

1 **Title**

2 **High-throughput automatic training system for odor-based cognitive behaviors**  
3 **in head-fixed mice**

4

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14

## 1 **Abstract**

2       Understanding neuronal mechanisms of cognitive behaviors requires efficient  
3 behavioral assays. We designed a high-throughput automatic training system (HATS)  
4 for olfactory behaviors in head-fixed mice. The hardware and software were  
5 constructed to enable automatic training with minimal human intervention. The  
6 integrated system was composed of customized 3D-printing supporting components,  
7 an odor-delivery unit with fast response, Arduino based hardware-controlling and  
8 data-acquisition unit. Furthermore, the customized software was designed to enable  
9 automatic training in all training phases, including lick-teaching, shaping, and  
10 learning. Using HATS, we trained mice to perform delayed non-match to sample  
11 (DNMS), delayed paired association (DPA), Go/No-go (GNG), and GNG reversal  
12 tasks. These tasks probed cognitive functions including sensory discrimination,  
13 working memory, decision making, and cognitive flexibility. Mice reached stable  
14 levels of performance within several days in the tasks. HATS enabled an experimenter  
15 to train eight mice simultaneously, therefore greatly enhanced the experimental  
16 efficiency. Combined with causal perturbation and activity recording techniques,  
17 HATS can greatly facilitate our understanding of the neural-circuitry mechanisms  
18 underlying cognitive behaviors.

19

## 20 **Introduction**

21       Behavioral design and analysis are critical for understanding neural mechanism  
22 of cognition (Gomez-Marin et al., 2014), including working memory (Fuster, 1997;  
23 Baddeley, 2012), decision making (Gold and Shadlen, 2007; Lee et al., 2012), and  
24 reversal of learnt rules (Bunge and Wallis, 2008). Combined with novel  
25 neural-circuitry technologies, such as optogenetics (Fenno et al., 2011),  
26 chemogenetics (Armbruster et al., 2007), and imaging methods (Deisseroth and  
27 Schnitzer, 2013), well-designed behavioral paradigms can greatly facilitate the circuitry  
28 level understanding of behavior. Reliable behavioral paradigms are also useful in  
29 pre-clinic studies such as target identification and mechanistic studies for brain  
30 diseases (Fernando and Robbins, 2011, Gotz and Ittner, 2008, Nestler and Hyman,  
31 2010).

32

33       Optimally, behavioral training systems should be automatic, ready to scale up,  
34 blind in design, and flexible in changing paradigms. Automatic training systems  
35 (Schaefer and Claridge-Chang, 2012) met well with these criteria. There was a long  
36 history of designing automatic behavior-training systems, for example in studies of  
37 operant conditioning (*e.g.*, Davidson et al., 1971). Automatic training systems are  
38 composed of monitoring and feedback controlling of behavior. In free-moving mice,  
39 automatic measurement has been implemented in characterizing visual performance  
40 (Benkner et al., 2013, de Visser et al., 2005, Kretschmer et al., 2013), evaluation of  
41 pain sensitivity(Kazdoba et al., 2007, Roughan et al., 2009), freezing behavior during  
42 fear conditioning(Anagnostaras et al., 2010, Kopec et al., 2007), home-cage

1 phenotyping(Balci et al., 2013, Hubener et al., 2012, Jhuang et al., 2010),  
2 anxiety(Aarts et al., 2015), diurnal rhythms(Adamah-Biassi et al., 2013), and social  
3 behavior(Hong et al., 2015, Ohayon et al., 2013, Weissbrod et al., 2013). With  
4 feedback controlling components, automatic training systems have been successfully  
5 implemented in multiple behavioral domains, including memory assessment (Reiss et  
6 al., 2014), operant learning (Remmelink et al., 2015), and training limb function  
7 (Becker et al., 2016). Automatic training systems with multiple cognitive behaviors  
8 requiring memory, attention, and decision making have been developed previously in  
9 free-moving rats (Erlich et al., 2011, Poddar et al., 2013) and mice (Gallistel et al.,  
10 2014, Romberg et al., 2013). Moreover, such systems were successful in dissecting  
11 neural-circuitry mechanisms underlying cognitive behaviors (*e.g.*, Brunton et al., 2013,  
12 Erlich et al., 2011, Hanks et al., 2015). Head-fixed mice (Dombeck et al., 2007; Guo  
13 et al., 2014) renders great flexibility in recording (Harvey et al., 2009; Boyd et al.,  
14 2012; Fukunaga et al., 2012; Kollo et al., 2014) and imaging (Boyd et al., 2015; Chu  
15 et al., 2016, Dombeck et al., 2007, Komiyama et al., 2010; Yamada et al., 2017)  
16 technologies. Moreover, free-moving and head-restricted mice exhibit similar ability  
17 of olfactory discrimination (Abraham et al., 2012). However, automatic training  
18 systems in head-fixed mice were not developed previously.

19

20 Olfaction is an important sensory modality for cognitive behavior (Doty, 1986;  
21 Ache and Young, 2005). Previous studies have demonstrated that rodents are very  
22 good at olfactory discrimination, memory, and decision (Abraham et al., 2004, Barnes  
23 et al., 2008, Cleland et al., 2002, Haddad et al., 2013, Hubener and Laska, 2001,  
24 Kepecs et al., 2007, Komiyama et al., 2010; Liu et al., 2014, Lu et al., 1993, Mihalick  
25 et al., 2000, Passe and Walker, 1985, Petrusis and Eichenbaum, 2003, Rinberg et al.,  
26 2006, Slotnick et al., 1991, Uchida and Mainen, 2003). Automatic behavioral systems  
27 have been developed for studying innate olfactory behaviors (Qiu et al., 2014).  
28 Olfactory behavioral testing has been developed in head-fixed rodents and greatly  
29 facilitates the understanding of neural circuits underlying olfaction (Verhagen et al.,  
30 2007; Wesson et al., 2008; Shusterman et al., 2011; Kato et al., 2013; Boyd et al.,  
31 2015) and odor-based cognition (Komiyama et al., 2010; Liu et al., 2014; Gadziola et  
32 al., 2015). However, fully automatic training systems for odor-based cognitive  
33 behaviors were not available for head-fixed mice.

34

35 We therefore designed a high-throughput automatic training system (HATS) for  
36 olfactory behaviors in head-fixed mice. Using the automatic step-by-step training  
37 procedures, we trained mice to perform olfactory delayed non-match to sample  
38 (DNMS), delayed paired association (DPA), Go/No-go (GNG), and GNG reversal  
39 tasks. Mice reached stable levels of performance within several days in the tasks.  
40 HATS can be an important tool in our understanding of the neural-circuitry  
41 mechanisms underlying odor-based cognitive behaviors.

42

## 1 **Material and Methods**

### 2 **Animals**

3 Male adult C57BL/6 mice (SLAC, as wild-type) were used for the current study  
4 (8-40 weeks of age, weighted between 20 to 30 g). Wild-type mice were provided by  
5 the Shanghai Laboratory Animal Center (SLAC), CAS, Shanghai, China. Mice were  
6 group-housed (4-6/cage) under a 12-h light-dark cycle (light on from 5 a.m. to 5 p.m.).  
7 Before behavioral training, mice were housed in stable conditions with food and water  
8 ad libitum. After the start of behavioral training, the water supply was restricted. Mice  
9 could drink water only during and immediately after training. Care was taken to keep  
10 mice body weight above 80% of a normal level. The behavioral results reported here  
11 were collected from a total of 25 wild-type mice. All animal studies and experimental  
12 procedures were approved by the Animal Care and Use Committee of the Institute of  
13 Neuroscience, Chinese Academy of Sciences, Shanghai, China.

14

### 15 **Animal surgery**

16 Mice were anesthetized with analgesics (Sodium pentobarbital, 10mg/mL, 80  
17 mg/kg body weight) before surgery. All surgery tools, materials, and  
18 experimenter-coats were sterilized by autoclaving. Surgery area and materials that  
19 cannot undergo autoclaving were sterilized by ultraviolet radiation for more than 20  
20 minutes. Aseptic procedures were applied during surgery. Anesthetized mice were  
21 kept on a heat mat to maintain normal body temperature. Scalp, periosteum, and other  
22 associated soft tissue over skull were removed. Skull was cleaned by filtered artificial  
23 cerebrospinal fluid (ACSF) with cotton applicators. After skull was dried out, a layer  
24 of tissue adhesive was applied on the surface of the skull. A steel plate was placed on  
25 the skull and then fixed by dental Cement.

26

### 27 **Behavior setups**

28 HATS was composed of a mouse containing, head-fix, odor delivery and reward  
29 delivery, Arduino based control, and data acquisition units (diagram in **Figure 1A**,  
30 photo in **Figure 1B**). All valves and motors were controlled by Arduino based  
31 processors and customized software. The 3d printing files, a step by step instruction  
32 for hardware assembling, the source code for behavior training and the data  
33 acquisition source code were publically available  
34 (<https://github.com/wwwweagle/serialj> ; <https://github.com/jerryhanson/frontiers/>)

35

36 Three-dimensional printing technique was used to generate the small  
37 components in the system (**Figure 1C**). The training tube was used to maintain the  
38 relative position of mouse body to the water- and odor-delivery ports. The motor slot  
39 held a direct-current motor to move the water port forward or backward. The water

1 tube slot held a metal needle with a blunt tip, from which mice obtained water as a  
2 reward. The odor-tube slot connected the odor tube from the odor-delivery unit.

3

4 A movable water port was connected to a peristaltic pump, which was controlled  
5 by an Arduino board. The volume of water reward was controlled by changing the  
6 duration of the output signal to peristaltic pump from the Arduino board. Peristaltic  
7 pumps of different setups were calibrated for the stable volume of water delivery in  
8 each trial ( $5 \pm 0.5 \mu\text{L}$ ).

9

10 Water- and odor-delivery units were both controlled by an Arduino board. During  
11 behavior training, detailed timing information of events was sent back to the computer  
12 via the USB-simulated serial-port interface and stored by a customized Java program.  
13 The stored events included an odorant valve on/off, peristaltic pump on/off, and  
14 licking start/end. Licking event was detected by a capacity detector. Infrared  
15 LED-based licking detectors were used for electrophysiological recording if required.  
16 An infrared camera was placed under the water port to monitor behavioral states of  
17 mice.

18

## 19 **Olfactometer**

20 The olfactometer was designed to efficiently and reliably mix and deliver odor.  
21 Air source was a pump that provided air flow with the flow rate of  $\sim 120$  L/min. The  
22 filter was applied to eliminate moisture and dust. Eight training setups shared one set  
23 of pump and filter. For each setup, pure air with the flow rate of 2 L/min is constantly  
24 delivered to mice during the entire process. The air input to each air route could be  
25 turned on and off by a manual valve (labeled as “M” in **Figure 2A** and **2B**). The flow  
26 rate was adjusted by a needle valve (labeled as “V” in **Figure 2A** and **2B**). As shown  
27 in the **Figure 2B**, one type of odorant in liquid state was stored in one airtight bottle.  
28 The air-in tube was placed right above the surface of the liquid odorant. Two-way  
29 solenoid valves were used to switch the odor to either mouse or flow meter. In the  
30 standby state (no odor was delivered, **Figure 2A**), the valve to odorant bottle (labeled  
31 as “O”) was closed, and that to the flow meter (labeled as “F”) was opened. Therefore,  
32 no odor will be mixed with pure air and delivered to the mouse. In the working state  
33 that odor was delivered (**Figure 2B**), “O” was open and “F” was closed. Therefore  
34 odor was mixed with constant air and delivered to the mouse. Four kinds of odorants  
35 were used in the behavior tasks, 1-Butanol, Methyl butyrate, Hexanoic acid, and  
36 Octane. The relative volume ratios of these odorants in the pure air were 10%, 2.5%,  
37 15% and 5%, respectively. The difference was due to the distinct evaporation pressure  
38 of different odorant molecules at room temperature (see **Table 1** for detailed  
39 rising/decay and residual time of the odorants). The odor tubes after “O” valves and  
40 before mixture chamber had an inner diameter of 0.5 mm. The odor tube for constant  
41 air before mixture chamber had an inner diameter of 2.5 mm.

42

## 1 **Behavior training**

### 2 **Water restriction**

3 Mice were allowed at least seven days for recovery after surgery for head-plate  
4 implantation. Before the start of formal training, mice were water restricted for 48~72  
5 hours, in which licking for water was allowed (less than 1.0 ML per day, exact  
6 amount was not monitored). Throughout the training, the daily intake of water was at  
7 least 0.6 ML per day (as in Guo et al., 2014) and typically 1.0 ML per day. Body  
8 weight was closely monitored and a steady increase in body was observed after initial  
9 decrease following 24 hour restriction.

10

### 11 **Habituation phases**

12 The habituation phase started thirty minutes before the start of the training phase  
13 and only occurred once. A training tube was placed into the home cage. Mice could  
14 explore the tube freely to be familiar with it. This step was designed to decrease the  
15 stress level of mice on the first day.

### 16 **Automatic licking teaching phase**

17 This phase was designed to teach mice to lick freely from the water tube. A  
18 mouse was fixated on the head plate to a holding bar connected to the training tube.  
19 The animals were transferred from homecages to the apparatus and headfixed  
20 manually by experimenters. The total time spent in transition was less than a minute.  
21 Then the training tube was placed into and fixated to sliding sockets in the  
22 sound-attenuated box (the typical decrease from background noise was 15dB).  
23 Initially, the tip of the water port was placed five millimeters away from the mouse  
24 mouth. By using a program-controlled movable water port, the initiation of a teaching  
25 bout was associated with the forward movement of the water port. During each day,  
26 this phase was divided into three bouts to facilitate the association between movement  
27 of the water port and delivery of water. In each bout, water port moved forward firstly  
28 to seduce mouse to lick. After two seconds, water port will be reset back to the  
29 original place. Once mouse licked, one water drop (volume of ~5  $\mu$ L) was delivered  
30 for every three licks. This bout ended when mice did not lick continuously for two  
31 seconds, or rewarded size is larger than 200  $\mu$ L from this bout. The daily reward size  
32 could vary between each mouse (typically 0.6 ML and less than 1.0 ML). This phase  
33 lasted for three days. Mice stayed in training apparatus for 1-2 hours per day in all  
34 training phases.

### 35 **Automatic shaping phase**

36 This phase was designed to teach mice to lick for water only in the response  
37 window, which was from 0.5 to 1.5 sec after the offset of the second odor delivery.  
38 Only rewarded condition was applied, which were non-matched pairs for DNMS task,

1 paired odors for DPA task, or go cue for GNG task, respectively. Mice could lick in  
2 response window to trigger water reward from every trial. During this phase, water  
3 port may or may not move while water was delivered. If mice missed several trials,  
4 lick-teaching would resume, in which the water port was moved forward and water  
5 was delivered during the response window. The reward in lick-teaching was  
6 program-controlled and was not triggered by lick. Two types of trials were defined for  
7 this phase, the self-learning (**Figure 5, left**) and program-teaching (**Figure 5, right**)  
8 trials, which switched automatically under the condition introduced below. The water  
9 port was moved forward during the response window in the program-teaching trials,  
10 in which the water delivery was automatic without triggered by licks. In the  
11 self-learning trials, however, reward delivery was licking-triggered, and water port did  
12 not move. The condition for switching from self-learning to program-teaching trials  
13 was that mice missed five times within 30 trials or missed during the last  
14 program-teaching trial. The condition for switching from program-teaching trial to  
15 self-learning trial is that mice licked in response window and obtained a reward from  
16 the last teaching trial. Daily shaping phase ended when mice performed 100 hit trials  
17 in total. This phase lasted for three days.

## 18 **Full task training phase**

### 19 **DNMS task training**

20 In the DNMS task, a sample odor was delivered at the start of a trial, followed by  
21 a delay period (4-5 seconds) and then a test odor, same to (matched) or different from  
22 (non-matched) the sample (**Figure 6**). Two kinds of odorants were used in DNMS  
23 task, 1-Butanol, and Methyl butyrate. The relative volume ratios in the pure air were  
24 10% and 2.5%, respectively. Odor-delivery duration was one second. Mice were  
25 trained to lick in the response window in non-match trials. The response window was  
26 from 0.5 to 1.5 sec after the offset of the second odor delivery. Licking events  
27 detected in the response window in the non-match trials were regarded as Hit and will  
28 trigger instantaneous water delivery (a water drop around 5  $\mu$ L). The false choice was  
29 defined as detection of licking events in the response window in the match trials.  
30 Mice were not punished in the False Choice trials. Mice were neither punished nor  
31 rewarded for the Miss (no-lick in a non-match trial) or the Correct rejection (CR,  
32 no-lick in a matching trial) trials. Behavioral results were binned in blocks of 24 trials.  
33 There was a fixed inter-trial interval of 10 seconds between trials. After training ended  
34 each day, mice were supplied with water of at least 300  $\mu$ L and up to 1 mL daily  
35 intake. This phase lasts for four to five days. The well-trained criterion was set to the  
36 existence of three continuous correct-rates larger than 80%, calculated using a sliding  
37 window of 24 trials. The reason to use 24 trials as a block is to maintain the  
38 consistency of different trial types between different tasks, with the need to be  
39 commonly divided by four and eight types of odor sequence for different tasks (4 for  
40 DNMS, 4 for DPA). It was intended to facilitate the comparison of the performance  
41 in the different tasks in the the current study. It can be easily modified according to  
42 the needs of .

## 1 **DPA task training**

2 For the DPA task, a sample and a test odor were delivered, separated by a delay  
3 period (**Figure 7**). Four kinds of odorants were used, 1-Butanol (S1), Methyl butyrate  
4 (S2), Hexanoic acid (T1) and Octane (T2). The relative volume ratios in pure air were  
5 10%, 2.5%, 15% and 5%, respectively. Odor delivery duration was one second.  
6 Delay period between two odors in a trial was 8-9 seconds. Response window was set  
7 to 0.5-1 second after the offset of the test odor in a trial. Mice were trained to lick to  
8 obtain water reward only after the paired trials (S1-T1 or S2-T2). Licking events  
9 detected in the response window in paired trials were regarded as Hit and will trigger  
10 instantaneous water delivery. The false choice was defined as detection of licking  
11 events in the response window in non-paired trials (S1-T2 or S2-T1), and mice were  
12 not punished in False Choice trials. Mice were neither punished nor rewarded for  
13 Miss (no-lick in the paired trial) or Correct rejection (CR, no-lick in a non-paired trial)  
14 trials. Behavioral results were binned in blocks of 24 trials. There was a fixed  
15 inter-trial interval of 16 seconds between trials. After training ended each day, mice  
16 were supplied with water of at least 300  $\mu$ L and up to 1 mL daily intake. This phase  
17 lasts for four to five days. The well-trained criterion was set to the existence of three  
18 continuous correct-rates larger than 80%, calculated using a sliding window of 24  
19 trials.

## 20 **GNG and GNG reversal task training**

21 For the GNG task, mice were trained to lick for water only after the Go cue but  
22 not No-go cue. Hexanoic acid and Octane were used as Go and No-go cues,  
23 respectively. The relative volume ratios in the pure air were 15% and 5%, respectively.  
24 Odor-delivery duration was one second. Response window was 0.5-1.5 second after  
25 the offset of a cue. Licking events detected in the response window in Go trials were  
26 regarded as Hit and triggered instantaneous water delivery. The false choice was  
27 defined as the detection of licking events in the response window in No-go trials.  
28 Mice were not punished in the False Choice trials. Mice were neither punished nor  
29 rewarded for the Miss (no-lick in a Go trial) or the Correct rejection (CR, no-lick in a  
30 No-go trial) trials. Behavioral results were binned in blocks of 24 trials. There was a  
31 fixed inter-trial interval of 5 seconds between trials. After training ended each day,  
32 mice were supplied with water of at least 300  $\mu$ L and up to 1 mL daily intake. This  
33 phase lasts for three days. The well-trained criterion was set to the existence of three  
34 continuous correct-rates larger than 80%, calculated using a sliding window of 24  
35 trials.

36  
37 In the third day of training, the GNG reversal task began, in which the  
38 odor-reward relationship was reversed.

39



## 1 **Data analysis**

2 The performance of the correct rate (referred to as “performance” in labels of  
3 figures) of each bin was defined by:

4

5 Performance correct rate = (num. hit trials + num. correct rejection trials) / total  
6 number of trials

7

8 Hit, False choice, and Correct rejection rates were defined as follows:

9

10 Hit rate = num. hit trials / (num. hit trials + num. miss trials)

11 False choice rate = num. false choice trials / (num. false choice trials + num.  
12 correct rejection trials)

13 Correct rejection rate = num. correct rejection trials / (num. false choice trials +  
14 num. correct rejection trials)

15

16 Mean correct rate (CR rate / FA rate) was calculated as an averaged correct rate  
17 (CR rate / FA rate) between different mice.

18

19 Error bars from the mean value of the correct rate (CR rate / FA rate) was  
20 calculated by the standard error of the mean. N represents the number of mice.

21

22 The licking rate was calculated as lick numbers within each time bin (bin  
23 size:100 ms). The curve was smoothed by smooth function from Matlab with a span  
24 size of 5 bin.

25

26 Discriminability ( $d'$ ) was defined by:

27

28  $d' = \text{norminv}(\text{Hit rate}) - \text{norminv}(\text{False choice rate})$ . The norminv function was  
29 the inverse of the cumulative normal function. Conversion of Hit or False choice rate  
30 was applied to avoid plus or minus infinity (Macmillan and Creelman, 2005). In  
31 conversion, if Hit or False choice rate was equal to 100%, it was set to  $[1 - 1/(2n)]$ .  
32 Here, n equals to a number of all possible Hit or False choice trials. If Hit or False  
33 choice rate was zero, it was set to  $1/(2n)$ .

34

35 Licking efficiency = rewarded licking number / (rewarded licking number +  
36 unrewarded licking number).

37

38 A number of trials to criterion was calculated as the trial numbers before  
39 reaching 80% correct rate for 24 consecutive trials. “NRC” in **Figure 6-8** represented  
40 Not Reaching Criterion, which indicated that mice did not reach the above criterion  
41 for that day.

42

## 1 **Results**

### 2 **Overview of Hardware, Software, and protocol**

3 In our previous study (Liu et al., 2014), mice were manually taught to lick for  
4 water and shaped for a DNMS task. The goal of the current study was to allow fully  
5 automatic training. The only things human operators need to perform were to fixate  
6 mice onto head-fix bars, close doors of training boxes, and run computer software  
7 controlling training protocols. The current study fulfilled the goal by designing HATS  
8 for olfactory and odor-based cognitive behavior in head-fixed mice. HATS was  
9 composed of a mouse containing, head-fix, odor- and water- delivery, Arduino based  
10 control, and data acquisition units (diagram in **Figure 1A**, photo in **Figure 1B**,  
11 3D-printed parts in **Figure 1C**). Optogenetic, chemogenetic, recording, and imaging  
12 methods can be easily integrated into HATS. All valves and motors were controlled by  
13 Arduino based processors and customized software. The daily routine was composed  
14 of system adjustment, head-fixation of mice, choosing a protocol, and training mice a  
15 given behavior (**Figure 1D**).

### 16 **Fast odor delivery**

17 In studying olfactory behaviors, it is critical to have fast rise and decay for odor  
18 delivery.

19 Our olfactometer exhibited fast response and stable performance. The reaction  
20 time constant for the onset of these odors was between 11-71 ms (**Table 1**), measured  
21 with a photoionization detector (PID). Another key parameter was the time constant  
22 for decay after the offset of the odor-delivery unit, which was especially important in  
23 working memory-related tasks. The current odor-delivery unit exhibited fast decay  
24 (time constant: 20-41 ms, **Figure 2C**, **Table 1**). Moreover, odor concentration  
25 remained stable following more than 200 trials of odor delivery (**Figure 2F**), which  
26 was important for behavioral and recording experiments.

### 27 **Automatic training protocol**

28 To achieve fully automatic training, we developed a step-by-step training  
29 protocol. The protocol was separated into two preparatory steps (water deprivation  
30 and habituation) and three training phases (lick-teaching, shaping, and learning,  
31 **Figure 3A**).

32

33 The first step of training was to automatically teach licking freely from water  
34 tube (**Figure 4A-B**). Moveable water port (**Figure 4C-E**) was located 5 mm away  
35 from the mouth of a mouse. The flow chart of the lick-teaching protocol was plotted  
36 in **Figure 4A**. At the start of a teaching bout, water port would deliver 10  $\mu$ L water  
37 and then moved forward until contacting the mouth, thus encouraging the licking. If  
38 mouse licked, 4  $\mu$ L water would be rewarded for every three licks. After no licking  
39 was detected for consecutive 2 sec or water of 200  $\mu$ L was delivered, one bout of

1 teaching was completed, and the water port was moved back to the initial position.  
2 The teaching bout was repeated for several times until water of 400  $\mu$ L was rewarded  
3 in total. The volume of water rewarded in each day was plotted in **Figure 4B**.

4  
5 The second step of training was shaping for a specific task. This phase was  
6 designed to allow mice to be familiar with the temporal structure of the tasks and the  
7 involved sensory stimuli, without experiencing the full task. In shaping, only the trials  
8 with water reward were applied. Specifically, for DNMS task, only non-matched odor  
9 pairs were applied to mice (**Figure 5A-B**). For DPA task, only paired trials were  
10 applied. For GNG task, only Go cue was applied. Two types of trials were designed,  
11 self-learning and teaching trials. In self-learning trials, water delivery was triggered  
12 by licking in the response window (**Figure 5C** left box). In teaching trials, water port  
13 moved forward and delivered water automatically during response window (**Figure**  
14 **5C** right box). These two types of trials were designed to switch automatically. The  
15 condition for switching from learning to teaching trial was that mice missed five trials  
16 in 35 trials. The condition for switching from teaching to learning trial was that mice  
17 licked within the response window in the last teaching trial. Daily shaping phase  
18 ended when mice performed 100 hit trials in total. This phase lasted for three days.

### 19 **Training the DNMS task**

20 We trained eight head-fixed mice to perform an olfactory DNMS task(Liu et al.,  
21 2014) (**Figure 6A**). In this design mouse needed to temporally maintain information  
22 during the delay period before behavioral choices and motor planning. After the  
23 shaping protocol, we added the non-rewarded matched trials, which induced false  
24 choice and reduced performance to chance level (**Figure 6B**). Gradually the  
25 performance, correct rejection, and discriminability ( $d'$ ) progressively increased,  
26 whereas the hit rate remained at a ceiling level (**Figure 6B-E**). After the training of  
27 five days (600 trials), the performance showed significant increase (ANOVA,  
28  $p < 0.0001$ ,  $F = 775.89$ ). Mice experienced a certain level of relearning each day, with a  
29 decreased number to criteria (defined as a correct rate above 80% in 24 consecutive  
30 trials) each day through learning (**Figure 6F**). Most of the licking responses were  
31 associated with non-match odor and expectation of water reward (**Figure 6G**). There  
32 were licks associated with the first odor delivery in the early phase of learning  
33 (**Figure 6G** black curve), which were declined through learning (**Figure 6G** blue  
34 curve). Also, the licking efficiency (defined as the ratio of successful licks resulting  
35 water reward) was increased progressively through learning (**Figure 6H**).

### 36 **Training the DPA task**

37 The second set of head-fixed mice was trained to perform an olfactory DPA task  
38 (**Figure 7A**). As in the DNMS task, the performance, correct rejection, and  
39 discriminability ( $d'$ ) progressively increased, whereas the hit rate remained at ceiling  
40 level (**Figure 7B-E**). After the training of five days (600 trials), the performance  
41 showed significant increase (ANOVA,  $p < 0.0001$ ,  $F = 1139.03$ ). Mice also experienced

1 a certain level of relearning each day (**Figure 7F**). Most of the licking responses were  
2 associated with paired odor and expectation of water reward (**Figure 7G**). There were  
3 licks associated with the first odor delivery in the early phase of learning (**Figure 7G**  
4 black curve), which were declined through learning (**Figure 7G** blue curve). Such  
5 early licks associated with the sample odor were lower than that in the DNMS task.  
6 The licking efficiency also increased progressively through learning (**Figure 7H**).

## 7 **Training the GNG and reversal tasks**

8 The third set of head-fixed mice was initially trained to perform an olfactory  
9 GNG task (**Figure 8A** above), then subsequently sensory-cue reversal task (**Figure**  
10 **8A** below). The performance, correct rejection, and discriminability ( $d'$ ) progressively  
11 increased, whereas the hit rate remained at ceiling level (**Figure 8B-E**). After the  
12 training of two days (200 trials), the performance showed significant increase  
13 (ANOVA,  $p < 0.0001$ ,  $F = 3455.17$ ). Mice also experienced a certain level of relearning  
14 each day (**Figure 8F**). Most of the licking responses were associated with paired  
15 odor-pair and expectation of water reward (**Figure 8G**). The licking efficiency also  
16 increased progressively through learning (**Figure 8H**). After two-days of GNG  
17 training, the odor-reward relationship was reversed (**Figure 8A** below). The  
18 performance, correct rejection, discriminability ( $d'$ ), and licking efficiency were  
19 decreased initially, and then progressively increased (**Figure 8B-E**). The hit rate  
20 remained at ceiling level (**Figure 8D**) and relearning was evident from the number of  
21 trials to criteria (**Figure 8F**).

## 22 **Discussion**

23 Automated, quantitative, and accurate assessment of behaviors is critical for  
24 understanding mechanisms underlying cognition. Here we presented HATS, a new  
25 integrated hardware and software system that combined fast olfactometer, 3D-printed  
26 components, step-by-step automatic training, for automatic training of cognitive  
27 behaviors in head-fixed mice. The robustness of the system was validated in multiple  
28 olfactory and odor-based tasks. The involved tasks require cognitive abilities  
29 including working memory (Fuster, 1997; Baddeley, 2012), decision making (Gold  
30 and Shadlen, 2007; Lee et al., 2012), and reversal of learnt rules (Bunge and Wallis,  
31 2008), all of which are required in more naturalistic environment and vital for  
32 survival.

33 An obvious limitation is that free-moving mice cannot be trained with HATS.  
34 Another limitation is that HATS only monitor the lick as behavioral readouts,  
35 therefore is more suited for large-scale screening of optogenetic. Although the  
36 head-movement was restrained in the current design, one would like to monitor the  
37 muscles controlling head or chewing movement to further eliminate the potential  
38 artifacts in electrophysiological recording. To obtain deep understanding of neural  
39 circuit underlying these behavior, one would also like to integrate more monitoring  
40 systems for behavioral events, such as sniffing (Kepecs et al., 2007; Verhagen et al.,  
41 2007; Wesson et al., 2008; Shusterman et al., 2011; Deschenes et al., 2012; McAfee et

1 al., 2016), pupil size (Reimer et al., 2014; McGinley et al., 2015; Vinck et al., 2015;  
2 Bushnell et al., 2016; Reimer et al., 2016), and whisker movement (Orbach et al.,  
3 1985; Friedman et al., 2006; Birdwell et al., 2007; O'Connor et al., 2010; Deschenes  
4 et al., 2012; Petreanu et al., 2012; Moore et al., 2013).

5 In designing HATS, we tried to fasten the training history, therefore aiding the  
6 dissection of neural circuit. However, this fast training in animals would only  
7 sufficiently model fast learning in humans. Indeed, many human behaviors and  
8 human learning are slow in learning and require extensive training, such as fine motor  
9 skill (*i.g.*, driving, playing piano) and sensory discrimination (*i.g.*, wine tasting). Thus,  
10 automations achieved in HATS have limitations to what kinds of behavioral and  
11 neural processes are being effectively modeled.

12 Nevertheless, HATS allowed for rapid, automated training of cognitive behaviors  
13 across diverse experimental designs. Our approach can also support high-throughput  
14 behavioral screening. In summary, the newly developed HATS is well-suited for  
15 circuitry understanding of odor-based cognitive behavior.  
16

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34

## 35 **Figure and Table Legends**

### 36 **FIGURE 1 | Components and operational processes for HATS.**

37 (A) Schematics showing the components of HATS. (B) Photos of HATS hardware. a,  
38 sound-attenuated box; b-c, odor containers; d, flow meter; e, needle valve; f, training  
39 tube for restraining mouse body; g, camera; h, holder for the odor- and water-delivery  
40 unit and motors; i, capacitance detector for licking; j, 3D-printed odor and water  
41 delivery unit; k, ventilator. (C) 3D-printed components. 1, mouse-body tube; 2, a

1 socket for odor-delivery tubes; 3-4, slots for the motor of the moveable water port; 5,  
2 a holder for the odor- and water-delivery unit and motors; 6, a socket for the training  
3 tubes. **(D)** Schematics of the operational processes for HATS.

#### 4 **FIGURE 2 | Design, implementation, and reaction time of the olfactometer.**

5 **(A)** Schematic showing the “standby” condition of the olfactometer. Diagram for the  
6 two-odor delivery unit was shown. The flow meter was designed for monitoring  
7 potential system failure. The flow rate was labeled as the numbers with the unit  
8 “L/min.” Arrows indicated for the direction of air flow. **(B)** Schematic showing the  
9 “working” condition when one odor was delivered (through “r2”). Reduction of the  
10 readout from the flow meter indicated for normal operation. **(C)** Photo of the  
11 flow-controlling unit for the olfactometer. **(D)** Photo of the tubing unit and mixing  
12 chamber. Thin tubes were used for fast reaction for odor delivery. Mixing chamber  
13 was designed for a maximal mixture of pure air (from “r1” in **B**) and the delivered  
14 odor (from “r2” in **B**). **(E)** Fast response of the olfactometer. Readout from PID was  
15 plotted in the log scale for main figure and linear scale for inset (Mean  $\pm$  SEM,  
16 standard error of the 0mean, unless stated otherwise; calculated from odor application  
17 of 200 trials). Rising/decay time constant and time with residual-odor were shown in  
18 **Table 1**. **(F)** Odor stability across trials.

#### 19 **FIGURE 3 | Step-by-step automatic training procedure.**

20 **(A)** Step-by-step automatic training procedure. Duration in a given step was labeled to  
21 the right.

#### 22 **FIGURE 4 | Automatic lick-teaching protocol.**

23 **(A)** Flow chart for the automatic lick-teaching protocol. **(B)** Daily consumed water  
24 volume in the lick-teaching phase. **(C)** Diagram of the moveable water port. **(D-E)**  
25 Diagram showing relative position between water port and mouse mouth in  
26 self-learning **(D)** and teaching **(E)** phases.

#### 27 **FIGURE 5 | Automatic shaping protocol.**

28 **(A)** Design paradigm and time line for the DNMS shaping. Only non-matched trials  
29 were applied. **(B)** Licking performance in the shaping phase. **(C)** Flow chart for the  
30 DNMS shaping. Left: self-learning trials. Right: teaching trials.

#### 31 **FIGURE 6 | Automatic DNMS training protocol and behavioral results.**

32 **(A)** Design paradigm and time line for the DNMS training. Both non-matched and  
33 matched trials were applied. **(B)** Performance of mice in the DNMS training phase.  
34 Bin size: 24 trials. **(C-E)** Correct rejection (CR) rate, hit rate, and  $d'$  in the DNMS  
35 training, respectively. **(F)** Re-learning in each day of the DNMS training, measured by  
36 the number of trials to criterion (defined as more than 80% performance in 24

1 consecutive trials). NRC, not reaching criteria. Mice that were NRC in the 2<sup>nd</sup> and 3<sup>rd</sup>  
2 days were not included. (G) Licking rates for training day 1 and 5. (H) Licking  
3 efficiency in the DNMS training. Licking efficiency was defined as the ratio of  
4 successful licks resulting water reward.

5 **FIGURE 7 | Automatic DPA training protocol and behavioral results.**

6 (A-H) As in Figure 6 A-H.

7 **FIGURE 8 | Automatic GNG and reversal training protocol and behavioral**  
8 **results.**

9 (A-H) As in Figure 6 A-H.

10

11 **TABLE | 1. Rising and decay properties of odorants. Time was in a millisecond.**

12

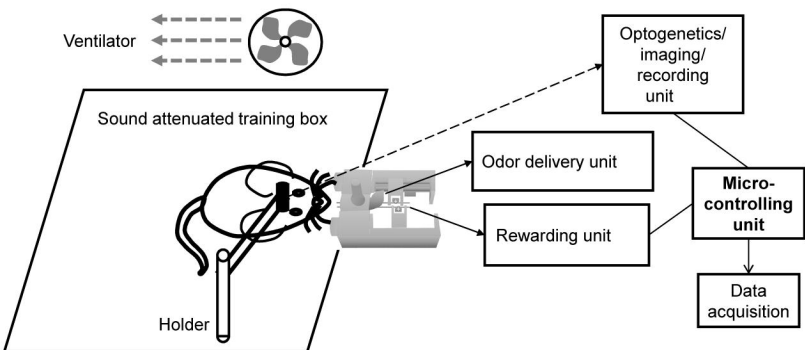
Odorant Name	Relative Volume Ratio in Air (%)	Rising Latency (95% of peak)	Decay Constant Time (1/e of peak)
1-Butanol	10	18 ± 1	20 ± 1
Methyl butyrate	2.5	17 ± 1	22 ± 1
Hexanoic acid	15	31 ± 1	41 ± 1
Octane	5	71 ± 1	31 ± 1

13 (mean ± standard error of the mean)

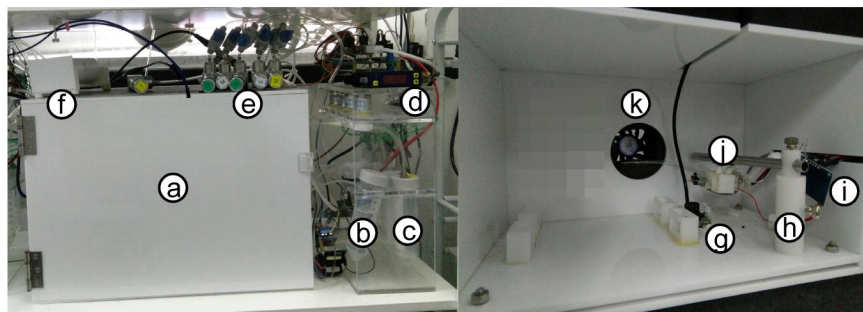
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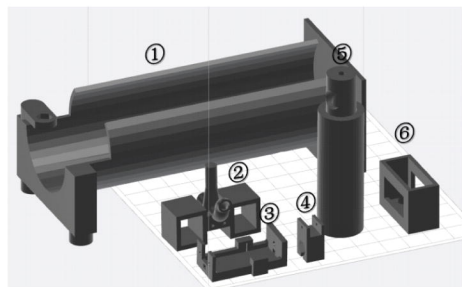
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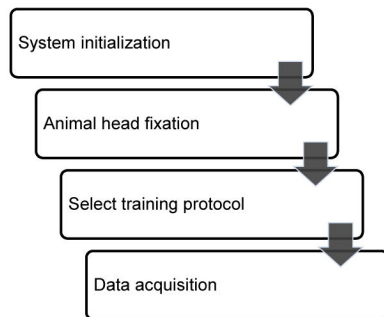
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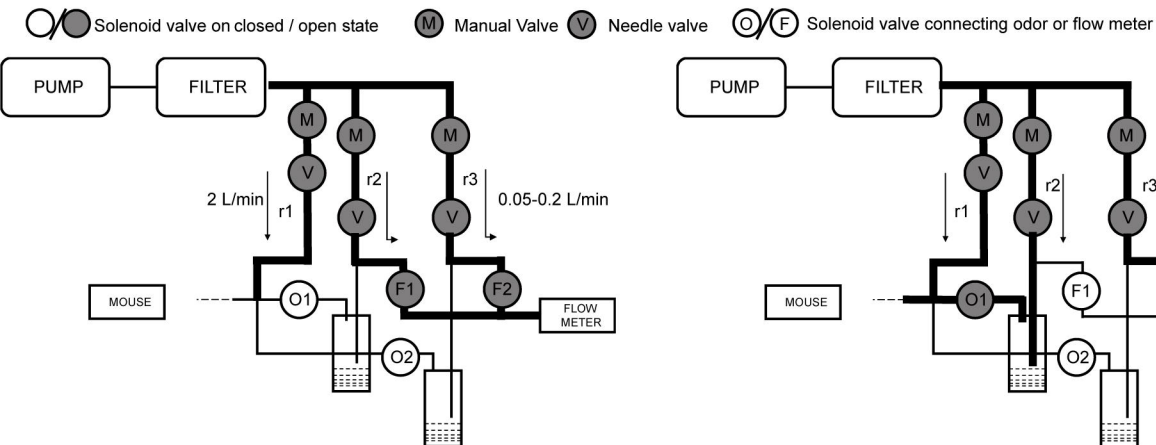
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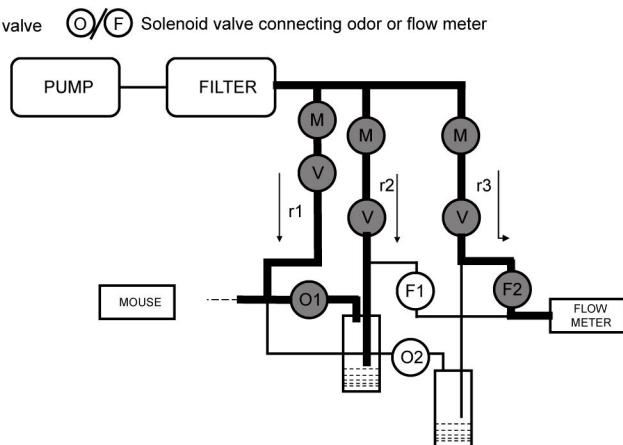
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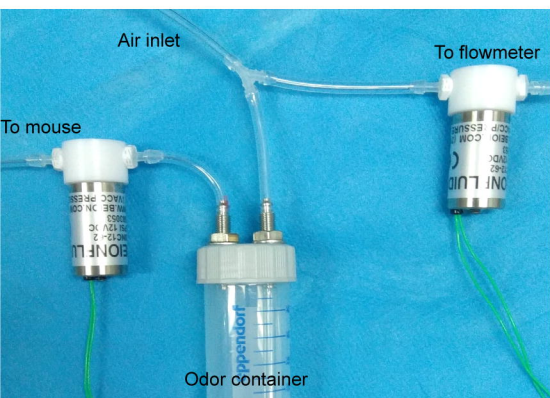
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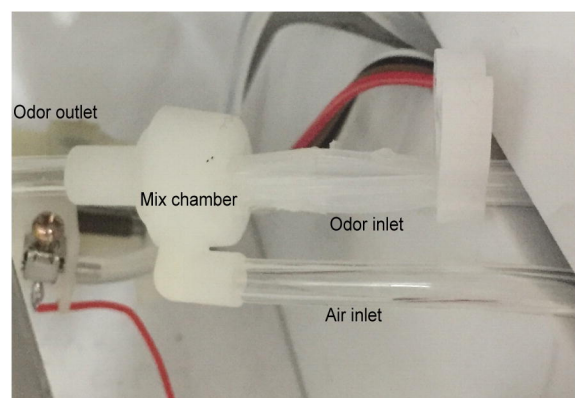
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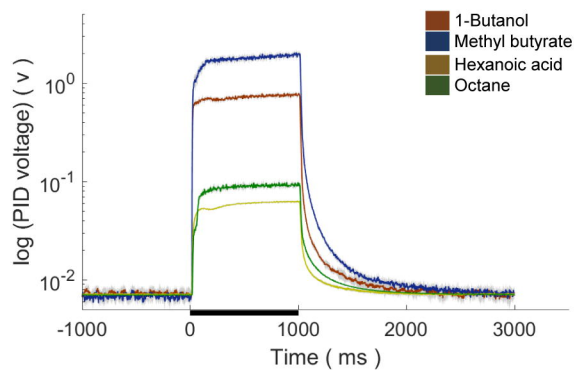
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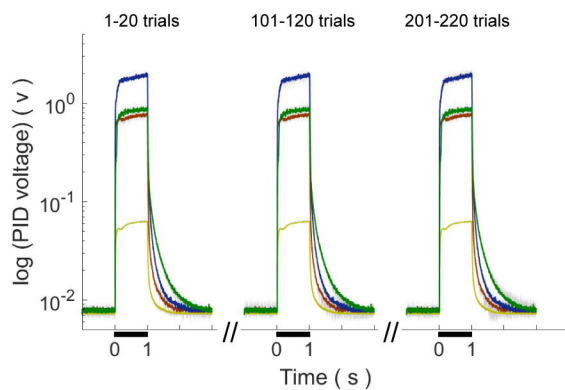
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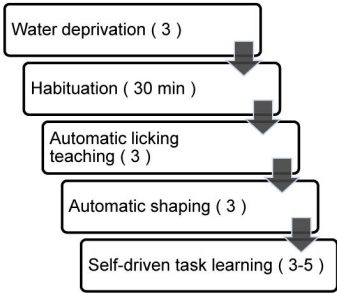
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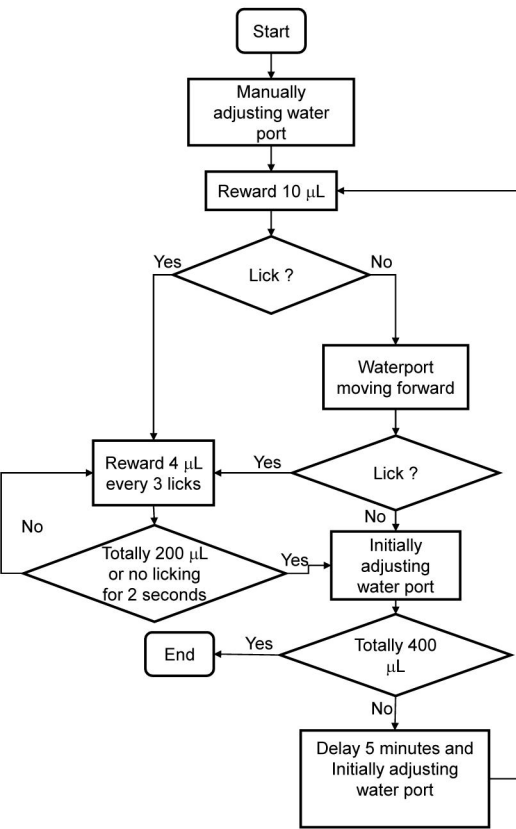
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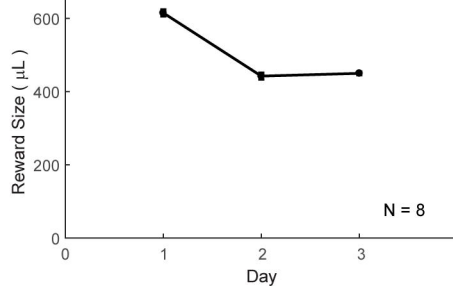
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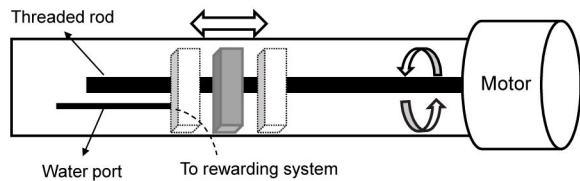
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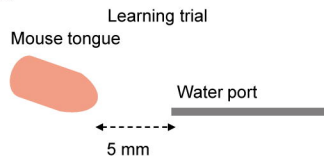
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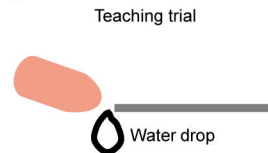
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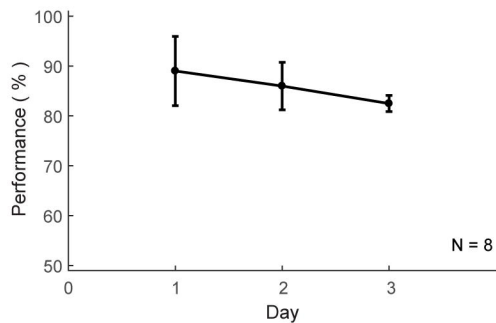
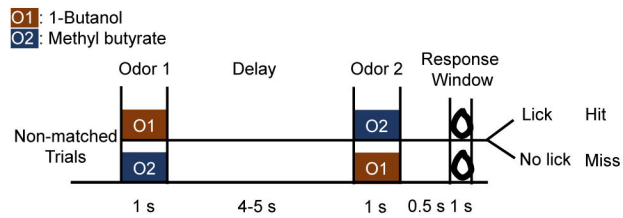


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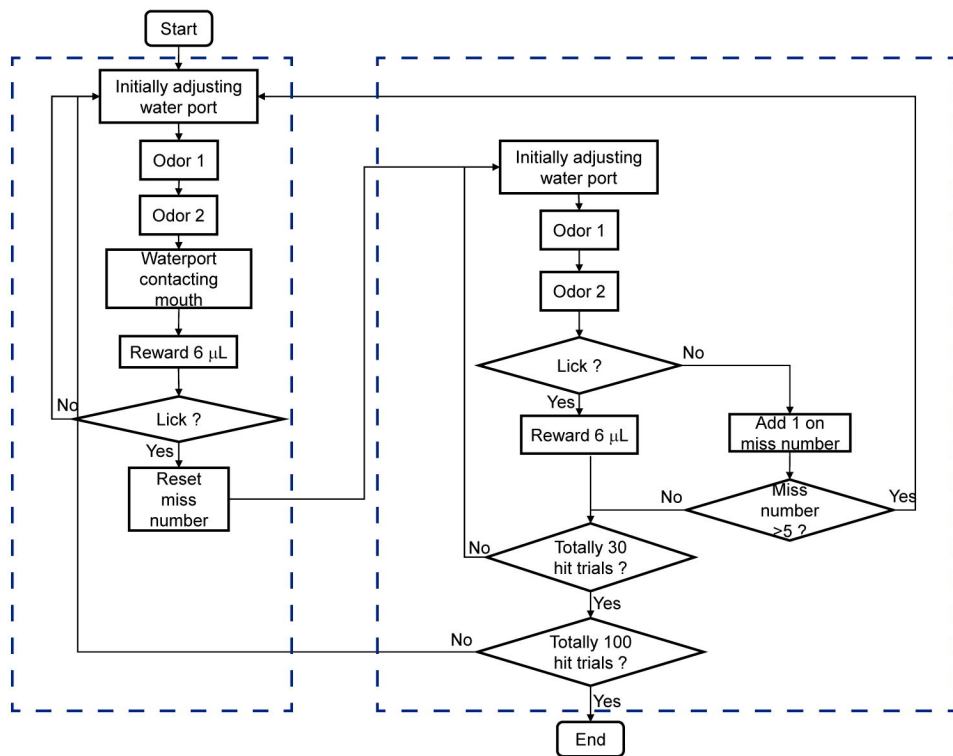




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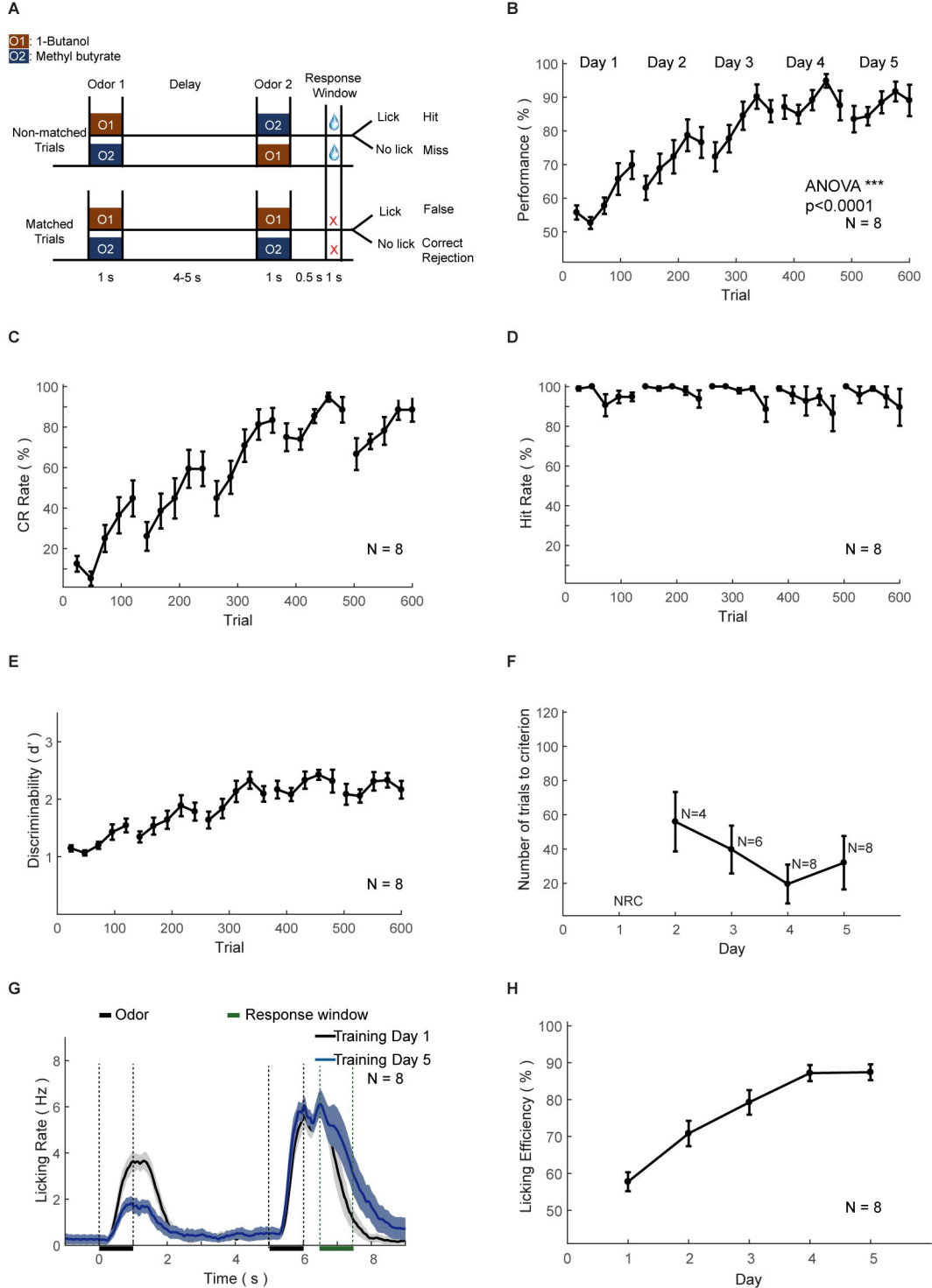


Figure 6 ( Han et al., 2017)

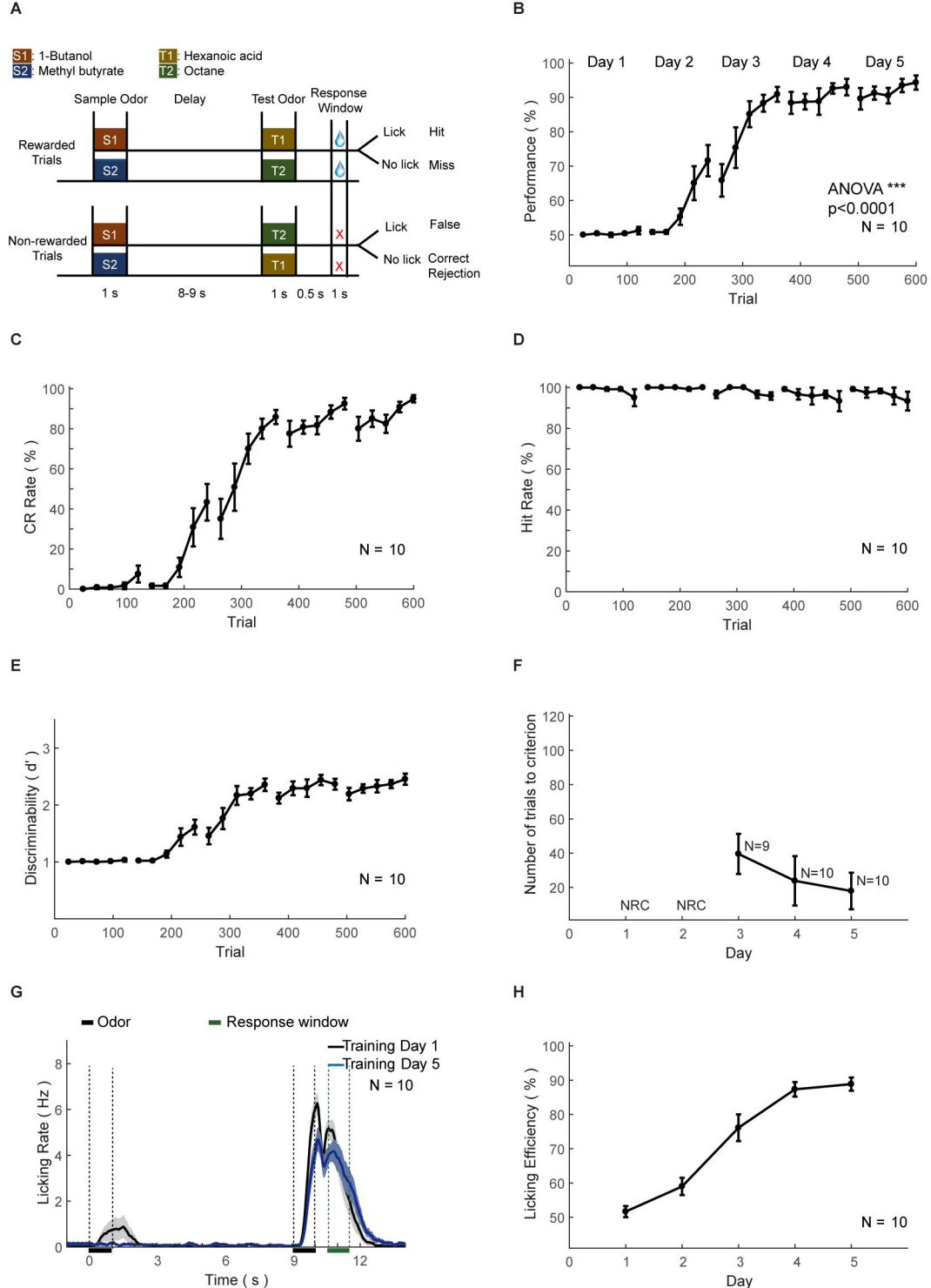


Figure 7 ( Han et al., 2017)

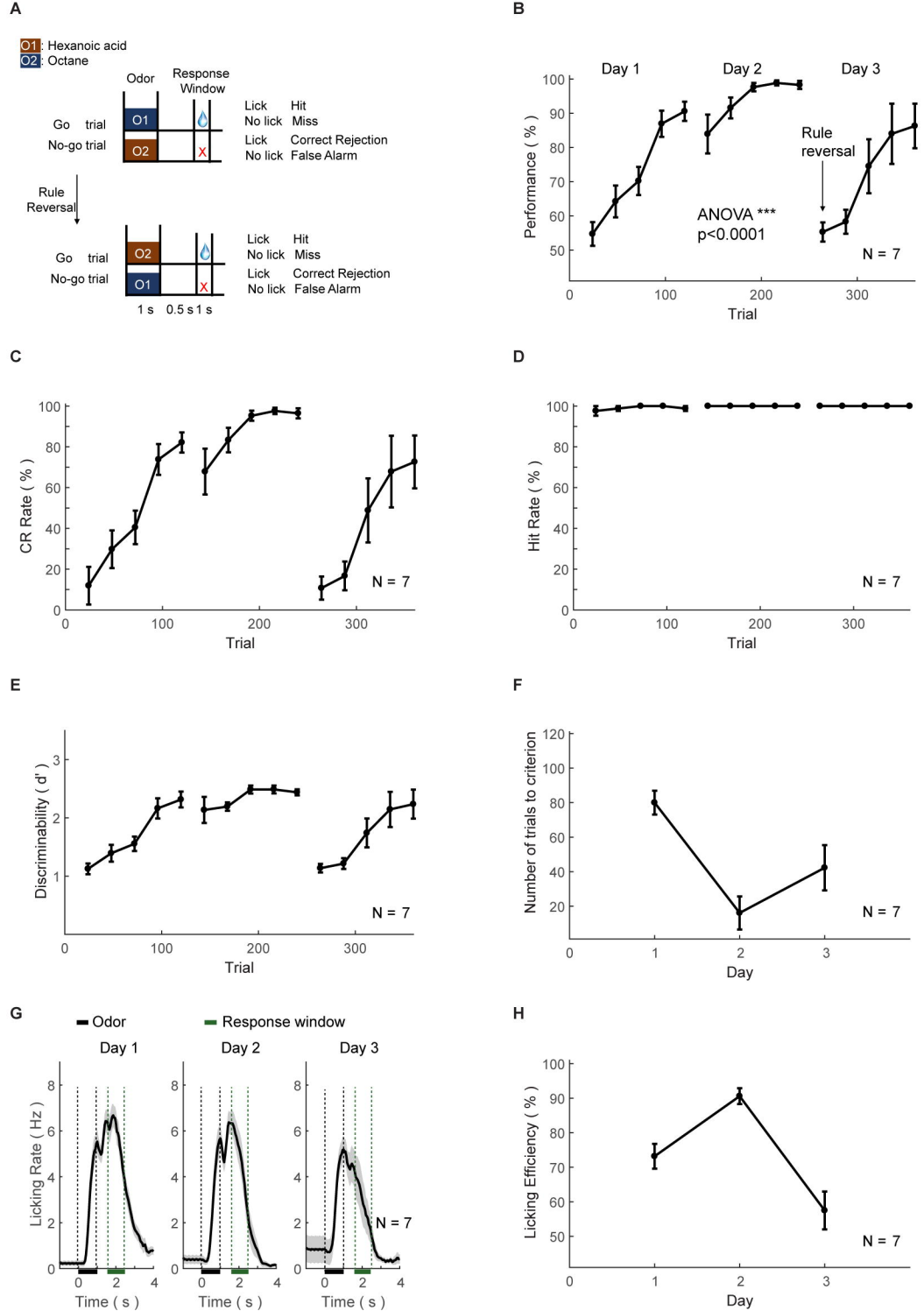


Figure 8 ( Han et al., 2017)