Odor identification in novel olfactory environments is selectively impaired in a mouse model of autism

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Abstract

Aversion to novel stimuli in autism affects quality of life. We developed a behavioral paradigm to study the effect of novel background odors on odor discrimination in mouse models of autism. We trained wild type mice to discriminate target odors in known background odors. When tested, mice could discriminate known targets in novel background odors, a task similar to the visual CAPTCHA used to distinguish humans from computers. Using glomerular imaging data, we showed that WT mice used an algorithm that required less training data than a linear classifier or nearest neighbor classifier. The *Cntnap2*^{-/-} mouse model of autism matched wild type mice performance in the presence of known backgrounds, but performance fell almost to chance levels in the presence of novel backgrounds. Wild-type mice use a robust algorithm for detecting odors in novel environments and this computation is selectively affected in a mouse model of autism.

Introduction

Autism Spectrum Disorders (ASD) can present with olfactory hypersensitivity to odors(Wicker, Monfardini, and Royet 2016) and deficits in odor valence discrimination(Rozenkrantz et al. 2015) that might underlie food neophobia(Luisier et al. 2015) affecting quality of life. Autistic people have an “insistence on sameness” according to DSM-V, and mouse models of autism avoid entering environments with novel background odors(Brunner et al. 2015; Gordon et al. 2016) suggesting computational problems with novel odors.

Wild type (WT) mice in their natural environment need to identify target odors in environments with novel background odors. Odorants activate olfactory receptors expressed in the olfactory epithelium. These receptor neurons project to specific glomeruli in the olfactory bulb. Glomeruli activation is processed by the olfactory bulb circuitry and is conveyed by mitral and tufted cells to different brain areas(Fukunaga et al. 2012a; Nagayama et al. 2010; Igarashi et al. 2012a). Critically, the glomerular representations of mixtures with different weak targets but the same strong background odor are dominated by the background odor and are very similar. The olfactory bulb circuitry can separate these similar patterns(Barnes et al. 2008); however, mixtures that include novel background odors do not have directly learned associations and mice need to generalize to produce an appropriate action—a function that is affected in ASD. Since novel odors produce larger mitral cell activation compared to familiar odors (Kato et al. 2012), discrimination might require enhanced cortical recruitment of inhibition to suppress novel background odor activity compared to familiar background odor activity. Mouse models of autism have reduced GABA activation(Coghlan et al. 2012); hence, olfactory cortical responses in mouse models of autism might be more easily overwhelmed by novel background odors. Odor identification in novel environments might be a function that WT mice can perform but might be affected in mouse models of autism.

WT mice can be trained over hundreds of trials to detect target odors in mixtures where mice are familiar with the target and the background odors (Rokni et al. 2014). However, we do not know if WT mice can

generalize and successfully recognize a known target odor on the first presentation with a novel background odor, nor what algorithm WT mice employ to generalize. Mouse models of autism have reduced neural reliability and reduced inhibition (Geramita et al. 2020; Peñagarikano et al. 2011); therefore, olfactory discriminations that employ algorithms that need these functions to be intact are more likely to be selectively affected in mouse models of autism.

Several algorithms can identify odors in the presence of known background odors and may generalize to novel background odors. The simplest mechanism uses a dedicated receptor activated specifically by a target odor. Activation of particular glomeruli would indicate the presence of this target odor even in the presence of background odors. Although some odors trigger innate responses and activate selective receptors (Pacífico et al. 2012; Ferrero et al. 2011; Dewan et al. 2018), this mechanism does not generalize to arbitrary odors that do not have such specialized receptors.

Unsupervised algorithms (Hopfield 1991) can use differences in the temporal profile between the background odor and target odor to segment them. These differences could also be onset delays (Sehdev and Szyszka 2019) between the target and the background (Verhagen et al. 2007). These algorithms do not require previous knowledge of the odors and are intrinsically able to handle novel background odors, but they require a temporal gap between the presentation of the background and the target odors.

The mouse olfactory system can also implement simple supervised algorithms using the feedforward connections between the olfactory bulb and its targets, including the olfactory tubercle and piriform cortex. These supervised algorithms can be trained to classify mixtures of background odors and target odors. The simplest supervised algorithm is the linear classifier, which can be implemented by an output neuron (Mathis et al. 2016) that receives glomerular activation as input. The synaptic weights are adjusted, based on training examples, to detect the presence of the target odors. Linear classifiers, using glomerular data from the dorsal surface of the olfactory bulb, can match the performance of WT mice on known background odors (Mathis et al. 2016). However, we do not know if linear classifiers would match the performance of animals for mixtures that include novel background odors.

The expansion in the number of neurons between the olfactory bulb and the piriform cortex can also implement a simple non-linear classifier—the nearest neighbor classifier (NNC) (Dasgupta, Stevens, and Navlakha 2017). NNC determines the closest match to the input in the training data. NNC can be implemented as multiple parallel linear classifier neurons, with the best matching neuron to the glomerular input suppressing the activity of other non-matching neurons. Although the piriform cortex has the required recurrent inhibition (Large et al. 2016; Franks et al. 2011) to implement this “winner-take-all” function (Bolding and Franks 2018), we do not know if the NNC would be a good match for WT mice behavior in novel background odors.

An alternative approach to the supervised algorithms is to deconvolve the individual odors that are present in a mixture. Deconvolution algorithms proposed for sensory systems (Koulakov and Rinberg 2011; Grabska-Barwińska et al. 2017; Li and Hertz 2000; Otazu and Leibold 2011) assume knowledge of a large dictionary of known odors. These algorithms estimate a combination of odors from the dictionary

that produces the best match to the observed pattern of glomerular activation. Interestingly, it has been hypothesized that failure to create an estimate of the sensory input (Sinha et al. 2014) is one of the key computational deficits in ASD and could affect the performance of these deconvolution algorithms.

Here we show that WT mice are able to recognize immediately target odors in the presence of novel background odors and familiarity with the backgrounds was not necessary. We show that WT use a sophisticated algorithm to identify odors in novel environments that is less sensitive to the number of training examples than simple supervised algorithms. This computation was selectively affected in the *Cntnap2*^{-/-} mouse model of autism whereas odor identification in known backgrounds was spared.

Results

Olfactory CAPTCHA

In order to test responses to novel background odors, we used the olfactory equivalent of the visual CAPTCHA (Von Ahn et al. 2003) employed for human verification tasks, which also serves as a benchmark for testing image recognition algorithms (George et al. 2017). CAPTCHA requires a user to identify letters (known targets) superimposed on distracting stimuli (novel backgrounds). Animals exposed to a novel background as part of an olfactory CAPTCHA task have to identify target odors present permitting an unambiguous evaluation of olfactory function. In contrast, it is harder to evaluate olfactory function of animals freely exploring novel background odors (Brunner et al. 2015; Gordon et al. 2016) because there is no task assigned. We hypothesize that WT mice will be able to identify known odors in the presence of novel background odors, since this situation mimics what they experience in the wild and that mouse models of autism will not, because CAPTCHA requires generalization and robust responses to novelty, which are affected in ASD.

Mixtures of target and background odors were divided into a training set and a test set. Animals were trained with the training set and their performance was evaluated with the test set. The training set consisted of 16 mixtures of 3 odors, 1 target odor that solely determined reward availability (4 possible odors), 1 contextual background odor (4 possible odors), and 1 fixed background, (s)-(-) limonene. Both (s)-(-) limonene and the contextual background odors were presented at a higher concentration (0.1% of saturated vapor) than the target odors (0.025%). The contextual background odors and the (s)-(-) limonene were the known background odors. The test set also consisted of mixtures of 3 odors that included the same target odors and contextual background odors, but the (s)-(-) limonene was replaced by one of 11 possible novel background odors, which were also presented at a higher concentration (0.1%). We used a go/no-go design, with two of the target odors indicating the presence of a water reward upon licking the water tube. We chose a relatively complex training set (eight go mixtures and eight no-go mixtures) to promote generalization. We chose a go/no-go licking task because it is easy for WT mice to learn (Komiyama et al. 2010) and it can also be learned by the *Cntnap2*^{-/-} mouse model of autism (Rosario et al. 2020).

Olfactory stimulus consisted of weak target odor embedded in strong background odors

We built a triple air-dilution odor machine to deliver mixtures of three odors (see **Methods**). We used intrinsic optical imaging of WT mice to quantify the pattern of glomerular activation of the target and background odors at the same concentrations used in the task (see Fig. 1A and **Sup. Figure 1**). The four target odors had lower levels of average glomerular activation compared to the four contextual background odors (see Fig. 1B,C). The 11 novel background odors had intermediate levels of average glomerular activation between the target and the contextual background odors. Glomerular patterns of mixtures of odors were dominated by the background odors (see Fig. 1D) resulting in go and no-go mixtures with known odors that were quite similar (correlation = 0.85 ± 0.07) but within range of previous stimuli that rodents were able to discriminate (Gschwend et al. 2015; Uchida and Mainen 2003; Abraham et al. 2004).

WT mice could identify target odors among novel background odors

Four head-fixed water-deprived WT mice were trained to detect the presence of target odors and were rewarded with water if they licked in response to the target go-odors (see Fig. 1E,F). If they licked before the target go-odors appeared or if they licked in response to no-go target odors, odor delivery was stopped and the mice were given a time-out. Mice were trained for ~ 9 days (see training protocol) to detect targets among known backgrounds. Once animals displayed consistent performance at the final concentration ($> 80\%$ correct for more than 50 trials), trials with novel background odors were introduced. Presentation of the novel background odor and the contextual background odor preceded the onset of the target odor by 0.75 s and 1.5 s respectively (see **Sup. Figure 2**). This asynchronous odor presentation emulated a situation where the animal is in a novel environment when the target appears and should be easier for the animals because of sensory adaptation to the novel background (Verhagen et al. 2007). We used a long inter-trial interval to avoid receptor adaptation effects and to permit the airflow to clean the odor delivery system: the separation between trials was 30.4 ± 3.3 seconds (1885 trials).

The trials with different novel background odors were separated by five or six trials with known background odors. These trials with known background odors were included to maintain the animal's motivation with an easier task and to reinforce the identity of the target odors. Each presentation of a novel background odor was separated by other presentations with the same background odor by ~ 25 minutes. Each novel background odor was presented, at most, four times per day on two separate days, giving a maximum of eight presentations per novel background odor per animal to prevent learning of the novel background odors.

WT mice performance for known background odors was high (87.4%, $n = 1563$ trials), and significantly higher than the 50% chance level ($p = 1.24 \times 10^{-215}$, binomial test) (see Fig. 2A). WT mice almost never failed to detect the targets (0.7% of the trials were false rejections), as with other go/no-go behavioral tasks (Rokni et al. 2014; Kuchibhotla et al. 2019). WT mice were able to solve the olfactory CAPTCHA and

successfully identify targets in the presence of novel background odors at higher than chance levels (76.9%, $n = 334$ trials for the novel background trials, $p = 3.08 \times 10^{-24}$, binomial test). Moreover, WT mice were able to identify target odors among novel background odors, even on the first presentation on day 1 (see Fig. 2B), with WT mice performing at 79% ($p = 6.4 \times 10^{-5}$, binomial test, $n = 44$ trials, 4 animals). There was no systematic increase in performance as the animals familiarized themselves with a novel background odor, and there was no significant positive linear correlation between a background odor presentation number and performance ($r = -0.48$, $p = 0.22$). Interestingly, when the novel background odors were used, animals had a significant increase in the number of false rejections (5% from 0.7%, $p = 6.91 \times 10^{-7}$, Fisher exact test) suggesting increased difficulty in detecting the target odor in novel backgrounds compared to known backgrounds (see Fig. 2C, D).

To further test whether familiarity with the novel background odors increased performance, we trained a different cohort of five WT mice in the same task (see **Supplementary material 1**). This new cohort had been exposed to 5 of the 11 novel background odors in their home cages for 30 minutes over 6 days before behavioral training started. The performance of the new cohort of animals in response to the previously exposed 5 odors was 79.6% (211 trials, see Supplementary Material), which was not significantly different ($p > 0.1$, Fisher exact test) from the original group performance on these odors (78.8%, 132 trials, 4 animals). Both exposure to the same novel background odor during the task and longer exposure times outside the task context did not produce systematic performance increases, indicating that WT mice employed an algorithm that did not require previous knowledge of the background odor to detect target odors among novel background odors.

WT recognized the novel background odors after a single presentation

We wanted to determine how quickly the mice familiarized themselves with a novel background odor by measuring the sniffing response. Sniffing rate increases as an animal explores novel odors (Wesson et al. 2008; Verhagen et al. 2007). We monitored non-invasively animal sniffing during the olfactory CAPTCHA task using a flow sensor attached to the odor delivery tube (Bolding and Franks 2017) (see Fig. 3A,B). On the first presentation of the novel background odors, the sniff rate increased compared to the trials with the known background odors (1.37 ± 0.36 extra sniffs per second, $p = 0.00067$ binary test) indicating that the novel odors were perceived as novel. The sniff rate was also elevated on the first presentation on the second day (day 2, 0.95 ± 0.34 , $p = 0.007$, binary test). Interestingly, average sniff rates dropped to rates similar to known background trials ($p > 0.1$) on further exposures in a session, indicating that the animals recognized the novel background odor (see Fig. 3C). This was quite remarkable, because the novel background odors were presented with different targets and contextual odors, and subsequent presentations of the same novel background odor were separated by at least 25 minutes.

We also quantified the animal familiarity to the novel odors using the number of sniffs that it took from target onset to lick response. During the known background trials, the animals took 2.93 ± 0.06 sniffs

before licking (842 responses). Animals took a larger number of sniffs (5.8 ± 1.11 , 20 responses) on the first exposure of a novel background and it was significantly higher than the number of sniffs for known backgrounds (Wilcoxon rank-sum test, $p = 0.0023$). Consistently with increasing familiarity after the first exposure and reduction of exploratory behavior, the number of sniffs before licking fell after the first presentation of a novel odor to a value that was similar to the number of sniffs in the presence of known backgrounds. Animals increased their sniff rate and number for novel odor but this increase disappears after a few exposures as they recognized the novel background odor. Surprisingly, there was no increase in performance even when WT mice recognized the novel background odor, suggesting that WT mice used an algorithm that required no knowledge of the background odor.

WT mice did not require background adaptation to detect odors in novel environments

In the first set of experiments, the target odor appeared 0.75 s after the onset of the novel background odor. Due to adaptation to the early-appearing novel background odor, the target could become more salient, enhancing its detectability. In order to directly test whether sensory adaptation was necessary for identifying odors in novel environments, we trained a different group of five animals in a condition where the target and background odors were presented synchronously (synchronous case), rather than the target odor appearing after the novel background odor (asynchronous case) (see Fig. 4A). This synchronous odor presentation emulated a situation where the animal is suddenly presented with a mixture that includes a novel odor. In the synchronous case, both the target and the novel background odors endured the same process of adaptation, reducing the impact of differential adaptation to the background odor. For the known background odors, animals in the synchronous case performed at a high level (88.5%, $n = 1,191$ trials, $p = 4e-176$, binomial test), similar to that in the asynchronous case.

WT mice were able to identify the target odors with the synchronously presented novel background odors at higher than chance levels ($p = 7.4e-21$, binomial test, see Fig. 4B). Although the mice had an increase in false rejections (see Fig. 4C), the total performance for the synchronous presentation (77.2%, $n = 277$ trials, 5 animals) was almost identical (Fisher exact test, $p > 0.9$) to the asynchronous case performance for the previous group of 4 animals (76.9%). Sensory adaptation did not make a significant contribution to odor identification in novel environments. Animals identified the target odors among novel backgrounds (74.3%, 39 trials) at higher than chance levels ($p = 0.001$, binomial test), even on the first presentation of a novel background odor (see Fig. 4D). There was no significant correlation between the odor presentation number and the performance of the mice ($r = -0.02$, $p = 0.95$); hence, as with the asynchronous case, there was no improvement of performance with further exposures to a novel background odor.

Animals performing the synchronous task also reacted to the first presentation of a novel background odor by increasing their sniffing rate (see Fig. 4E), and this response was also adapted after two

presentations, but the number of extra sniffs before a response did not increase for novel background odors (see Fig. 4F, **Supplementary Material 3**, and **Sup. Figure 4**).

Fast sniff rates correlated with increased performance on novel background odors

We wondered whether rapid sniff rates would correlate with increased performance as described for other olfactory tasks (Kepecs, Uchida, and Mainen 2007). When animals increased their sniff rates, inhalations become shorter (Jordan et al. 2018). Brief inhalations correlated with higher performance only on novel background odors, suggesting that rapid inhalation induced adaptation (Verhagen et al. 2007) might contribute to odor identification in novel environments (see **Supplementary material 2** and **Sup. Figure 3**); however, the lack of difference in performance between the synchronous and asynchronous case showed that adaptation is not the only mechanism used in odor identification in novel olfactory environments, and other circuit mechanism might play a role.

Linear classifier matched WT mice's performance in novel background odors

WT mice performance in odor identification for mixtures of known odors can be matched by linear classifiers, trained using calcium imaging of glomerular activation patterns of the dorsal surface of the olfactory bulb (Mathis et al. 2016). The very simple circuit implementation of a linear classifier is less likely to be affected in mouse models of autism. We wanted to determine whether linear classifiers, trained with glomerular activation images from mixtures from the training set, were sufficient to generalize and match WT mice performance in response to novel background odors. Could a linear classifier predict the WT mice's performance in response to individual novel background odors?

We compared the behavioral performance of nine WT mice for individual novel background odors with the performance of a linear classifier trained using intrinsic imaging (see Fig. 5A). We measured the glomerular activation patterns in response to individual odors in WT mice and created virtual odor mixtures, including different levels of Gaussian noise and an experimentally measured saturation nonlinearity (Mathis et al. 2016) (see **Sup. Figure 5**). The linear classifier was trained using the 16 mixtures of the training set to determine if the target odor in the training mixture was a go or a no-go. To evaluate the performance for each of the 11 individual novel background odors, the linear classifier was tested using the 16 mixtures where (s)-(-)-limonene was replaced by an individual novel background odor. The linear classifier trained with intrinsic images was able to identify odors at a level that matched or exceeded the performance of the animals in novel backgrounds similar to measures using glomerular calcium imaging (Mathis et al. 2016). However, the performance of the linear classifier for a given novel background odor was not a good predictor of the WT mice's performance for that novel background odor (see Fig. 5B,E,F).

Nearest neighbor classifier (NNC) predicted WT mice performance in response to novel background odors

We wondered if WT mice performance for individual novel background odors could be predicted using a non-linear classifier. The NNC is a non-linear classifier that determined which of the 16 training mixtures that included (s)-(-) limonene was the best match to the observed mixture with a novel background odor. If both the best match and the observed mixture contained the go (or no-go) target odors, the classification was a success (see Fig. 5C). We trained the NNC using the same imaging data from the dorsal surface as used for the linear classifier. The NNC correlated significantly with the mice behavior in response to individual novel background odors (see Fig. 5D,E,F) demonstrating that it was possible to predict WT mice performance using dorsal glomerular data.

The virtual odor mixtures used did not capture other types of nonlinear receptor activation in response to odor mixtures (Zak et al. 2020; Inagaki et al. 2020) that might affect an algorithm performance. To control for these potential confounders, we used imaging data from real mixtures to train the linear classifier and the NNC. The comparison with the behavioral responses of nine animals yielded similar results as the responses of the classifiers calculated using the virtual odor mixtures: the NNC predicted WT mice performance in response to individual novel background odors, whereas the linear classifier was not a good predictor (see Fig. 5G,H). Animals used an algorithm that was better approximated by the NNC than the linear classifier. The NNC requires a recurrent inhibitory circuit in olfactory cortex to perform the winner-take-all computation, and its performance might be affected by reduced inhibition in mouse models of autism (Coghlan et al. 2012).

WT mice responses were delayed in novel backgrounds

We wondered if mice might be performing a different computation when challenged with novel background odors and whether this extra computation might be reflected in increased reaction times, as shown for other behavioral tasks (Abraham et al. 2004). Previous work (Rokni et al. 2014) had shown very little increase in response latency with task difficulty for animals identifying odors among known background odors.

WT mice had slower reaction times for novel background odors compared to known background odors for both the synchronous and the asynchronous task. The lick reaction time for the asynchronous case for novel background odors was 651.3 ± 37 ms and it was significantly slower ($n = 151$ lick responses, 4 animals, $p = 0.013$, Wilcoxon rank-sum test) than the response for the known background odors (571.4 ± 11.9 ms, 812 lick responses, see Fig. 6A). The reaction time for novel background odors for the synchronous case was 844.8 ± 46.3 ms, and it was also significantly slower (110 lick responses, 5 animals, $p = 0.026$, Wilcoxon rank-sum test) than the reaction time for known background odors (739.8 ± 16.5 ms, $n = 577$ trials). This increase in response time in the CAPTCHA task suggested that identifying

odors among novel background odors might further involve recurrent computations compared to odor identification in known background odors.

WT mice performance was less sensitive to amount of training data than the NNC

The NNC was a good predictor of WT mice performance in novel background odors. The NNC requires a training set that includes an appropriate match to a new mixture; otherwise, the NNC performance might decrease (see Fig. 6B). To confirm this, we reduced the number of training examples, from 16 training mixtures (4 contextual background odors x 4 target odors x (s)-(-)limonene) to 8 mixtures (see Fig. 6C). The task became harder because not only the test mixture included a novel background odor, but also the test mixture was a novel combination of known contextual background and target. As expected, the performance of the NNC decreased significantly ($p = 0.017$, single tailed fisher exact test) from 79.3% when trained with the full set to 70.4% when trained with the restricted set. The performance of a linear classifier also significantly decreased ($p = 0.017$, single tailed fisher exact test) from 76.7% for the full set to 64.7% for the restricted set. If animals employed these algorithms, their performance would be affected by reducing the number of training examples.

In order to test this, we trained a new batch of three WT mice using the restricted set with asynchronous presentation of the odors (see Fig. 6D). The WT mice performance on novel backgrounds was 74.12% (228 trials) and not significant lower ($p = 0.25$, single tailed fisher exact test) than the previous group of animals trained with the full set of training mixtures (76.9%, 334 trials, 4 animals, asynchronous task). WT mice used the training data more efficiently than the linear classifier or the NNC and are able to generalize to novel combinations of known odors in the presence of a novel background odor.

Sparse deconvolution algorithms(Koulakov and Rinberg 2011; Grabska-Barwińska et al. 2017; Li and Hertz 2000; Otazu and Leibold 2011) use the training data more efficiently than the NNC and might be a plausible type of algorithm used by WT mice. Sparse deconvolution algorithms decompose the signal into contributions selected from a very large dictionary of known odors, while minimizing the number of odors used, permitting generalization to any combination of known odors. We could not directly test the performance of these algorithms for individual novel background odors because the performance in response to a novel background odor depends on the glomerular representation of all odors that an animal knows, which is hard to estimate. However, we performed simulations (see **Supplementary material 4**) to determine whether a standard sparse representation algorithm, the Lasso(Tibshirani 1996), could identify the target odors in the presence of novel background odors that are not part of a simulated dictionary. The Lasso solved the behavioral task at a performance of > 90% suggesting (see **Sup. Figure 6**) that deconvolution algorithms are a plausible hypothesis as the algorithms used by WT mice. The recurrent computations required to generalize, given the reduced size of the training set, might make these algorithms more sensitive to the diverse circuit defects observed in mouse models of autism.

***Cntnap2*^{-/-} mouse model of autism odor detection was selectively affected in novel background odors**

We tested the *Cntnap2*^{-/-} mouse model of autism (Poliak et al. 2003) (JAX Stock No: 017482) because *Cntnap2*^{-/-} mice have either equal (Levy et al. 2019) or better (Peñagarikano et al. 2011) performance than WT mice in simple olfactory tasks, suggesting a functional olfactory system. Nevertheless, *Cntnap2* is expressed in olfactory receptor neurons (Gordon et al. 2016) and its absence might reduce neural excitability, affecting glomerular representations. However, the glomerular activation patterns of WT mice and *Cntnap2*^{-/-} mice were very similar (see Fig. 7A). We compared odor responses across 190 odor pairs and found a significant correlation in similarity between the odor pairs calculated using WT and *Cntnap2*^{-/-} mice imaging data ($R = 0.46$, $p = 1.774 \times 10^{-11}$, Pearson linear correlation) (see Fig. 7B). We also compared the performance of the linear classifier trained using the 16 mixtures with known background odors, using WT and *Cntnap2*^{-/-} imaging data. The performance of the classifiers in mixtures that included novel backgrounds (176 mixtures) was almost identical for the WT and *Cntnap2*^{-/-} data (see Fig. 7C), indicating that *Cntnap2*^{-/-} and WT mice had similar responses at the sensory periphery.

We trained four *Cntnap2*^{-/-} mice using the restricted training set with asynchronous presentation of the odors. Animal training proceeded similarly to that for the WT mice, reaching similar performance levels on well-known background odor after ~ 9 days of training and indicating that learning was not affected in the context of our task. The *Cntnap2*^{-/-} mice performance on the standard background odors, just before the presentation of the novel odors started, was 85.5% (220 trials) and not significantly different ($p = 0.75$, Fisher exact test) from the performance of the WT mice (87.1%, 140 trials, 3 animals) (see Fig. 7D). However, for novel background odors, the *Cntnap2*^{-/-} mice performance dropped to 61.13% (283 trials), which was significantly lower ($p = 0.00242$, Fisher exact test) than the performance of WT mice (74.1%, 228 trials) (see Fig. 7E).

The *Cntnap2*^{-/-} mice poor performance might have been due to their hyperactivity (Peñagarikano et al. 2011), possibly causing uncontrolled licks in response to the novel background odors. However, *Cntnap2*^{-/-} mice did not make many early licks nor false detections; in fact, the main type of error in the presence of novel background odors were false rejections; that is, the mice failed to lick in response to the target go-odor (see Fig. 7F).

Lack of odor exploration in *Cntnap2*^{-/-} mice (Brunner et al. 2015; Gordon et al. 2016) might explain their poor performance for novel background odors; however, *Cntnap2*^{-/-} mice strongly increased their sniffing rate in response to novel background odors, similar to WT mice, suggesting that *Cntnap2*^{-/-} mice explored the novel background odor (see Fig. 7G). Although *Cntnap2*^{-/-} mice were reluctant to lick for target odors among novel background odors, the lick reaction time for the asynchronous case for the novel background odors was 664.8 ± 68 ms and not significantly slower ($n = 49$ lick responses, 4 animals, $p = 0.85$, Wilcoxon rank-sum test) than the response time for the known background odors ($632.5 \pm$

17.5 ms, 462 lick responses). *Cntnap2*^{-/-} mice did not delay their responses for novel background odors as WT mice did, suggesting a deficit in recruiting recurrent computations necessary to perform the task (see Fig. 7H). The circuitry required for robust sensory identification in novel environments might be selectively affected in this mouse model of autism, while performance of well-rehearsed mixtures was spared.

Discussion

We have developed an olfactory CAPTCHA for mice. We showed that WT mice could identify odors in novel environments, even on the first presentation. Further exposure to an initially novel background did not improve performance, and adaptation to the novel background odor was not necessary for correct target identification. By using intrinsic images of the dorsal glomeruli, we showed that WT mice performance was more consistent with the use of the non-linear NNC than a linear classifier. WT mice performance was unaffected by a reduction in the number of training mixtures, whereas the NNC performance was affected. *Cntnap2*^{-/-} mice glomerular activation were similar to WT mice and their performance for known backgrounds was indistinguishable from WT. However, *Cntnap2*^{-/-} mice performance for novel backgrounds was significantly lower than that of WT mice, indicating that the circuitry that performed robust odor identification was selectively affected in this mouse model of autism.

Animal performance in our task was very similar when novel background odors were synchronous with the target presentation, or when the novel background odor preceded the target onset, showing that adaptation was not necessary in novel background odors. Adaptation to the preceding background odor might play a greater role in situations with longer time gaps between background odor onset and target odor onset (Sehdev and Szyszka 2019). Faster sniffing correlated with improved performance and might constitute an additional mechanism for odor detection in novel backgrounds, which may act by suppressing the novel background odor (Verhagen et al. 2007). *Cntnap2*^{-/-} mice might rely more on sniffing induced suppression as a mechanism to identify odors in novel backgrounds.

The NNC was able to predict the behavior of WT mice in response to novel background odors using only ~ 200 glomeruli from the dorsal surface of the olfactory bulb. This was surprising to us, as these glomeruli represented just ~ 10% of the total number of bulb's glomeruli. Nevertheless, the dorsal glomeruli might be enough to capture the presumably low dimensional olfactory perceptual space (Markus Meister 2015). We also averaged glomerular responses over a 7 second window, an order of magnitude slower than the animal reaction time, which ignored the rich temporal dynamics exhibited by the glomeruli (Spors et al. 2006). WT mice might achieve faster reaction times by using glomeruli with higher affinity for the presented odors that might be located in the ventral surface of the olfactory bulb. Mice might also use the glomerular temporal dynamics for a fast readout of the olfactory receptor response (Wilson et al. 2017).

The linear classifiers' performance matched the average WT animals' performance, but did not predict performances on individual novel background odors, whereas the NNC did. Linear classifiers are simple

and fast: they do not require recurrent or feedback connections. Why did the animals not use them in novel environments? Linear classifier performance decreased significantly when we tested the WT mice with novel combinations of known odors and novel background odors. Natural scenes with novel odors might be very different to mixtures that animals have experienced before, and animals might apply complex deconvolution algorithms when they detect a novel background.

Linear classifier implementation does not require recurrent inhibition or corticobulbar feedback. The olfactory tubercle lacks both an association fiber system(Haberly and Price 1978) and feedback projections to the olfactory bulb(in 't Zandt et al. 2019). Tufted cells, which are not modulated by cortical feedback(Otazu et al. 2015), project strongly to the olfactory tubercle(Igarashi et al. 2012b; Fukunaga et al. 2012b; Igarashi et al. 2012a) and olfactory tubercle neurons have enhanced representations of reward-associated odorants(Millman and Murthy 2019; Gadziola et al. 2015), creating a possible circuit substrate for fast discrimination of known odor mixtures using linear classifiers.

WT mice responses to novel background odors were delayed by ~ 80 ms compared to responses to known background odors, suggesting the involvement of recurrent circuitry. This delay was not present in the *Cntnap2*^{-/-} mice. Deconvolution algorithms proposed for the olfactory system use cortical feedback and require computations that require recurrent connections.(Koulakov and Rinberg 2011; Grabska-Barwińska et al. 2017; Li and Hertz 2000; Otazu and Leibold 2011) The piriform cortex sends feedback axons to the olfactory bulb and has a rich association fiber system(Haberly and Price 1978) that could implement the necessary computations. Mitral cells preferentially target the piriform cortex(Igarashi et al. 2012b; Nagayama et al. 2010), creating a recursive circuit between mitral cells and the piriform cortex. We hypothesize that the recursive circuit in piriform cortex is recruited for novel olfactory backgrounds and might be selectively affected in mouse models of autism.

Cntnap2^{-/-} mice and WT mice had similar glomerular odor responses. Accordingly, the performance of *Cntnap2*^{-/-} mice in known background odors were undistinguishable from WT mice, consistent with previous report of *Cntnap2*^{-/-} mice even outperforming WT mice on buried food task(Peñagarikano et al. 2011). *Cntnap2*^{-/-} mice olfactory deficits appeared only with novel background odors. Olfactory bulb neuron responses are less reliable in *Cntnap2*^{-/-} mice(Geramita et al. 2020). Reduced reliability might be compensated by the enhanced exposure to known background odors(Jones et al. 2008), but it could become critical with a novel background odor. *Cntnap2*^{-/-} mice also have reduced expression of several type of inhibitory interneurons(Peñagarikano et al. 2011). Enhanced mitral cell responses to novel background odors(Kato et al. 2012) might require increased inhibitory activity, which would be a problem for the *Cntnap2*^{-/-} mice, affecting their implementation of selective inhibition. Reduced local spine density in cortex(Lazaro et al. 2019) might also affect the implementation of robust algorithms used to identify odor in novel background odors.

In environments where novel odors are present, *Cntnap2*^{-/-} mice might not be able to detect ethologically relevant odors. This olfactory blindness might explain the reluctance of freely moving *Cntnap2*^{-/-} mice to

enter into environments where a novel odor is present (Brunner et al. 2015; Gordon et al. 2016). Inability to detect odors of interest in complex natural mixtures might also contribute to *Cntnap2*^{-/-} mice lack of interest in social interactions (Peñagarikano et al. 2011). Urine smell is a variable mixture of different volatile compounds (Brennan and Zufall 2006), and only some of the compounds relay social information. *Cntnap2*^{-/-} mice failure to explore conspecific urine (Levy et al. 2019) might be caused by a failure to detect the socially relevant volatile compounds in urine. The olfactory CAPTCHA will permit to elucidate the circuit mechanism that affect the computations affected in ASD sensory deficits.

Methods

Odor delivery

We used an air-dilution machine (Gupta, Albeanu, and Bhalla 2015) that delivered mixtures of target odors embedded in higher concentration of background odors. We used a triple serial air-dilution to achieve reliable low odor concentrations. Each of the three groups of odorants (targets, contextual backgrounds, and novel background plus (s)-(-)-limonene) had a single air valve to direct the odorized air to the animal. The same valve opened for the go target odor as well as for the no-go target odors so it was not possible to solve the task by using the valve sound.

We used relatively low odor concentrations, so that the background odors were stronger than the target odors, without having high absolute concentration that might be aversive to the animals. Odor mixtures were composed of 3 odors; a target odor, and two background odors. On each trial, the target odor was selected from 4 possible odors and was presented at a relatively low concentration (0.025% of saturated vapor). Both the algorithmic and behavioral performance were based on the discrimination of the target odors. Two of the target odors were labelled go-odors and were associated with water reward in the behavioral part of the experiment and the other two target odors were the no-go odors that were not be rewarded with water. The second odor in the mixture was a contextual background odor, which was selected to be one out of four odors and will be presented at a higher concentration (0.1% of saturation vapor). This contextual background odor made the task harder and prepared the animals to identify the target odors in variable backgrounds. The third odor in the mixture was selected from 12 possible background odors and was also presented at the higher concentration (0.1% of vapor pressure). In the case of the training examples, which were used to train the algorithms and the animals, the third odor was always (s)-(-)-limonene. The combination of 4 targets, with the 4 contextual background odors and the 12 variable backgrounds resulted in 192 possible odor mixtures. Of these, the 16 mixtures that include limonene were the training set and were used for algorithm training and the animal's behavioral training.

The test set consisted of mixtures of a target and contextual background, but instead of (s)-(-)-limonene, they contained one out of the other 11 novel background odors, which will result in 176 possible test mixtures. The odors were selected for their non-toxicity as well as their capability to activate dorsal glomeruli (Ma et al. 2012; Soucy et al. 2009), and absence of reported innate responses (Kobayakawa et al. 2007).

Behavioral training: Adult female C57BL/6J mice (WT) and *Cntnap2*^{-/-} mice (2–4 month old, original weight 20–24 gr) were implanted with titanium head bars (Otazu et al. 2015) and water deprived for at least 7 days before training started. Animals were weighted every day. Every day after training, water was supplemented such as animal's weight reached 80 to 85% of their initial weight. The water-deprived mice were trained to detect odors in 2 stages before **Testing: Target training** and **Target in known background training** (see attached file for training schedule table).

Target training: On the first training stage, mice were exposed only to the target odors (no background odors). After animal's heads were fixed, animals were let to rest for at least 5 minutes before starting the training session. Immediately after head-fixation, animals inhalation patterns were symmetrical with long inhalation and long exhalations. As animals relaxed, their sniff pattern changed to deeper shorter inhalations and shallower exhalations, indicating that animals were ready for the start of the training. Animals were initially exposed to one of the go target odors at high concentration (isopropyl butyrate, 0.5% of saturated vapor) and odor deliver was coupled with a small amount of water (4 µl) to elicit licking, delivered after 1 s of odor exposure. Once the animals reliably licked for 10–20 trials, the direct delivery of water was stopped and animals were required to lick the water tube in response to the odor for water to be delivered. The target odor was presented for 3 seconds. Animals continued to perform using the go odor. Once they performed > 100 trials without early licks (reaction time > 300 ms, suggesting they were reacting to odor and not to valve clicking sounds), a no-go target odor (isobutyl propionate, 0.5% saturation vapor) trials was initially introduced with a probability of 10%, so as not to discourage the animal from continue performance of the task. The minimal reaction time constrain played a role only on the first days of training because animals naturally evolved a response time of ~ 700 ms from valve opening onset. Once the animal showed robust continuous responses even in the presence of the no-go stimuli, the probability of the no-go stimuli was increased to 50%. Most animals learned to discriminate the first odor pair (one go odor versus one no-go odor) on the first day of training. On day 2, a similar procedure was used for the second odor pair (go odor: propyl butyrate, no-go odor: ethyl propionate 0.5% saturation vapor pressure). On days 3 and 4 both odor pairs were introduced simultaneously and the concentration was reduced to reach the target concentration of 0.025%. As described in other go/no-go tasks (Kuchibhotla et al. n.d.), animals had a tendency to make false alarm errors by licking for the no-go stimuli. In order to discourage that behavior, the probability of no-go stimuli was set to 75% at the beginning of each training session. Once animals stopped responding to the no-go stimuli, the go-stimuli probability was returned to 50%. Care was taken to return the go-stimuli probability to 50% before the reaction time of the animals increased to over 1 second, as this indicated that the animal were losing interest and were about to stop the behavior altogether. All behavioral measurements reported here were done at a go-stimuli probability of 50%. Early licks or false alarms produced increased inter-trial intervals (see **Sup. Figure 2** for a diagram of the time out schedule).

Target in known background training

Mixtures that include background odors were introduced in this phase. The background odors (one of the four contextual background odors and (s)-(-)limonene) were introduced initially at a concentration of

0.025%, the same concentration as the target odors. Odor delivery started with the delivery of the contextual background odor, followed 0.75 later by (s)-(-) limonene. The target odor was presented 1.5 s after the onset of the contextual background odor. The background odor were continuously presented during the 3 second target odor presentation. Continuous white noise of ~ 70 db SPL was delivered through a speaker to mask the sound of the background odor valve opening to avoid having animals reacting to the odor by licking on the onset of the background odors. Animals easily generalized to the background odors. Over several days the concentration of the background odors was increased in 3 steps (see **supplementary table** for a detailed training schedule). Animals performed at the final concentrations (target 0.025%, background 0.1%) for at least 2 sessions (> 400 trials), before the introduction of the novel background odors. All animals, except the animals performing the reduced training set task, were trained using all 16 odor mixtures. For the animals doing the reduced training set, they were exposed only to the 8 odor mixtures specified in Fig. 6C for their entire training.

Testing: The 11 novel background odors were presented over 3 consecutive days. Each day animals started a pre-training session on the known background odors (training set) until their performance reached over 80% for more than 50 trials. Once reliable performance was obtained, animals were switched to the test trial session that included a small number of novel background odors mixture trials. Each novel background odor was presented at most 4 times per training session and each novel background odor presentation was separated from presentations of the same novel background odor by at least 25 minutes. On day 1 of testing, only five different novel background odors were presented. On day 2, the other six novel background were presented. On day 3, all 11 novel background odors were presented. The performance on the known background odors interleaved with the novel odors, should be above 80%, or the session was discarded. Only 1 session from 63 sessions across 21 animals had to be discarded because of low performance on the known background odors. **Synchronous trial training and testing:** For the five animals that performed the synchronous trials, training was identical. Animals did two extra sessions with the training set using the final concentrations of the target and background odor, but where the backgrounds preceded the onset of the target by only 50 ms. The 50 ms delay was introduced to reduce the possibility that the target odor might reach the animal's olfactory epithelium before the background odors arrived.

Odor pre-exposure

Before starting on water deprivation, a group of 5 animals were exposed to the group E. Briefly, we put a filter paper soaked in 0.1 ml of each group E odorant in an empty cage. Animals were individually placed on the cage for five minutes at a time. Each day, this procedure was repeated for all five odors of group E. The same procedure was repeated for at least 5 days preceding the onset of the behavioral training. Odor pre-exposure was not done in the 24 hour period preceding the beginning of the testing phase.

Surgical procedure

Female mice > 60 days old, 20–25 gr were anesthetized using ketamine/xylazine (KX, initial dose 70/7 mg/kg), further supplemented to keep the pedal withdrawal reflex diminished. Lidocaine was

applied topically at the site of surgical incision. Respiration and lack of pain reflexes were monitored throughout the experiment. After the animal was deeply anesthetized, it was mounted in a stereotaxic frame with ear bars. Lidocaine and iodine were applied topically to skin (as analgesic and antiseptic, respectively). Aseptic technique was used, first clipping hair and prepping with betadine on the skin. A small incision (2–3 cm) was made into the skin above the surgical site. A titanium head-bar was cemented on the skull near the lambda suture using light-cured Vitrobond (3M). For animals used for imaging, we also implanted 3 mm windows over the olfactory bulb, as described in (Otazu et al. 2015). Animals were allowed to recover for 1 week before starting water deprivation.

During the imaging sessions, the animal's head were held firmly in place by mounting the titanium head bar onto a custom-built holder. The animal were awake during the imaging session.

Imaging: Intrinsic optical imaging was done following (M Meister and Bonhoeffer 2001). The surface of the bulb was illuminated using an infrared ($\lambda = 780$ nm) fiber-coupled LED (M780F2, Thorlabs) connected to a 1000 μm fiber of 0.50 NA (M59L01 - $\varnothing 1000$ μm , 0.50 NA, Thorlabs). The fiber output was collimated using a fiber collimation package $f = 8.00$ mm, NA = 0.50 (F240SMA-780–780, Thorlabs). Images were taken using a pair of back to back SLR lenses with a 50 mm f/1.4 lens used as objective and second lens Tamron AF 90 mm f/2.8 Di SP AF/MF 1:1 Macro Lens coupled to an sCMOS camera (CS2100M, Thorlabs). This setup resulted in 3.3 μm per pixel. Images were acquired at a rate of 40 Hz. Illumination was setup to reach 80% of the pixel saturation value. White light from a flashlight was used to find the surface of the olfactory bulb. Imaging plane was set between 200–250 μm below the vasculature. Single odors and odor mixtures were presented for 9 seconds randomly interleaved.

Image acquisition

Image acquisition started 7 seconds before odor onset. An inter-trial interval of 40 seconds was used between odor presentations in order to avoid adaptation effects as well as to have time to remove residual odor contamination using a vacuum pump. Each stimulus (either single odors or mixtures) was repeated between 20 and 26 times.

Image analysis

Images in responses to the same stimulus were averaged. A normalized signal was calculated for the average image using as f_0 the average response of the 5 seconds period preceding odor onset. In order to remove the broad hemodynamic signal in response to odors, the images were convolved with a Gaussian of radius $\sigma = 40$ μm and this low-pass filtered signal was subtracted from the original signal. In order to remove high spatial frequency spatial noise, the resulting images were further convolved with a Gaussian of radius $\sigma = 12$ μm . In order to normalize this signal, a z-score was calculated for each pixel using the values of df/f_0 of the 2 seconds before the onset of the air period to calculate the mean and the standard deviation. Average z-score odor responses were calculated using the period between 2 and 9 seconds following odor onset. ROI were drawn manually using ImageJ (Schindelin et al. 2015) over activated

glomeruli across all odors presented. We quantify glomerular activations as the mean value of the z-score across all selected pixels in an ROI.

Measurement of saturation non-linearity

In order to measure the saturating nonlinearity of the intrinsic imaging signals, we presented 2 odors in separated trials as well as mixed together and compared the sum of the intrinsic signals (as z-score, see above) of the individual odors with the intrinsic signal produced by the mixture. We used ethyl butyrate (0.1%) and ethyl tiglate (0.1%) as well as ethyl valerate(0.1%) and ethyl tiglate(0.1%). We recorded from 143 glomeruli on two animals and found 68 glomeruli that responded to either the odors used or to the mixture (see **Sup. Figure 5**).

We calculated the deviation from the identity and found that there was a $39\% \pm 0.05$ ($n = 68$) deviation from the identity line for responses of individual glomeruli. As this measure includes both noise as well as saturation, we fitted the data using a saturating nonlinearity (shown in green) similar to (Mathis et al. 2016), that is $\sigma(R_o) = 2A/(1 + e^{-(R_o/s)}) - A$, where R_o is the sum of the z_score produced by the two odors measures separately and $\sigma(R_o)$ is the z_score measured for the mixture of both odors. A is the asymptote value and s is the saturation constant. The estimated saturating values were of the fitted model were $A = -8.0934$ $s = 0.2091$. We calculated the average difference between the fitted saturating nonlinearity and the sum of the glomeruli value as $-0.19 \pm 0.4\%$.

Creation of linear classifier and NNC using virtual odor mixtures from dorsal glomeruli: Virtual odor mixtures from the training and test set were created by adding the glomerular activation patterns (as a z-score produced by individual glomeruli) of individual odors. The 16 mixtures that compose the training set were generated by adding the contribution of three odors: a target odor (0.025% saturated vapor), one contextual background odor (one out of 4 odors, concentration 0.1% saturated vapor) and (s)-(-)-limonene (0.1% saturated vapor). The sum of activations was processed through the saturating nonlinearity as shown in **Sup. Figure 5**. Gaussian noise of several values were added to the generated mixtures. The classifiers were trained in detecting the presence of the target go odors in a mixture of background odors. Classification linear SVM model were fitted to predict the go and no-go trials using the Matlab function *fitclinear*. The function uses as input the glomerular activation patterns of the 16 combinations that included limonene. The fitted variable were a vector that had the value of +1 for the 8 mixtures that included a go odor, and a value of -1 for the 8 mixtures that included a no-go odor. The testing was done using the 176 mixtures that included the novel background odors. The output of the linear classifier (either a go or no go) was based on the sign of the output of the filter, with positive values considered a go stimulus.

For the nearest neighbor classifier, we used the 16 combinations with (s)-(-)-limonene as templates. Given a new mixture that included a novel background, a dot product was calculated between the templates and the tested mixture. The nearest neighbor was determined as the template with the largest dot product. The output of the algorithm (either a go or no go) was based on the target odor content of the nearest neighbor.

Creation of classifiers using real odor mixtures from dorsal glomeruli

For each novel background odor, we acquired images of target, contextual background, and (s)-(-) limonene (16 training mixtures) and the same mixtures where (s)-(-) limonene was replaced by one novel odor (16 testing mixtures per novel background odor). Each mixture was repeated between 20 and 25 times. Because of the long acquisition time required, we had to split some recording sessions in two for individual novel background odors. In those cases, on each day we presented either one go odor and one no-go odor with all 4 contextual background odors (8 training mixtures and 8 testing mixtures), or we presented the two go-odors and the two no-go odors with two of the contextual background odors (8 training mixtures and 8 testing mixtures). We trained the linear classifier and used as templates for the nearest neighbor classifier the mixtures that contained (s)-(-)limonene. For the split session, we report the performance of the classifiers calculated in the two individual recording sessions. We recorded data from 10 novel odors, one of the novel background odors, 2–3 pentanedione, stick to the odor tube and the large number of trials requires (over 150) clogged the odor valve.

Reduced training set

In order to test how reducing the training set affected performance of the linear classifier and the NNC, we used the intrinsic optical imaging data from 2 animals to create mixtures as shown in **Creation of linear classifier and NNC using virtual odor mixtures from dorsal glomeruli**. For each imaged animal, the level of Gaussian noise added to the linear classifier was set for a level that better approximated the performance of the animals in novel backgrounds (77%) when trained with the full data set of 16 mixtures.

The training of the algorithm was done using the restricted training set (8 mixtures that included limonene) and the testing was done using the testing set (8 mixtures combined with 11 novel odors = 88 test mixtures). For the behavioral measurements, three new animals were trained. The training phase **Target** training was the same as described above. However, from the **Target in known background testing phase**, only the restricted training set was used. During the testing phase, the animals were tested with the above described 88 mixtures that included the novel odors.

Comparison of intrinsic olfactory imaging between *Cntnap2*^{-/-} mice and WT mice

Imaging was performed as described on three *Cntnap2*^{-/-} mice (2 females, 1 male, ages between 3 and 4 months of age). Mice were implanted with 3 mm windows as described above and we imaged the responses to the 20 odors used in Fig. 1 at the concentrations used in behavioral testing. Glomerular odor evoked responses were quantified as z-scores as described above. In order to compare the odor evoked responses of the WT mice (2 animals) we constructed a vector of all the glomerular responses across all animals for each individual odor (352 glomeruli for the *Cntnap2*^{-/-}, 353 for the WT mice). We calculated the similarity between the odors as the dot product between the representations for all 190 comparisons between the 20 odors used. We repeated the analysis of **Creation of classifiers using virtual odor mixtures from dorsal glomeruli** with the data from the *Cntnap2*^{-/-} animals. We used 10 different repetitions for each level of noise added to the virtual mixtures. We report the 80% confidence intervals for the mean.

Sniff detection

Sniff was detected using an airflow sensor (1000 SCCM AWM300V, Honeywell) circuit connected opposite to the animal nose as shown in (Bolding and Franks 2018). A soft viton o-ring (ID = 3/16 inches, McMaster Carr part number 1284N108) was glued to the odor delivery port to create a tight seal without discomforting the animal. The airflow signal was acquired after passing it through an antialiasing filter to remove high frequency turbulence associated noise. We used a low-pass RC filter with $R = 9.4 \text{ k}\Omega$ and $C = 0.3 \mu\text{F}$, given a cutoff frequency of 56.5 Hz. The signal was acquired at 1000 Hz using a NIDAQ USB board.

In order to determine the change in sniff rate produced by the novel odors, we calculate the number of inhalations in a 1 s window between 300 to 1300 ms following the onset of the novel odor or the (s)-(-) limonene odor. In order to have a comparison that takes into account the non-stationarity of the sniff rate, we compared the presentation of mixtures with novel background odor to the preceding presentation with known background odors. To determine the reaction time in response to a target odor, we assumed that the odor reached the olfactory epithelium at the onset of the first inhalation following the target odor valve opening. We measured the lick latency from this time point.

Supplementary Fig. 1. Correlation matrix of the glomerular representation of the odors used. The two target go-odors had very similar patterns of glomerular activation (correlation = 0.65) as well as the no-go target odors (correlation = 0.66). The cross-correlation between the go and no-go stimuli was lower (0.47 ± 0.06). The similarity between the target go odors and the target no-go odors and the difference between these groups facilitated learning.

Supplementary Fig. 2. Temporal profile of olfactory stimulus delivery during behavior for asynchronous task. Odor valves opened for 7 seconds. After 7 seconds, a valve near to the animal opened and routed the contextual background odor flow to the animal snout. After 0.75 s, the novel background odor was delivered, followed after 0.75 s by the target. Animal had to lick with a latency of $> 0.3 \text{ s}$ from target onset to get rewarded. The target appeared concurrently with the background odors for 3 s. If the target was a go-odor and the animal licked during the response window, the animal received water and 5 seconds were added to the intertrial interval. (correct detection). If the target odor was a no-go odor and the animal responded, it was a false alarm and 10 s were added to the intertrial interval. If the animal responded before the target onset or before the 0.3 s minimal latency response, extra 10 s were added to the intertrial interval. If the animal did not lick, correctly or a false detection, 5 s were added to the intertrial interval. There was an extra random interval between 12 and 16 s that was always present in the intertrial interval, resulting in interstimuli interval of $\sim 30 \text{ s}$.

Supplementary Fig. 3. Fast inhalations in response to novel background odors were associated with improved performance **A.** Example traces showing that in some trials animals did not change their inhalation width in response to a novel background odor (example in upper trace), whereas in other trials (example in lower trace) the second inhalation is briefer after the onset of the novel background. **B.** The first inhalation in response to novel background and (s)-(-)limonene was similar between correct and

incorrect trials. **C.** The second inhalation width reflects the animal reaction to novel background odors and it was significantly briefer in correct trials. There was not effect of briefer inhalation for known backgrounds.

Supplementary Fig. 4. Number of sniffs before a lick response in response to the target odor were elevated in the first presentation of a novel background odor. **A.** In the asynchronous case, animals increased the number of sniffs on the first presentation and on subsequent presentations values dropped to values closer to 3 sniffs, the number of sniffs used in known backgrounds. **B.** For the synchronous case, the number of sniffs before a response was elevated on the second presentation of the novel background.

Supplementary Fig. 5. Deviations of linear addition on imaging of odor mixtures **A.** Glomerular activation patterns of in odors presented separately and combined together as a mixture approximated as a linear combination **B.** The main observed effect was sublinear and we approximated as a saturating nonlinearity on individual glomerular activation.

Supplementary Fig. 6. Lasso can identify target odor in novel backgrounds. **A.** Example of estimated concentrations by the Lasso in response to a mixture that included a novel background odor. The Lasso used a dictionary of $N = 1500$ elements. The Lasso estimated a large concentration for the target and the contextual background odor. The novel background odor was not part of the dictionary and was represented by a series of small values distributed over multiple elements. **B.** Lasso determined a larger estimate for the correct target odor (mean and 90% confidence interval). **C.** Number of estimate for other dictionary elements that had estimated values larger than the target increased with larger regularization constant.

Declarations

Animal Ethics Statement

All procedures were approved by the Institutional Animal Care and Use Committee, NYIT College of Osteopathic Medicine (#: 2017-GOA-01A3).

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Supplementary Materials

Supplementary material 1: Familiarity with background odor did not increase target recognition in novel backgrounds

We wondered if animals could boost their performance on the novel background odors by having previous substantial exposure to those background odors outside the context of the task. During the task, animals got a total exposure to a novel background odor of at most 8 trials $\times$ 3.75 s=30 s. In order to increase the exposure time to a novel background odor, we divided our set of novel background odors into 2 odor groups, an exposed odor group (Group E) and a non-exposed odor (Group NE). Odor Group E consisted of 5 background odors (butyl propionate, ethyl tiglate, γ -terpinene, heptanal, and acetal) and the performance of our initial group of animals was 78.8% (4 animals, 132 trials). Odor Group NE consisted of six odors (2-3 pentanedione, ethyl benzoylacetate, hexanal, methyl pyruvate, 1-4 cineole, 4-methylanisole) that had a performance of 86.9% (4 animals, 176 trials). Before the start of behavioral training, a new group of 5 animals was pre-exposed to the odors in group E (5minutes $\times$ 5days=25 minutes total) and then they were trained on the behavioral task as described before. There was only a 0.8% increase in the performance of the odors (group E, 79.6%, 211 trials). The main effect was a reduction of the performance of the non-exposed group NE (75.2%, 250 trials).

Supplementary material 2: Faster sniff rates increased animal performance in novel environments

Animals increased their sniff rate and reduced the inhalation width on the second sniff after the onset of the novel background odor, as they acquired the information that a novel odor was present from the first inhalation and reacted to it on the second inhalation. We compared the second inhalation after the onset of the novel background odor between correct and incorrect trials to determine if fast sniffing was

correlated with better performance. This second sniff width was significantly shorter ($p=0.014$, Wilcoxon rank sum test, single tailed) in the correct trials (91.2 ± 2.0 ms, 552 trials, 9 animals) compared to the incorrect trials (102.0 ± 4.5 ms, 131 trials). Interestingly, fast sniffing was not correlated with better performance for known backgrounds; the second inhalation width after (s)-(-)limonene onset was not significantly shorter for known backgrounds ($p=0.9725$, Wilcoxon rank sum test, single tailed), with a width of 101.6 ± 1.03 (n=2816 trials) for the correct trials and 94.6 ± 2.7 ms (331 trials) for the incorrect trials. We wondered if the correlation between the second sniff width and performance might be caused by slow fluctuations in animal alertness. If so, the trend between sniff rate and performance should be noticeable from the first sniff after novel odor background onset. However, there was no difference in inhalation width ($p=0.4985$, single tailed Wilcoxon sum test) between correct trials (110.1 ± 2.5 ms, 552 trials) and incorrect trials (109.6 ± 5.3 ms, 131 trials). There was also no difference ($p=0.8510$, Wilcoxon test) for the known backgrounds, with 105.3 ± 1.08 ms (2816 trials) for the correct trials and 100.67 ± 2.8 ms (331 trials) for the incorrect trials. Faster sniffing increased odor identification performance only for novel background odors.

Supplementary material 3: Sniff responses for synchronous odor presentation

The base rate of sniffing before odor onset was slightly higher for animals that were doing the synchronous task (2.8 ± 0.04 sniffs per second, n=1191 trials,) compared to animals doing the asynchronous task (2.28 ± 0.03 , n=1563 trials). This suggests an enhanced level of baseline alertness for the synchronous task, consistent with the faster onset of target from the start of olfactory input. Once odors started, the sniff rate increased to 3.3 ± 0.05 sniffs per second, which is less pronounced than the increase in sniff rate seen for the asynchronous case. The novel background odors created an increase in the sniff rate in the first day for the first two presentations of novel odors (presentation 1: 1.17 ± 0.39 extra sniffs per seconds, $p=0.03$, binary test; presentation 2: 0.87 ± 0.29 extra sniffs per seconds, $p=0.0191$, binary test). On subsequent presentations the sniff rate did not increase compared to the presentation of limonene ($p>0.1$, binary test), indicating that animals had familiarized themselves with the novel background odors.

Supplementary material 4: Lasso deconvolution

The Lasso (least absolute shrinkage and selection operator) finds the combination of elements of the dictionary that could reconstruct the observed pattern of glomerular activation in the least square error sense. The Lasso finds a solution that minimizes the sum of the absolute value (or L1 norm) contribution of the dictionary elements multiplied by a regularization constant λ . In order to test if the Lasso could be used for identifying target odors in the novel backgrounds we created a large dictionary of glomerular activation patterns based on our glomerular imaging. This dictionary included the 9 individual odors used in animal training (4 targets+4 contextual background+ (s)-(-) limonene). We used data from 2 animals each with 217 and 136 glomeruli to create a dictionary of 353 glomeruli. In order to extend the dictionary to a larger number of odors we generated artificial patterns of glomerular activation by creating vectors of the same length as the measured glomeruli using independent Gaussian samples. Although we imaged

the odor responses at the concentrations used during the behavior, each dictionary element was normalized to unit variance. We generated 1000 and 1500 extra dictionary elements. The artificial dictionary did not include the 11 novel background odors that were part of our novel mixtures. In order to test the Lasso, we generated the virtual odor mixtures that included the novel background odors as described above. We used extra noise of $\rho=1$ and $\rho=1.5$ for the mixtures. The Lasso algorithm was implemented using the lasso function from Matlab 2017B (Mathworks). We tested the Lasso using 50 values of the regularization constant λ equally spaced in the logarithmic space between 0.01 and 1. Lasso estimated high concentrations for the target being present and the contextual background odor. The novel background odor was represented as small concentrations distributed over multiple other dictionary elements.

We evaluated the performance of the Lasso in two ways. The first one was to determine if the Lasso assigned a larger contribution to the correct target (go or no-go) than to the incorrect target. If the Lasso estimate did so, this was considered a correct discrimination. Lasso performance was >80% for all values of λ with performance improving with increasing values of λ . This criterion assumes that animals would be able to filter out the responses of other dictionary elements. The second criteria measured the number of estimated values that exceeded in value the contribution associated to the correct target element. Animals could perceive these other elements as being present and might affect the behavioral response because these other elements might have their own associated behavioral responses that might affect the task. The number of these other peaks was relatively small suggesting that deconvolution algorithms could be used by the mice to identify odors in novel environments.

Figures

Figure 1

Background odors had stronger glomerular activation pattern than target odors. A. Intrinsic optical imaging was used to measure glomerular activation in response to odors used during the behavior. B. The target odors had few activated glomeruli (dark spots) compared to the contextual background odors. The novel background odors had intermediate levels of glomerular activation. C. Average glomerular activation. D. Mixtures were dominated by the strong background odors, resulting in go and no-go mixtures that were very similar to each other. E. Stimuli used during training consisted of mixtures of 3 odors: a contextual background odor, (s)-(-) limonene, and a target background odor. The contextual background odor was delivered first, followed after 0.75 s by (s)-(-) limonene, and 1.5 s after by the target odor, which was presented for 3 seconds. Test stimuli were identical except that (s)-(-) limonene was replaced by one out of 11 novel background odors. Test set trials were separated by 4-6 training set trials. F. Head-fixed animals could lick and get rewarded with water after 0.3 s from target onset.

Figure 2

WT mice successfully solved olfactory CAPTCHA (asynchronous case). A. WT mice were able to identify the target odor in the presence of novel background odors. B. Performance was well above chance for the first presentation of the novel background odors and it did not increase with more exposure to a novel background odor. C.D Responses for novel background odors have increased number of failure to lick for the go target ("False rejection") compared to trials with known background odors. There was a small increase in early licks in response to novel background odors (7.8 %) compared to 3.9 % for known backgrounds).

Figure 3

Animals increased their sniff rate when initially presented with a novel background odor. A. Example respiratory signal. Animals slightly increased their sniff rate during the odor presentation in the training set. Introduction of a novel background odor introduced a further increase in sniffing. B. Mean $\pm$ s.e.m airflow signal for four WT mice for the first presentation of novel background odors compared to the presentation of the interleaved trials of the training set. Animals had a base rate of 2.28 ± 0.03 sniffs per sec ($n=1563$ trials) before the onset of the odors and the sniff rate ramped up as the target approached. After the onset of the contextual background odors, the sniff rate increased to 3.13 ± 0.07 s. The presence of the limonene odor caused a further increase to 3.98 ± 0.06 . After the target onset, the sniff rate further increased to 4.51 ± 0.06 . C. Increase in sniff rate for novel odor compared to preceding trial with (s)-(-) limonene is present only on the first trial of each day.

Figure 4

WT mice successfully solved olfactory CAPTCHA (synchronous case). A. Stimuli were the same as the asynchronous case but the target appear only 50 ms after the onset of the background odors. B. WT mice were able to identify the target odor in the presence of synchronously presented novel background odors at the same level as the asynchronous case. C. Animals had increased levels of false rejections indicating difficulty in detecting the targets in novel background odors. D. Performance in the synchronous case was well above chance for the first presentation of the novel background odors and it did not increase with following presentation. E. Animals also increased their sniff rate on the first presentation of the novel background odors. F. Sniff rate decreased after the second presentation of the novel background odor.

Figure 5

NNC using imaging data predicted WT mice performance in a novel background odor. A. The linear classifier used the training mixtures to create a hyperplane that separates the go mixtures from the no-go

mixtures and classified a test mixture that contained a novel background odor. B. The x coordinate of each circle represents the performance of each group of animals (synchronous and asynchronous task) on a novel background odor and the y coordinate represents the linear classifier performance obtained from two imaging sets using virtual odor mixtures, resulting in 44 points. The p value indicates the standard deviation of the Gaussian noise added to the mixtures. Linear classifier had better performance (>80%) than animal behavior, but it had no significant correlation with the animals performance for individual background odors. C. NNC determined the training mixture that most resembled a test mixture with novel background odor and classified it according to the target content of the best match. D. NNC performance was lower than the linear classifier but NNC was a better predictor of an animal's behavior for an individual novel background odor. E.F. Performance and correlation with behavior for classifiers with different levels of added Gaussian noise. Results of 200 simulations at each noise level. Error bars indicate the 90% confidence interval. E. Linear classifiers had higher performance than NNC. F. NNC was a better predictor of the animal behavior. G.H. NNC was a better predictor of animal performance than the linear classifier when trained with imaging data from actual odor mixtures.

Figure 6

A. WT mice lick response times were longer in the presence of novel background odors in both the asynchronous and the synchronous task. B. NNC and linear classifier performance on novel background odors might be affected by a reduction in the number of training mixtures. C. The reduced training set consisted of 8 mixtures. Each contextual background odor was presented with a target go odor and a target no-go odor to avoid creating biases for the contextual backgrounds. The novel backgrounds in the test set were presented with combinations of contextual background and target that were not part of the

training set. D. Linear classifier and NNC performance on novel backgrounds was reduced, whereas the performance of a new cohort of 3 mice was not different from the performance of 4 animals that trained with the full training set.

Figure 7

Cntnap2^{-/-} mice target discrimination in novel backgrounds was selectively affected. A. Example intrinsic imaging showing very similar pattern of glomerular activation in Cntnap2^{-/-} animals and WT mice. B. Glomerular similarity of odor pairs was highly correlated between Cntnap2^{-/-} mice and WT mice. C. Linear classifier performance was similar between WT mice and Cntnap2^{-/-} mice. D. Cntnap2^{-/-} mice performance on known backgrounds was undistinguishable from WT mice. Circles indicate performance of individual animals. E. Performance in novel background odors was lower than WT mice. F. Cntnap2^{-/-} failed to lick to the target in novel backgrounds. G. Cntnap2^{-/-} mice did increase their sniff rate when presented with a novel background odor for the first time. H. Cntnap2^{-/-} mice did not delay their lick response in novel backgrounds.

Supplementary Files

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