel to the spinal column. The incision was closed with 3 mm steel wound clips and treated with 4 % lidocaine and bacitracin. Animals were allowed at least four days of post-operation recovery before further experimentation.

Non-associated odorant exposure. In order to establish a non-associated familiar odorant as a control, rats were exposed to limonene in glass chambers at a flow rate of 1 l/m for 30 mins per day for four consecutive days. The chamber was cleaned with 70% ethanol before and after each exposure.

Mecamylamine injection. One week after mini-pump implantation subjects were given an injection of mecamylamine (Sigma-Aldrich, Saint Louis, MO; 2.0 mg/kg; i.p.) or saline

and placed into an observation chamber for 30 mins per day for four consecutive days. For paired and control rats, propyl butyrate was introduced into the chamber at a flow rate of 1.5 l/m. The number of withdrawal signs, such as cheek tremors, body shakes, genital licks, headshakes, teeth chatters, and yawns was recorded by the experimenter.

Olfactory hole-board apparatus. A polycarbonate custom-made circular olfactory-hole board (60 cm diameter) was constructed with six holes (3 cm diameter) spaced equidistantly around the periphery of the apparatus 3 cm from the perimeter (**Fig. 12**). Odor preference was operationalized as the number of photo beam breaks within and the amount of time spent surrounding a hole containing a given odorant. The hole-board was divided into seven zones with one zone around each of the six holes and a center zone. In a pilot study, 12 naïve rats were used to determine the validity of the hole-board as a measure of odor preference to propyl butyrate, limonene, or octadiene in addition to fox urine, a predator pheromone. In a follow-up experiment, the same three monomolecular odorants were placed in a 10 ml glass jar under even-numbered holes of the hole-board. Empty jars were placed under the remaining, odd-numbered holes. The position of the odorants under the even-numbered holes was counterbalanced across within-subject trials to minimize the bias of place.

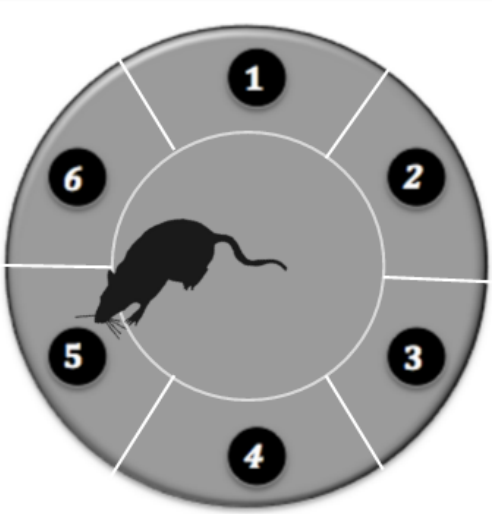


Figure 12: The olfactory hole-board apparatus. A custom, circular olfactory hole-board apparatus was constructed to assess the behavioral effects of a nicotine withdrawal-associated odorant on preference. Odorants were placed under even-numbered holes. The apparatus was divided into seven zones, each containing a central point.

Odor preference test. After nicotine exposure, rats were placed into the center of the hole-board facing between hole 5 and 6 and were allowed to freely explore for 10 mins in a low light environment. The number of head dips into each hole, the amount of time spent within each zone, and the distance from each point were measured by video tracking software. Movement of the animal's midpoint was tracked using ANY-maze (Stoelting, Wood Dale, IL) and head dips using Med-PC (Med Associates, Inc., Fairfax, VT). The apparatus was thoroughly cleaned with 70% ethanol before and after each trial.

III. RESULTS

The olfactory hole-board showed potential as a valid measure of odor preference

Pilot experiment assessing the validity of the hole-board apparatus as a measure of odor preference using 12 naïve rats. During the baseline session, there was no

difference in the number of head dips made into each hole, indicating no initial bias of place preference on the apparatus when no odorants are present (**Fig. 13, Panel A**). However, the head dips amongst even-numbered holes tended to be lower than those of odd-numbered holes, on average. During the experimental session, the number of head dips for even-numbered, odorized holes was lower than than for empty holes within the same session and between that for the same holes during baseline measures (**Panel B**). Among the experimental odorants, holes containing propyl butyrate tended to receive more head dips, on average. A linear regression model indicated a strong positive correlation between the average number of head dips and the average total distance travelled by each rat ($R^2 = 0.698$; data not shown).

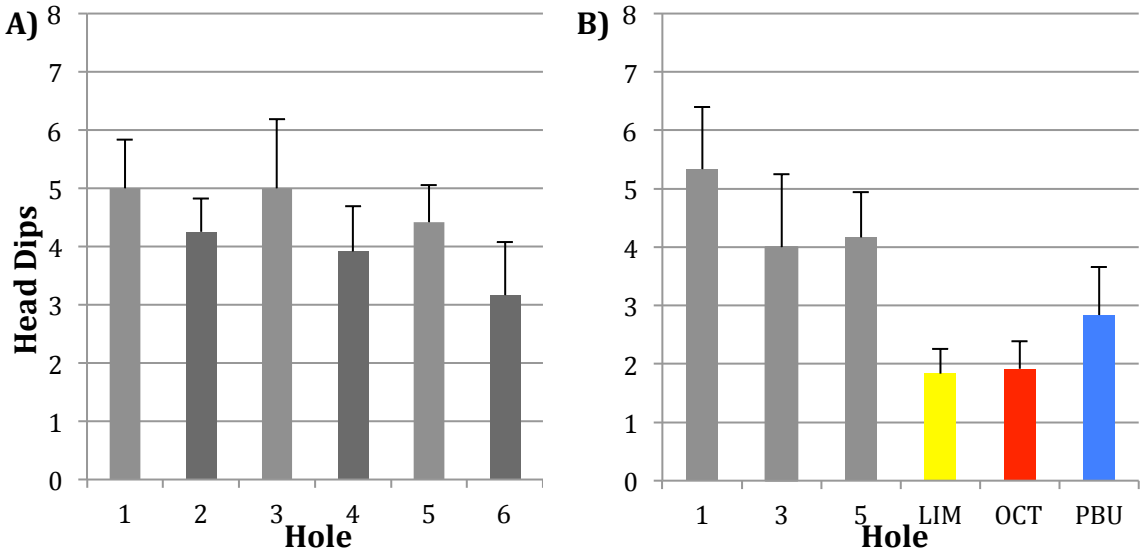


Figure 13: Naïve rats made fewer head dips into holes containing novel odorants. **A)** Number of head dips made into each of the six holes during a baseline session. When the holes contain no odorants the number of head dips made into each is invariable. **B)** When odorants are placed under even-numbered holes the number of head dips is considerably lower compared to those made into the odd-numbered, empty holes within the same session and into the same holes during the baseline session. LIM = limonene; OCT = octadiene; PBU = propyl butyrate. Error bars represent data ± SEM.

Rats also demonstrated thigmotaxsis during behavioral assays on the hole-board both with and without odorants as demonstrated by the average distance from the Center point at the middle of the apparatus (**Fig. 14**, **Panel A** & **B**). During experimental trials, rats remained closer to Hole 1 and further from Hole 3 compared to baseline. There was no difference in the distance from points corresponding to any of the other holes. Trackplot and occupancy plot diagrams also suggested this, as they showed a general preference for the periphery of the hole-board and an aversion to the center.

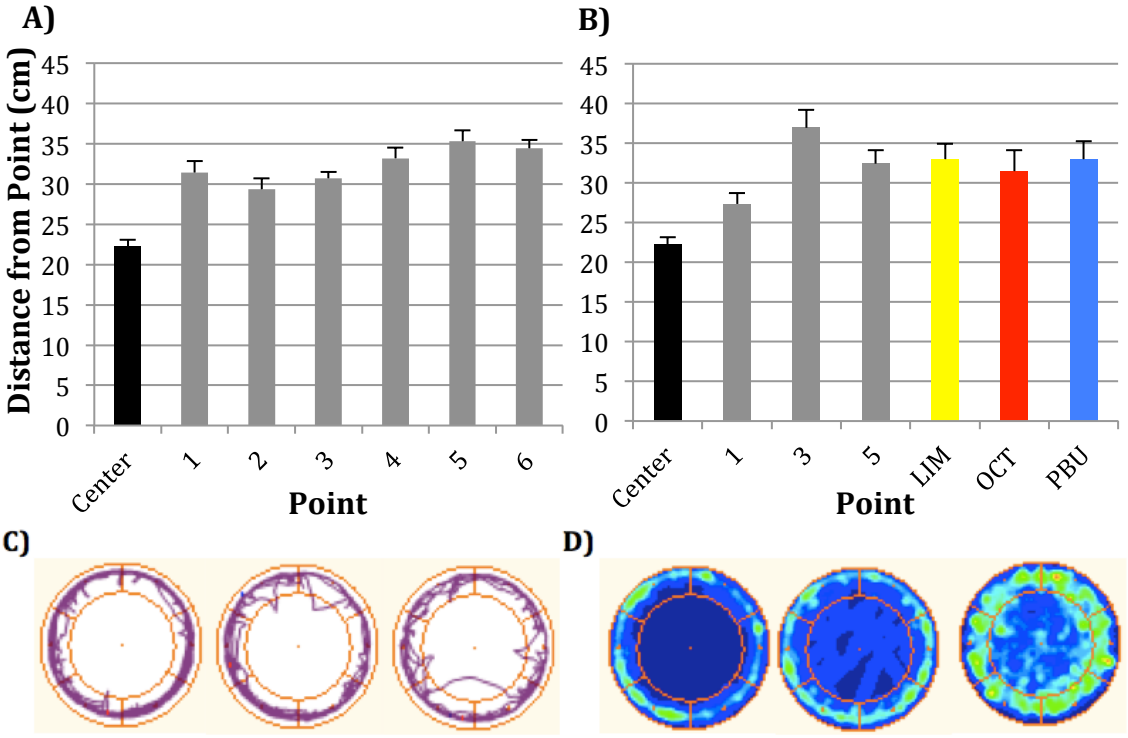


Figure 14: Rats demonstrated thigmotaxis during hole-board assays. Hole numbering runs clockwise with Hole 1 being in the rightmost zone. **A, B)** Rats remained close to the maximum distance from the Center point on the hole-board (r = 30 cm) regardless of whether or not odorants were present on the apparatus. There was no difference in the distance from any of the Hole points during baseline, but a decrease in distance from Point 1 and an increase in distance from Point 3 were seen during the experimental session. **C)** Trackplot of movement of three animals over the course of the trials. Movement traces can be seen to revolve around and avoid the Center zone. **D)** Occupancy plot of location of three animals over the course of the trials. Similarly,

location trace hotstops can be seen around the periphery of the hole-board. Error bars represent data ± SEM.

Rats showed an aversion during trials in which the predator pheromone fox urine replaced one of the monomolecular odorants on the hole-board (**Fig. 15**). This trend was also reflected in the time spend in each zone (**Panel B**). Interestingly, rats tended to make more head dips into holes that contained fox urine as opposed to those containing monomolecular odorants or nothing, on average (**Panel A**). Trackplots and occupancy plots show that rats spent more time in the zone directly opposite to the one containing fox urine, even when the location of the fox urine on the hole-board is changed (**Panels C & D**).

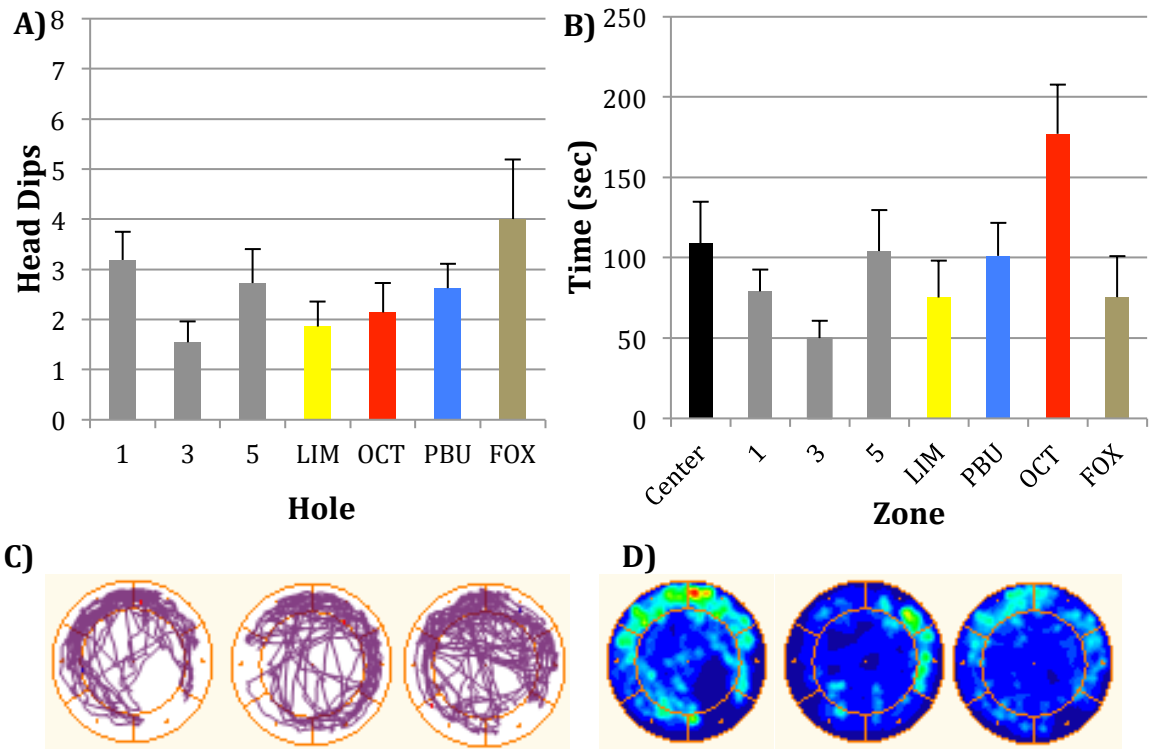


Figure 15: Rats showed an aversion to the predatory odor fox urine. A) Rats tended to make more head dips into holes that contained fox urine. There was no difference amongst those made into holes containg monomolecular odorants. **B)** Rats tended to

spend more time in the zone containing OCT when fox urine was present on the hole-board. **C)** Hole numbering runs clockwise with Hole 1 being within the rightmost zone. Trackplot of movement of three animals over the course of the trials when fox urine is placed under Hole 2, 4, and 2, respectively. Movement traces are shown to avoid the zone containing fox urine. **D)** Occupancy plot of location of the same three animals in **Panel C** over the course of the trials. Similarly, location trace hotstops can be seen around the zone opposite that which contained fox urine. Error bars represent data ± SEM.

II. The nAChR antagonist mecamylamine precipitated somatic signs of withdrawal

Rats implanted with osmotic subcutaneous mini-pumps containing either 8 mg/kg/day nicotine or saline demonstrated an array of somatic symptoms of withdrawal after i.p. injection of 2 mg/kg of the nAChR antagonist mecamylamine (**Fig. 16**). Withdrawal and paired rats displayed a greater number of body shakes, cheek tremors, head shakes, teeth chatters, and yawns compared to control rats, which were not nicotine dependent. There was no difference between the number of symptoms observed between paired and withdrawal rats. An apparent difference was seen in the number of teeth chatters between nicotine and saline rats.

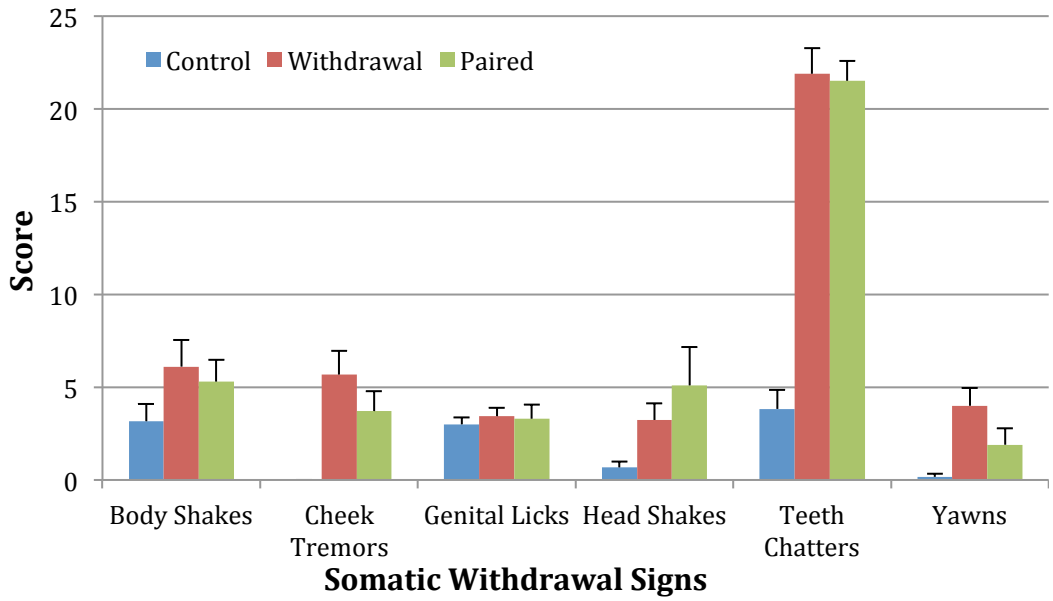


Figure 16: Mecamylamine precipitated somatic signs of nicotine withdrawal. Nicotine dependent rats displayed an increased number of withdrawal signs compared to controls. No difference was seen in the number of signs exhibited between paired and control rats. In particular, mecamylamine precipitated drastically more teeth chatters in nicotine-dependent rats compared to controls. Error bars represent data ± SEM.

III. Subjects did not show an aversion to the conditioned cue on the hole-board

Nicotine dependent rats that experienced precipitated nicotine withdrawal in the presence of the odorant propyl butyrate did not demonstrate an aversion during the hole-board assay in terms of the number of head dips made or time spent in zone (**Fig. 17**).

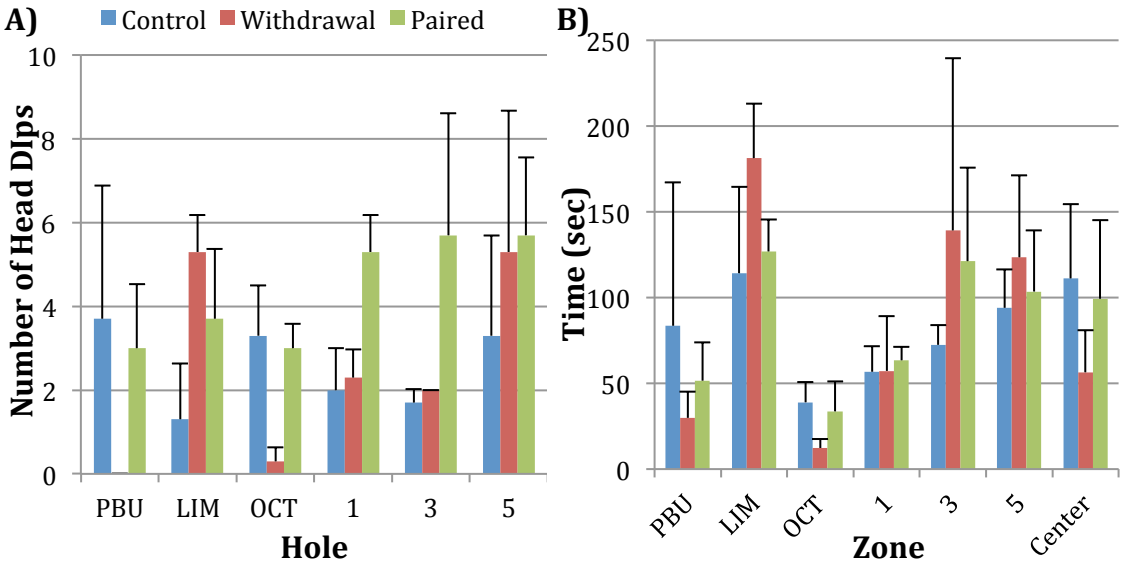


Figure 17: Paired rats did non demonstrate an aversion to the conditioned odorant. Error bars represent data ± SEM.

IV. DISCUSSION

I. The olfactory hole-board shows potential as a valid measure of odor preference

This study utilized a custom-built hole-board apparatus to assess the behavioral effect of a nicotine withdrawal-associated odorant on odor aversion. First, however, a pilot study had to be conducted in order to determine the validity of the hole-board as a

measure of odor preference and aversion. In order to assure that the assay would be as valid of possible, several considerations were made in the design of the apparatus. For example, the board was made circular rather than square or rectangular in order to reduce the bias of place that animals may have for corner holes (File & Wardill, 1975). Here, the six holes were spaced equidistantly around the periphery of the hole-board and odorants were placed under alternating holes to minimize odorant mixing. Indeed, rats exhibited thigmotaxis and tended to spend more time around the walls of the apparatus both with and without odorants on the board (**Fig. 14**). This is important for the validity of the assay since it shows that rats tend to spend more time around the perimeter of the board where holes are found and thus will be encouraged to make more head dips into them. As shown in **Fig. 13**, rats did in fact perform a considerable number of head dips into both odorized and empty holes, however there appeared to be fewer made into odorized holes, on average. Additionally, the floor and walls of the apparatus were colored dark and assays run in low lighting in order to allow for optimal animal contrast and video tracking performance as well as reduce animal anxiety.

When the predator odor fox urine replaced one of the monomolecular odorants, an aversion was seen in the form of less time spent around the corresponding hole and more time spent in the zone on the opposite side of the board (**Fig. 15**). This is expected, as predator odors are known to elicit unconditioned fear responses and aversion behaviors in rodents (Rosen, 2014). An aversion was not as apparent in terms of the number of head dips made into holes containing fox urine, however. This may be an indication that the hole-board assay is not adequate for the detection of odor aversions. In reconciliation of

this seemingly contradictory finding is the possibility that fox urine resulted in an aversion in the form of reduced exploratory behavior, as fewer head dips were made into all other holes whether odorized or not (**Fig. 15** compared to **Fig. 13**).

II. The nAChR antagonist mecamylamine precipitates somatic signs of nicotine withdrawal

Intraperitoneal injection of 2.0 mg/kg of the non-competitive nAChR antagonist mecamylamine was sufficient to precipitate somatic signs of withdrawal in nicotine-dependent rats (**Fig. 16**). Withdrawal and paired rats displayed a greater number of body shakes, cheek tremors, head shakes, teeth chatters, and yawns compared to control rats, which were not nicotine dependent. There was no difference between the number of symptoms observed between paired and withdrawal rats. Thus, the observation of somatic withdrawal signs confirmed that nicotine dependency had been instated by minipump implantation and that the odorant paired with withdrawal had the potential to gain a conditioned negative affective valence.

III. Rats do not show an aversion to the withdrawal-associated cue on the hole-board

Paired rats did not appear to show an aversion to the conditioned withdrawal-associated odorant propyl butyrate during behavioral assays on the olfactory hole-board apparatus in terms of the number of head dips made into or the amount of time spent around the hole containing the paired odorant (**Fig. 17**). In fact, the withdrawal group, which did not experience precipitated withdrawal paired with of propyl butryate seemed

to show an aversion to this odorant more so than the paired group. On average, more time was spent in the zone containing limonene, which was the familiar unconditioned odorant, for all groups. Overall, the wide variability of the data made interpretation difficult.

It is unclear why a conditioned response to the withdrawal-associated odorant was not observed in paired rats. Of the three experimental odorants, propyl butryate was chosen as the conditioned odorant since it was shown in a pilot study to evoke more head dips in naïve rats compared to limonene and octadiene, thus making any subsequent differences in head dips seen in paired rats more apparent. In order to strengthen the conditioned association, a future study might aim to use a higher dose of mecamylamine in order to precipitate more severe withdrawal symptoms or use a different nAChR antagonist completely, such as DHβE, which precipitates affective rather than somatic withdrawal symptoms (Kenny & Markou, 2001). It would also be advantageous to perform electrophysiological recordings in the OT of awake nicotine-dependent rats during olfactory hole-board assays in order to explicate how withdrawal-associated odorants are represented by OT neurons during discrimination tasks.

CONCLUSION

Taken together, these two experiments offer an electrophysiological and behavioral approach to understanding the effects of acute and chronic nicotine exposure on the neural systems of olfaction and reward that intersect within the olfactory tubercle. Given the OTs pivotal role in mediating processes of the two, I hypothesized that there

would be an interaction between nicotine and odorant exposure. In Experiment 1, electrophysiological recordings of urethane-anesthetized rats were conducted in OT while a series monomolecular odorants were presented before and after an acute intracerebroventricular infusion of nicotine.

Electrophysiological assessment revealed that a single instance of acute i.c.v. nicotine infusion was sufficient to significantly change the firing rate of OT neurons for a period of time, with a majority of these changes manifesting as a decrease in activity. Furthermore, the rate of odor-elicited activity following odor onset was altered by nicotine infusion while, in a subset of the sample, the rate of baseline activity preceding onset was unchanged. The findings of this experiment suggest that even upon initial exposure to nicotine, processes of valence attribution as carried out by the OT may have already begun to be affected.

These findings thus effectively set the stage for Experiment 2, which explored a chronic model of nicotine exposure and aimed to test the behavioral response to an odorant that was repeatedly paired with mecamylamine-precipitated withdrawal and that through conditioning would gain negative affective valence and thus evoke an aversive response. However, assays on the olfactory hole-board apparatus did not reveal an aversion to the conditioned withdrawal-associated odorant. Notwithstanding, there is still strong evidence for the OT to potentially dually mediate mechanisms of olfaction and drug addiction and thus be involved in representing drug- and withdrawal-associated cues. Further investigation of this research program will be imperative for the elucidation of processes through which drug reinstatement is induced and addiction, perpetuated.

REFERENCES

Balfour DJ, Wright AE, Benwell ME, Birrell CE (2000) The putative role of extra-synaptic mesolimbic dopamine in the neurobiology of nicotine dependence. Behav Brain Res 113:73–83.

Bissonette GB, Burton AC, Gentry RN, Goldstein BL, Hearn TN, Barnett BR, Kashtelyan V, Roesch MR (2013) Separate populations of neurons in ventral striatum encode value and motivation. PLoS One 8:e64673.

Burgstaller M, Tichy H (2011) Functional asymmetrics in cockroach ON and OFF olfactory cortical neurons. J Neurophysiol 105: 834-845.

Caggiula AR, Donny EC, White AR, Chaudhri N, Booth S, Gharib MA, Hoffman A, Perkins KA, Sved AF (2001) Cue dependency of nicotine self-administration and smoking. Pharmacology Biochem Be 70:515-530.

Dani JA, Heinemann, S (1996) Molecular and cellular aspects of nicotine abuse. Neuron 16:905–908.

Davison IG, Katz LC (2007) Sparse and selective odor coding by mitral/tufted neurons in the main olfactory bulb. J Neurosci 27:2091–2101.

File SE, Wardill AG (1975) Validity of head-dipping as a measure of exploration in a modified hole-board. Psychopharmacology 44:53-59.

Fitzgerald BJ, Richardson K, Wesson DW (2014) Olfactory tubercle stimulation alters odor preference behavior and recruits forebrain reward and motivational centers. Front Behav Neurosci 8:81.

Franks KM, Isaacson JS (2006) Strong single-fiber sensory inputs to olfactory cortex: implications for olfactory coding. Neuron 49:357-363.

Gadziola MA, Wesson DW (2016) The neural representation of goal-directed actions and outcomes in the ventral Striatum's olfactory tubercle. J Neurosci 36:548-560.

Gadziola MA, Tylicki KA, Christian DL, Wesson DW (2015) The olfactory tubercle encodes odor valence in behaving mice. J Neurosci 35:4515-4527.

Goniewicz ML, Knysak J, Gawron M, et al (2014) Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. Tobacco Control 23:133-139.

Ikemoto S (2003) Involvement of the olfactory tubercle in cocaine reward: intracranial self-administration studies. J Neurosci 23:9305-9311.

Ikemoto S (2007) Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens–olfactory tubercle complex. Brain Res Rev 56:27-78.

Imperato A, Mulas A, Di Chiara G (1986) Nicotine preferentially stimulates dopamine release in the limbic system of freely moving rats. Eur J Pharmaco 132**:** 337–338.

Kenny PJ, Markou A (2005) Conditioned nicotine withdrawal profoundly decreases the activity of brain reward systems. J Neurosci 25:6208-6212.

Lehmkuhle MJ, Normann RA, Maynard EM (2003) High-resolutionanalysis of the spatio-temporal activity patterns in rat olfactory bulb evoked by enantiomer odors. Chem Senses 28:499 –508.

Mansvelder HD, McGehee DS (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. Neuron 27**:**349–357.

Markou, A. 2008. Neurobiology of nicotine dependence. Phil Trans R Soc B 363: 3159–3168.

Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR, Craig CR, Collins AC, Damaj MI, Donny EC (2007) Guidelines on nicotine dose selection for in vivo research. Psychopharmacology 190:269-319.

Mayer, B (2017) How much nicotine kills a human? Tracing back the generally accepted lethal dose to dubious self-experiments in the nineteenth century. Archives Toxico l88.1:5-7.

Motokiza F, Yasyda N, Shimomura F (1988) The piriform cortex is not a direct olfactory relay to the mediodorsal thalamic nucleus in cats and rabbits. Neurosci Lett 8441-45.

Novotny TE, Hardin SN, Hovda LR*, et al* Tobacco and cigarette butt consumption in humans and animals *Tobacco Control* 2011;20:i17-i20.

O'Dell LE, Bruijnzeel AW, Smith RT, Parsons LH, Merves ML, Goldberger BA, Richardson HN, Koob GF, Markou A (2006) Diminished nicotine withdrawal in adolescent rats: implications for vulnerability to addiction. Psychopharmacology 186:612-619.

Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. San Diego: Academic Press; 1997.

Payton CA, Wilson DA, Wesson DW (2012) Parallel odor processing by two anatomically distinct olfactory bulb target structures. PLoS One 7:e34926.

Pidoplichko VI, Noguchi J, Areola OO, et al. 2004 Nicotinic cholinergic synaptic mechanisms in the ventral tegmental area contribute to nicotine addiction. Learn. Mem 11: 60-69

Rosen J (2004) The neurobiology of conditioned and unconditioned fear: A neurobehavioral system analysis of the amygdala. Behav Cog Neuro R 3(1):23-41.

Sosulski DL, Bloom ML, Cutforth T, Axel R, Datta SR (2011) Distinct representations of olfactory information in different cortical centres. Nature 472:213-216.

Stettler DD, Axel R (2009) Representations of odor in the piriform cortex. Neuron 63:854 – 864.

Striano BM, Barker DJ, Pawlak AP, Root DH, Fabbricatore AT, Coffey KR, Stamos JP, West MO (2014) Olfactory tubercle neurons exhibit slow- phasic firing patterns during cocaine self-administration. Synapse 68: 321–323.

Voorn P, Jorritsma-Byham B, Van Dijk C, Buijs RM (1986) The dopaminergic innervation of the ventral striatum in the rat: a light- and electron-microscopical study with antibodies against dopamine. J Comp Neurol 251:84 –99.

Wernecke KEA, Fendt M (2015) The olfactory hole-board test in rats: a new paradigm to study aversion and preferences to odors. Front Behav Neurosci 9:1-9

Wesson DW, Wilson DA (2010) Smelling sounds: olfactory–auditory sensory convergence in the olfactory tubercle. J Neurosci 30:3013-3021.

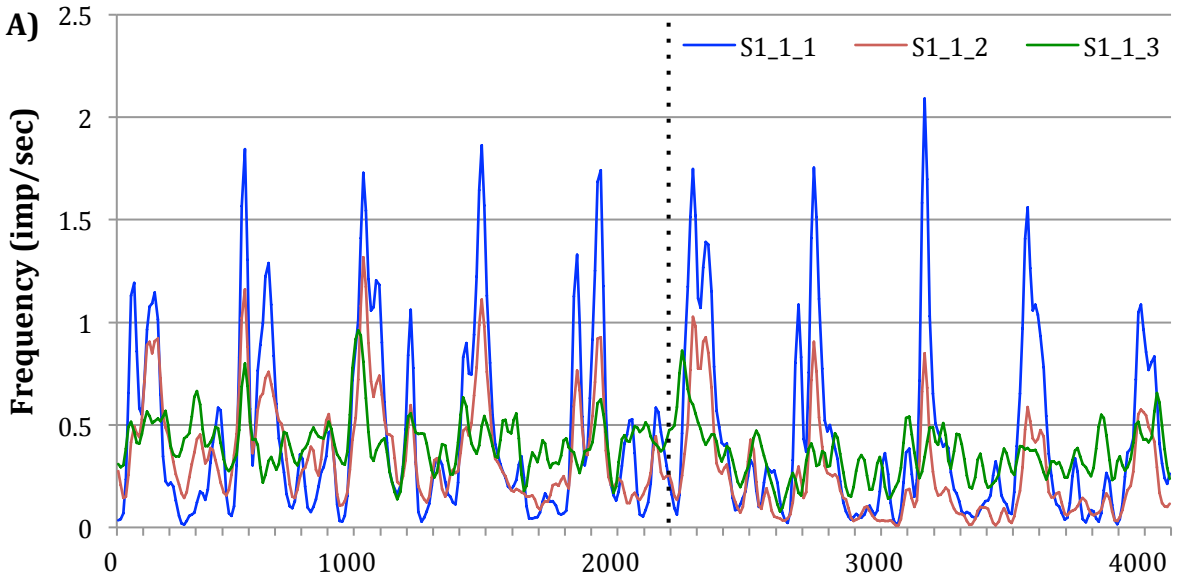
Wesson DW, Wilson DA (2011) Sniffing out the contributions of the olfactory tubercle to the sense of smell: hedonics, sensory integration, and more? Neuroscience Biobehav Rev 35:655-668.

Xiu L, Caggiula AR, Yee SK, Nobuta H, AF, Pechnick RN, Poland RE (2007) Mecamylamine attenuates cue-induced reinstatement of nicotine-seeking behavior in rats. Neuropsychopharmacology 32, 710 – 718.

Xu W, Wilson DA (2012) Odor-evoked activity in the mouse lateral entorhinal cortex. Neuroscience 223:12-20.

Zhou FM, Liang Y, Dani, JA (2001) Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. Nat Neurosci 4**:** 1224–1229.

APPENDIX



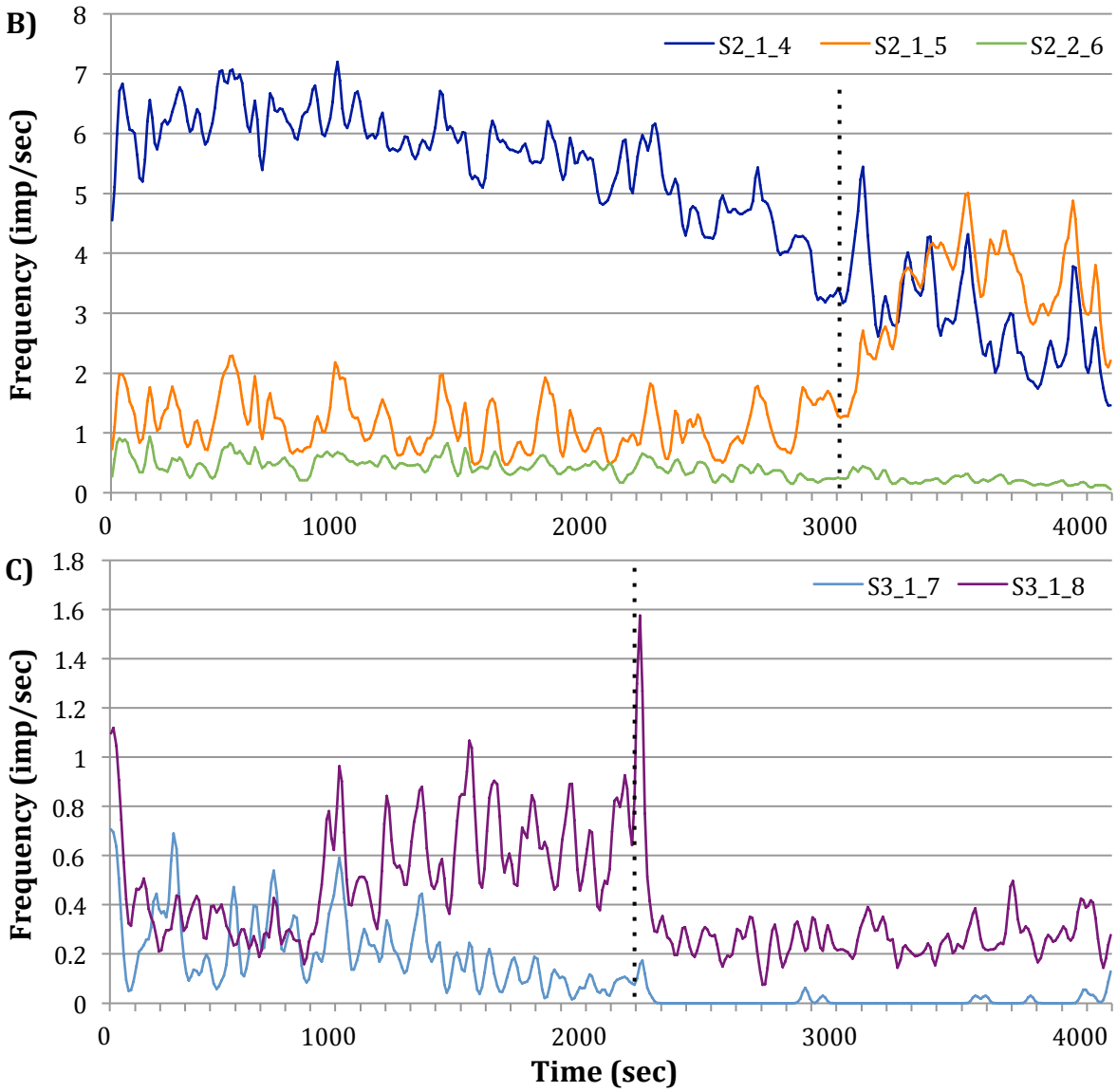


Figure 4: Olfactory neurons exhibit changes in neuronal firing rate after nicotine infusion. Rate histograms of impulses per second over time. Nicotine infusion at time indicated by black dotted line. **A)** Three simultaneously recorded adjacent cells demonstrate similar patterns of activity over the course of the recording session. The NFR of S1_1_2 decreased during the post-infusion period. **B)** Nicotine had strong, yet varying effects on cells recorded on the same electrode, although cells still displayed similar peak-trough occurrences. **C)** Nicotine had a potent, lasting inhibitory effect and completely silenced neuronal firing in S3_1_7 for several hundred seconds after infusion.

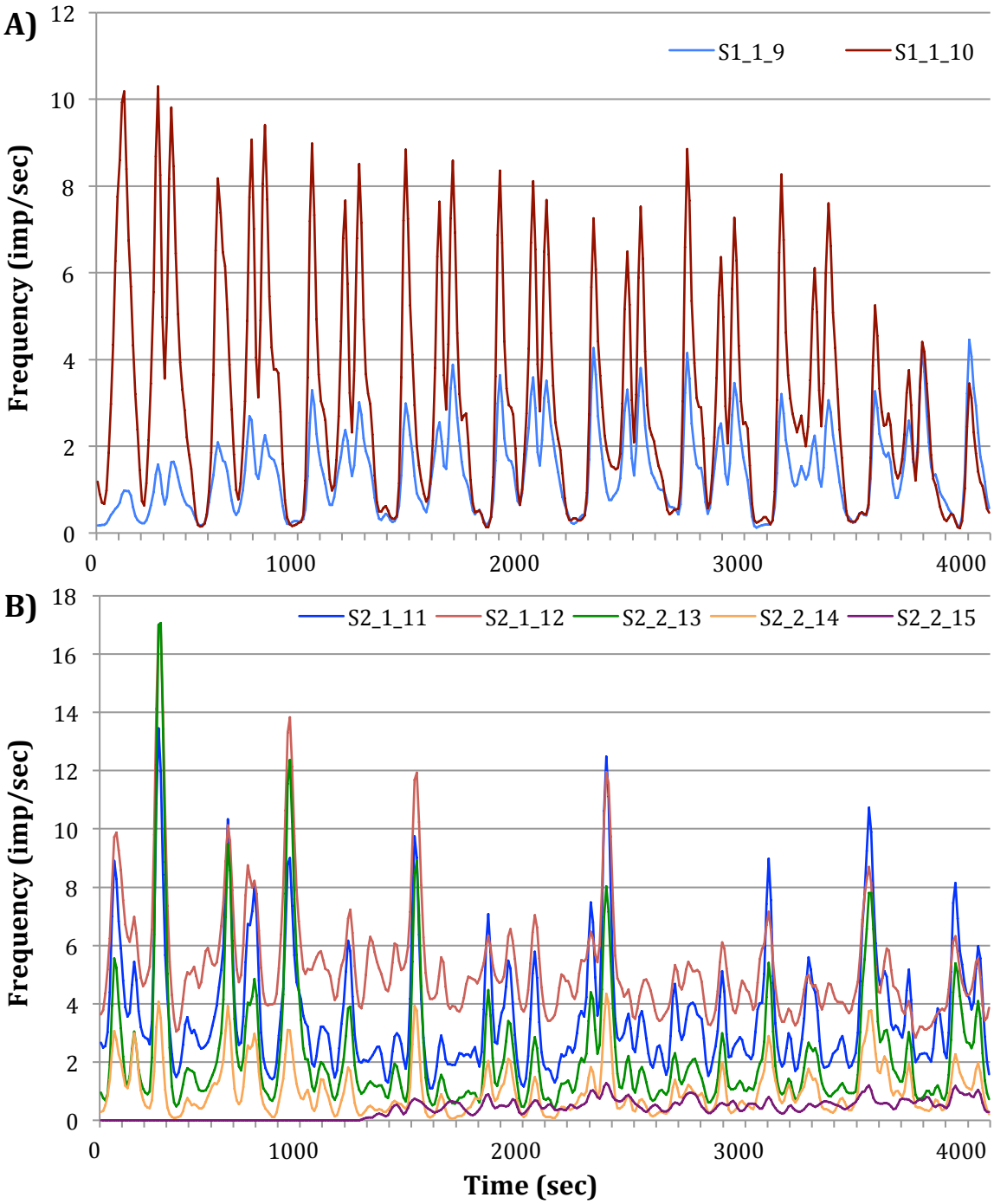


Figure 8: Olfactory neurons exhibit similar patterns of activity during recordings with odor presentations. Rate histograms of impulses per second over time. **A)** Two simultaneously recorded cells from the same electrode in S1. The timing of peaks and troughs are in synchrony throughout the recording session. **B)** Five simultaneously recorded cells from two separate electrodes in S2. Cells from adjacent electrodes also exhibited similar patterns of activity. Spike data for S2_2_15 before t = 1400 not sorted.