Pupillary Dynamics of Mice Performing a Pavlovian Delay Conditioning Task Reflect Reward Predictive Signals.

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Abstract

Pupils can signify various internal processes and states, such as attentio n, arousal, and working memory. Changes in pupil size are reportedly associ ated with learning speed, prediction of future events, and deviation from p rediction in human studies. However, the detailed relationship between pupi l size change and prediction is unclear. We explored the dynamics of the pu pil size in mice performing a Pavlovian delay conditioning task. The head-f ixed experimental setup combined with deep learning-based image analysis en abled us to reduce spontaneous locomotor activity and to track the precise dynamics of the pupil size of behaving mice. By manipulating the predictabi lity of the reward in the Pavlovian delay conditioning task, we demonstrate d that the pupil size of mice is modulated by reward prediction and consump tion, as well as body movements, but not by the unpredicted reward deliver y. Furthermore, we clarified that the pupil size is still modulated by rewa rd prediction, even after the disruption of body movements by intraperitone al injection of haloperidol, a dopamine D2 receptor antagonist. These resul ts suggest that the changes in the pupil size reflect the reward prediction signals and do not reflect reward prediction error signals, thus we provide important evidence to reconsider the neuronal circuit computing the reward prediction error. This integrative approach of behavioral analysis, image a nalysis, pupillometry, and pharmacological manipulation will pave the way f or understanding the psychological and neurobiological mechanisms of reward prediction and the prediction errors essential to learning and behavior.

Manuscript contributions to the field

Predicting upcoming events is essential for the survival of many animals, including humans. Accumulating evidence suggests that pupillary responses reflect autonomic activities modulated by noradrenergic, cholinergic, and serotonergic neurotransmission. However, the relationship between pupillary responses and reward prediction and reward prediction error remains unclear. This study examined changes in pupil size while water-deprived mice performed a Pavlovian delay conditioning task using a head-fixed setup. The head-fixed experimental setup combined with deep learning-based image analysis enabled us to reduce spontaneous locomotor activity and to track the precise dynamics of the licking response and the pupil size of behaving mice. A well-controlled, rigid behavioral experimental design allowed us to investigate behavioral states modulation induced by reward prediction. Pharmacological manipulation allowed us to differentiate the reward prediction signal itself and the signal modulated by body movements. This study integrated behavioral analysis techniques, image analysis, pupillometry, and pharmacological manipulation. We revealed that the changes of the pupil size (1) reflect reward prediction signals and (2) do not reflect reward prediction error signals. These results provide important evidence to reconsider the neuronal circuit computing reward prediction errors. The approach used in this study will pave the way for understanding the psychological and neurobiological mechanisms of the prediction and the prediction error that are essential in learning and behavior.

Keywords

dopamine, reward prediction error, pupil, licking, Pavlovian conditioning, mice

Introduction

Predicting future events from current observations helps organisms obtain rewards and avoid aversive events in a given environment. Pavlovian conditioning is a widely used experimental procedure when investigating predictive abilities of animals. For example, water-restricted mice are exposed to an auditory stimulus followed by the water reward. After several training sessions, mice develop anticipatory responses to the auditory stimulus. Pavlovian conditioning involves both behavioral and physiological responses. In appetitive conditioning, a conditioned approach response to a stimulus that signals food (Hearst and Jenkins, 1974) or to the location where the food is presented (Boakes, 1977) is observed. In fear conditioning, freezing responses (Estes and Skinner, 1941) are induced by a stimulus that signals aversive events. Physiological responses, such as salivary response, changes in skin conductance, heart rate, pupil dilation, body temperature, and respiration are also acquired through Pavlovian conditioning (Pavlov, 1927; Notterman et al., 1952; Wood and Obrist, 1964; Öhman et al., 1976; Esteves et al., 1994; Leuchs et al., 2017; Lonsdorf et al., 2017; Pietrock et al., 2019; Ojala and Bach, 2020). Thus, accumulating evidence in the field of psychological and physiological studies of animal learning demonstrates that Pavlovian conditioning is a valuable technique for studying the function and mechanism of prediction.

Although the use of pupillometry in Pavlovian conditioning dates back more than half a century, its reliability as an indicator of learning has recently been reevaluated (Finke et al., 2021). It has been reported that changes of pupil size occurs as a reactive response to a conditioned stimulus in fear and appetitive conditioning in humans (Leuchs et al., 2017; Lonsdorf et al., 2017; Pietrock et al., 2019; Ojala and Bach, 2020). The relationship between the pupil size and theories of learning, such as prediction errors in temporal difference learning (Sutton and Barto, 2018), the Rescorla-

Wagner model (Rescorla and Wagner, 1972), as well as attention to the stimuli in the Pearce-Hall model (Pearce and Hall, 1980) have also been discussed (Koenig et al., 2017; Pietrock et al., 2019; Vincent et al., 2019). Changes in the pupil size are associated with various internal states, including arousal level, attention, working memory, social vigilance, value of alternatives in choice tasks, and uncertainty in diverse research fields (Ebitz et al., 2014; Ebitz and Platt, 2015; Van Slooten et al., 2018; Larsen and Waters, 2018; Vincent et al., 2019; Zénon, 2019; Joshi and Gold, 2020; Finke et al., 2021). These findings suggest that pupil size is not only a reactive response to a conditioned stimulus, but is an active modulator of sensorimotor processing that affects the prediction (Ebitz and Moore, 2019).

Despite the potential usefulness of pupillometry in understanding the neurobiological mechanism of behavior, there have been only a few attempts to record pupillary changes in rodent research (Reimer et al., 2014; Lee and Margolis, 2016; Nelson and Mooney, 2016; Privitera et al., 2020; Cazettes et al., 2021; Wang et al., 2022). This can be attributed to two technical issues. First, conventional behavioral tasks designed for rodents use experimental apparatuses in which animals move freely, making it impossible to precisely record pupil size. Second, pupil size is also modulated by body movements (Nelson and Mooney, 2016; Cazettes et al., 2021). This makes its interpretations more complex than human studies that allow participants to remain in the experimental setup. Recent experimental setup and machine learning developments have enabled researchers to overcome these technical limitations. By combining a head-fixed setup and image analysis techniques such as DeepLabCut (Mathis et al., 2018; Nath et al., 2019), several studies have successfully quantified pupils and eyelids size of mice performing behavioral tasks (Privitera et al., 2020; Kaneko et al., 2022)

In this study, we explored the dynamics of licking and pupillary responses of mice performing a Pavlovian delay conditioning task with a head-fixed experimental setup. In Experiment 1, we trained the head-fixed mice on

the Pavlovian delayed conditioning task in which an auditory stimulus was presented before the delivery of a sucrose solution reward while recording their licking and pupil response. In this task, we designed contingent and non-contingent groups to manipulate the predictability of the delivery of the sucrose solution by the auditory stimulus. In the contingent group, the auditory stimulus was followed by the delivery of the sucrose solution, setting that the auditory stimulus signaled the arrival of the sucrose solution. In the non-contingent group, the auditory stimulus and the delivery of the sucrose solution were independent and randomized, setting that the auditory stimulus provided no predictive information about the arrival of the sucrose solution. By measuring the licking and pupillary responses while the mice perform the Pavlovian delay conditioning task, we investigated the dynamics of licking and pupillary response in predictable and unpredictable situations. In addition, bout analysis of the licking responses allowed us to unveil the detailed relationship between the licking and pupillary responses. In Experiment 2, we examined the pupil dynamics by suppressing body movements with systemic administration of haloperidol, an antagonist of dopamine D2 receptors that has been reported to inhibit anticipatory and consummatory licking (Fowler and Mortell, 1992; Liao and Ko, 1995) and spontaneous movements in an open-field experiment (Strömbom, 1977; Bernardi et al., 1981; Conceição and Frussa-Filho, 1996; Arruda et al., 2008), thus excluding the possibility that body movements affect the changes in pupil size.

Methods

Subjects

Eight adult male C57BL/6J mice were used. All mice were naive and eight weeks old at the start of the experiment. The mice were maintained on a 12:12 light cycle. All the experiments were conducted during the dark phase of th

e light cycle. The mice had no access to water at their home cage and were provided with water only during experimental sessions. The mice were allowed to consume sufficient sucrose solution during the experiment. The mice's body weight was monitored daily. They were provided with additional access to water at their home cage if their body weight fell below 85% of their normal body weight measured before the start of the experiment. The mice were allowed to feed freely in their home cages. The experimental and housing protocols adhered to the Japanese National Regulations for Animal Welfare and were approved by the Animal Care and Use Committee of Keio University.

Surgery

Mice were anesthetized with 1.0% to 2.5% isoflurane mixed with room air and placed in a stereotactic frame (942WOAE, David Kopf Instruments, Tujunga, CA, USA). A head post (H.E. Parmer Company, Nashville, TN, USA) was fixed at the surface of the skull using dental cement (Product #56849, 3M Company, Saint Paul, MN, USA) to allow the mice to be head-fixed during the experiment. The mice were group-house (four mice per cage) before the experiments and two weeks of recovery were allowed between the surgery and experiment commencement.

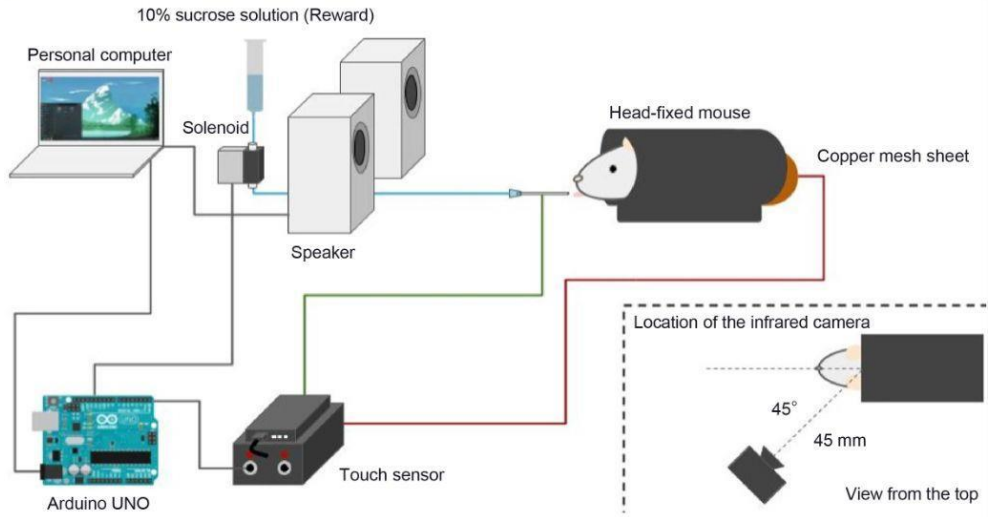
Procedure

Mice were habituated to a head-fixed experimental setup (Figure 1A; Toda et al., 2017; Yamamoto et al., 2022; Kaneko et al., 2022) the day before the experiment commenced. During habituation, mice were head-fixed in the apparatus and randomly presented to 10% sucrose solution through a drinking steel spout and a pure tone of 6000Hz at 80dB from a set of two speakers placed 30cm in front of the platform. Mice were head-fixed on a tunnel-like, covered platform by clamping a surgically implemented head plate on both sides (i.e., left and right from the antero-posterior axis of the skull). The clamps

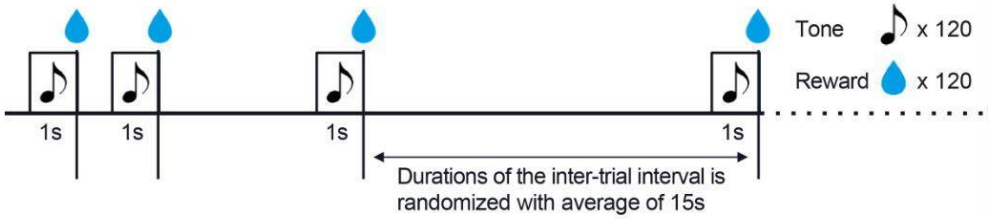
were placed on a slide bar next to the platform and adjusted to an appropri ate height for each mouse. The floor of the platform was covered with a cop per mesh sheet, and a touch sensor was connected to the mesh sheet and stee l spout.

After habituation, we conducted a Pavlovian delay conditioning task. Figure 1 (B and C) shows the experimental procedure. Mice were assigned to two experimental groups, contingent (Figure 1B) and non-contingent(Figure 1 C), with four mice in each group. In the contingent group, a pure tone of 6 000Hz at 80dB was randomly presented for 1s as the conditioned stimulus (C S), followed immediately by a 4μl drop of 10% sucrose solution (Figure 1B). The CS presentations interval was random, ranging from 10s to 20s, and the mean value was set to 15s. In the non-contingent group, the CS and reward w ere independently presented (Figure 1C). The CS and reward presentation int ervals were random, ranging from 10s to 20s. One session comprised 120 rewa rd presentations for both groups. The training lasted for eight days. The C S and reward presentation, response and video recording were controlled usi ng a custom-made program written in Python 3 (3.7.8). The experiment was co nducted in a soundproof box with 75dB of white noise in the laboratory to m ask external sounds.

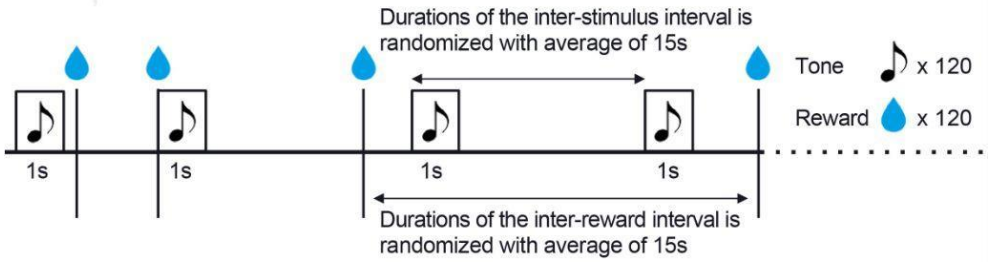
A. Head-fixed experimental setup



B. Contingent group : reward is predictable (N =4)



C. Non-contingent group : reward is unpredictable (N =4)



D. Quantification of pupil size with DeepLabCut

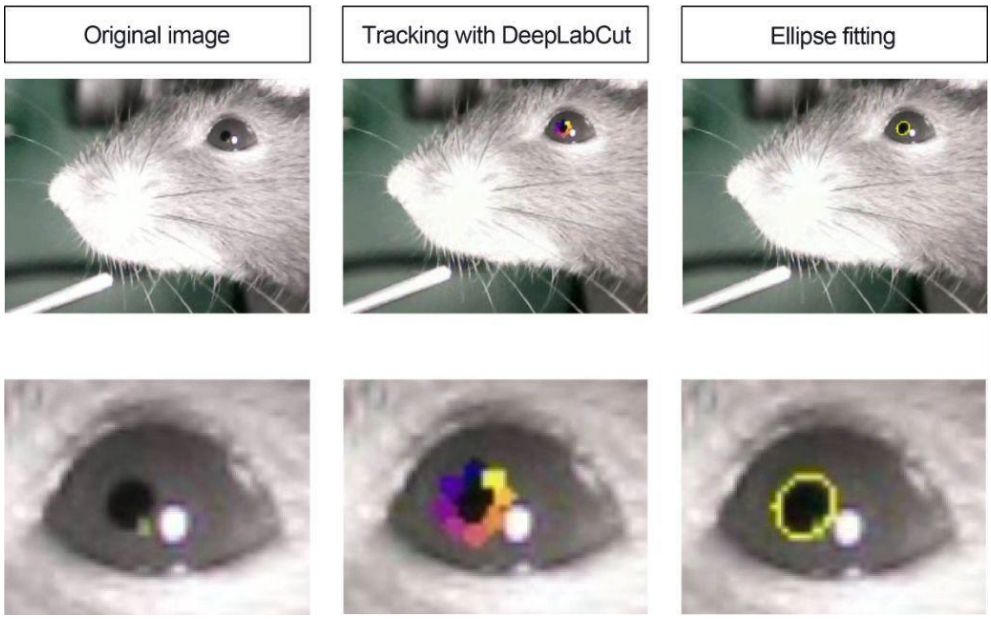


Figure 1. Schematic representation of head-fixed apparatus, Pavlovian delay conditioning task and pupilmetory.

A. Schematic representation of head-fixed experimental apparatus and the custom-made experimental control system. B. Contingent group. In this group the one second auditory stimulus (6000 Hz tone) is followed by a reward delivery of a 4 μL drop of 10% of sucrose solution, and the auditory stimulus signals the upcoming reward. C. Non-contingent group. In this group, the auditory stimulus and the reward are presented independently and semi-random fashion and so as to prevent the development of reward predictive value of the auditory stimulus. D. The left panel shows an image of a mouse's eye taken by the infrared camera in mice performing the Pavlovian delay conditioning task. The center panel shows an image with eight tracked points using DeepLabCut. The right panel shows an image of an ellipse fitted to the points and an example of the temporal change in pupil size.

Drug

Pharmacological manipulations were conducted after training the Pavlovian conditioning task to suppress licking response in mice. Six blocks were conducted for all individuals, each lasting two days. On Day 1, all mice were intraperitoneally administered saline solution 15 min before the experiment commenced. On Day 2, 15 min before the experiment commenced, haloperidol (Selenase Injection 5 mg, Sumitomo Pharma) 0.1, 0.2, and 0.5 mg/kg was intraperitoneally administered. This has been reported to inhibit licking (Fowler and Mortell, 1992; Liao and Ko, 1995) and spontaneous movements (Strömbom, 1977; Bernardi et al., 1981; Conceição and Frussa-Filho, 1996; Arruda et al., 2008), dose-dependently. After Day 2, mice were allowed to drink water freely for one hour. On Day 3, mice were not allowed access to water at all, and the experiment was not conducted to avoid residual effects of the drug. All individuals received each concentration of haloperidol twice. Halop

eridol was diluted in a saline solution. We administered haloperidol to mice via intraperitoneal injection at the dose of 10 mL/kg.

Pupillometry

To measure the pupil size of mice performing the Pavlovian delay conditioning task, we used an infrared camera (Iroiro1, Iroiro House) to capture a video of mice's heads during the task. The camera was placed at 45° from the midline of the mouse (antero-posterior axis) and 45 mm from the top of the head (Figure 1A). The room's brightness was set to 15lux using a luminaire device (VE-2253, Etsumi). The pupil size was extracted from videos. Figure 1D shows the flow of the pupil size analysis. DeepLabCut, a deep-learning tracking software (Mathis et al., 2018; Nath et al., 2019), was used to track the pupil edge at eight points. An ellipse was fitted to the eight points obtained by tracking, and the estimated parameters (major and minor diameters) were used to calculate the area of the ellipse. This area was used as pupil size.

Licking bout analysis

Animal responses occur as bouts, characterized by bursts of responses and pauses that separate each bout (Gilbert, 1958; Shull et al., 2001). Conditioned responses (CR) also occur as bouts (Kirkpatrick, 2002; Harris, 2015; Toda et al., 2017). Since the CR has such a temporal pattern, Individual licking can be classified into two types: those that occur within bursts and during pauses. In previous studies, such a bout-and-pause pattern was described by the mixture distribution of two exponential distributions (Killeen et al, 2002): $p\,(IRT = \tau) = qe^{-b\tau} + (1-q)e^{-w\tau}$. In the equation, q denotes the mixture ratio of the two types of responses and w and b denote the speed of the responses within bouts and the length of the pauses respectively. We fitted the equation to the empirical data to estimate the parameters q, w, a

nd b. Under the estimated parameters, individual licking was classified bas ed on the likelihood whether it occurred within bursta or during pauses.

Results

In the contingent group, the stimulus signaled the reward delivery. Licking and pupil responses increased after the auditory stimulus presentation and the sucrose solution delivery (Figure 3). In the non-contingent group, the auditory stimulus did not signal the reward delivery. Licking and pupil responses did not change after the auditory stimulus presentation but increased after the sucrose solution delivery (Figure 3). We set three periods for analysis of licking and pupil size, 1s before the presentation of the auditory stimulus (Pre CS period), one second during the presentation of the auditory stimulus (CS period), and one second after the reward presentation (US period). We performed a Dunnett test for each group to examine whether licking and pupil size increased during CS and US periods compared to Pre CS period. As the tests were performed for each group, we set the significance level at $\alpha = 0.025$ (0.05/2) according to the Bonferroni correction. In the contingent group, licking responses increased after the presentations of the auditory stimulus (Pre CS period vs. CS period, Pre CS vs. CS, $t(11) = 15.57$, $p < 0.001$) and reward (Pre CS period vs. US period, Pre CS vs. US, $t(11) = 17.80$, $p < 0.001$) compared to the Pre CS period. Pupil size did not increase after the presentation of the auditory stimulus (Pre CS vs. CS, $t(11) = 2.553$, $p = 0.0289$); however it increased after the sucrose solution delivery (Pre CS vs. US, $t(11) = 7.335$, $p < 0.001$). In the non-contingent group, licking responses increased after the sucrose solution delivery (Pre CS period vs. US period, Pre CS vs. US, $t(11) = 31.159$, $p < 0.001$), but not after the auditory stimulus presentation (pre-CS period vs. CS period, Pre CS vs. CS, $t(11) = -0.127$, $p = 0.988$). Pupil size did not change after either the auditory stimulus or the reward presentation (Pre CS vs. CS, $t(11) = 0.628$, $p = 0.757$; Pre CS vs. US, $t(11) = 1.526$, $p = 0.235$).

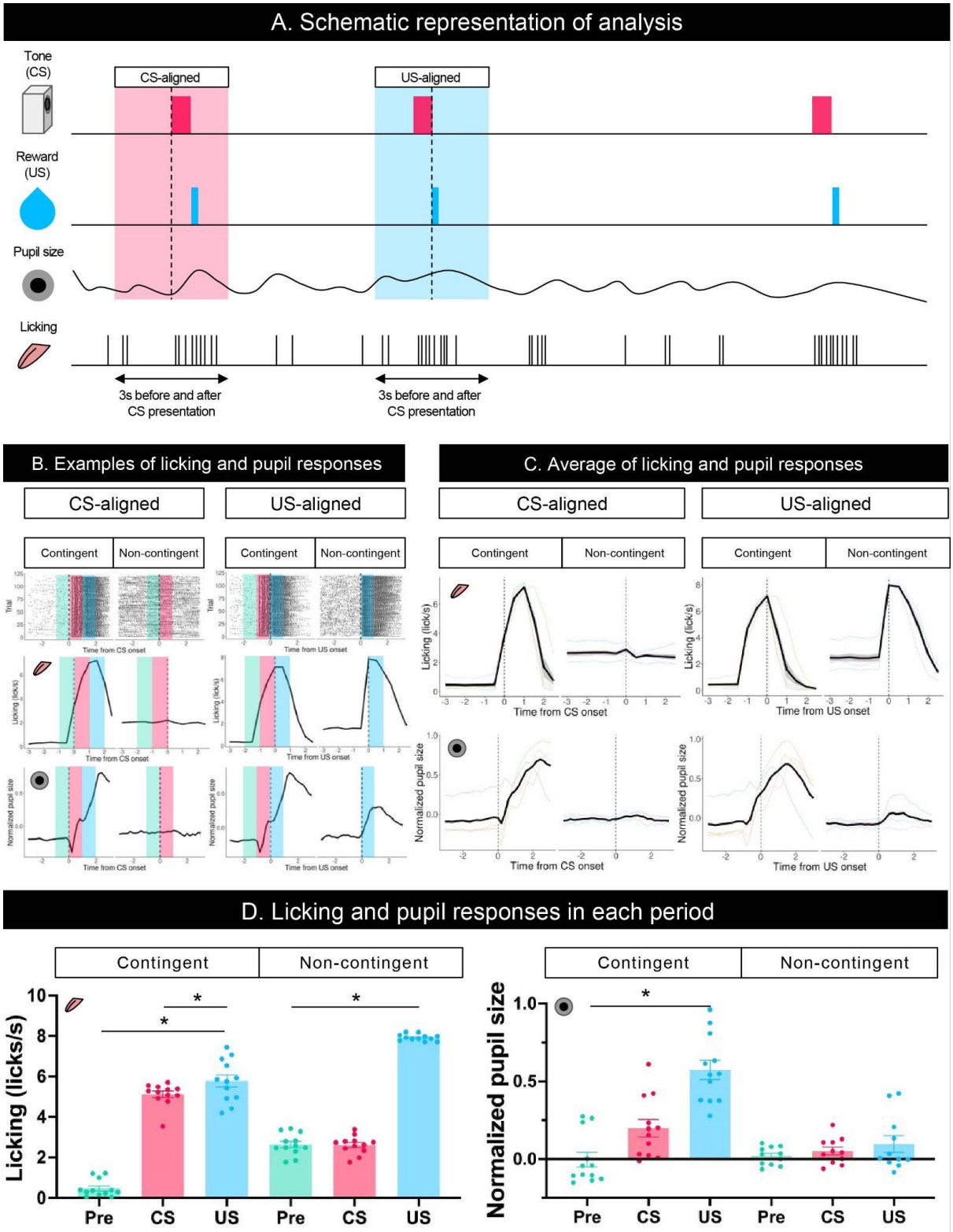
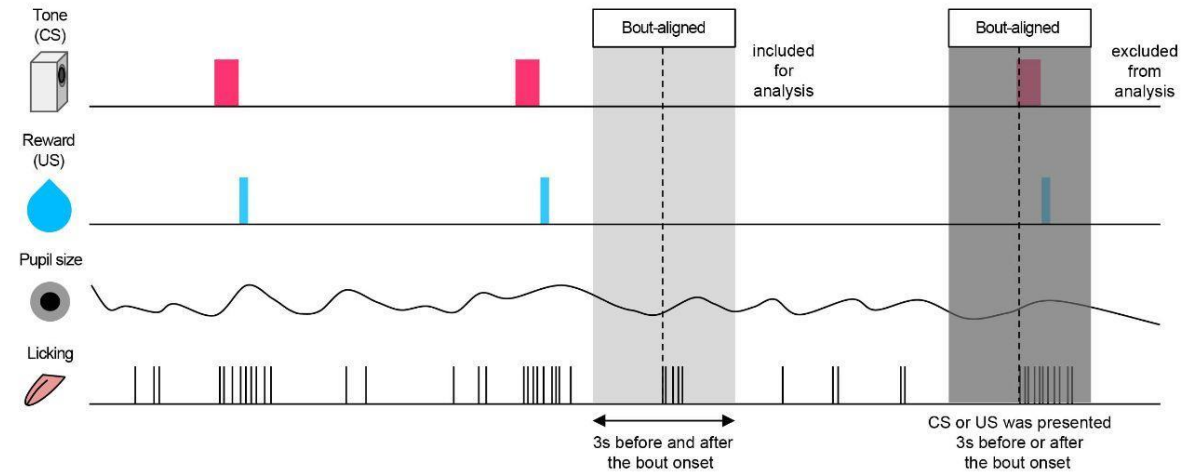


Figure 2. Results of the Pavlovian conditioning training.

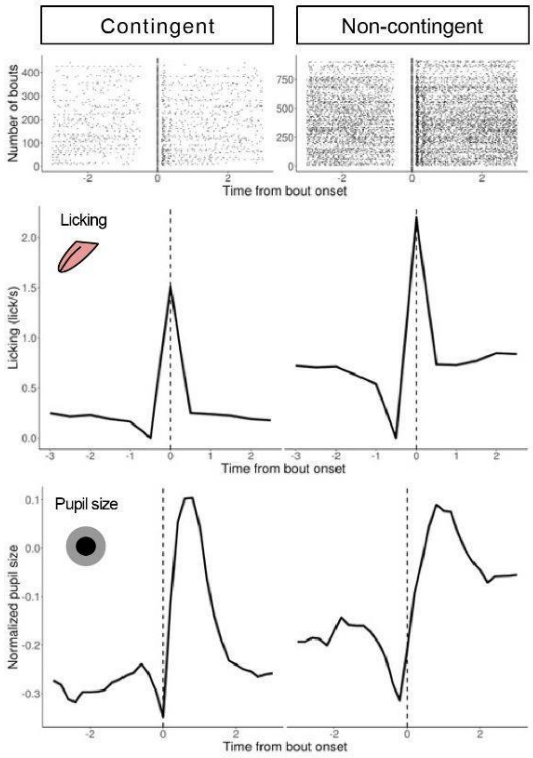
(A) Schematic representation of analyzed time windows. Presentation of CS o r US was set as 0, and the 3 seconds before and after from presentation wer e used for analysis. (B). An example of licking responses and pupil respons

e during each group’s Pavlovian delay conditioning task. Raster plot (top), temporal change in licking and pupil size (middle and low) of a representative individual from each of the contingent and non-contingent groups (N = 1 for each group, 120 trials each). 1s before the auditory stimulus presentation (Pre), during the auditory stimulus presentation (CS), and immediately after the reward presentation (US) are shown in green, red, and blue, respectively. (C) Mean temporal changes in licking frequency and pupil size before and after CS and US presentations. Solid black lines indicate means; gray-covered areas indicate standard error of the mean (N = 4 for each group, 3 sessions each). Thin, colored lines indicate individual data. (D) Licking frequency (left) and pupil size (right) at 1s before, during, and immediately after CS presentations (N = 4 for each group, 3 sessions each). Each time window corresponds to the area covered by green, red, and blue in (A).

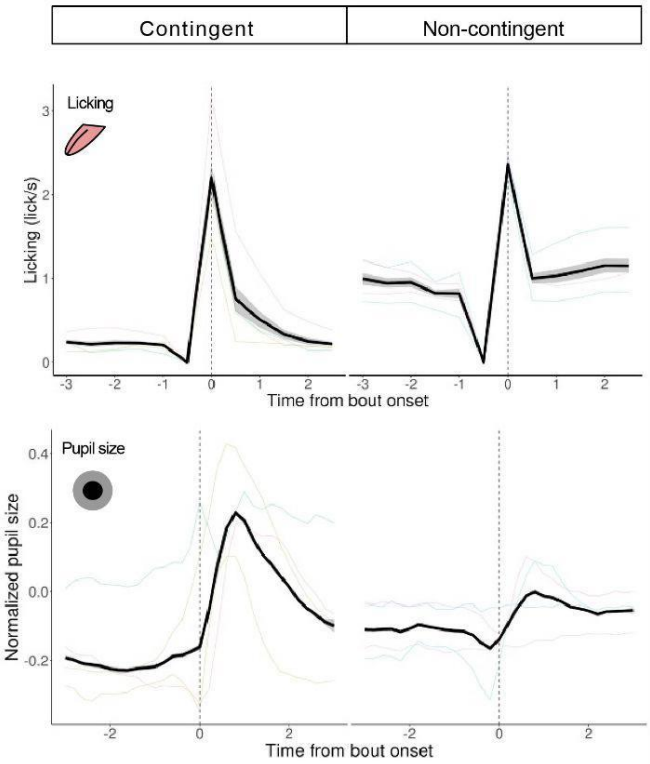
A. Schematic representation of analysis



B. Examples of licking and pupil responses



C. Averaged licking and pupil responses



D. Licking and pupil responses aligned with bout onset

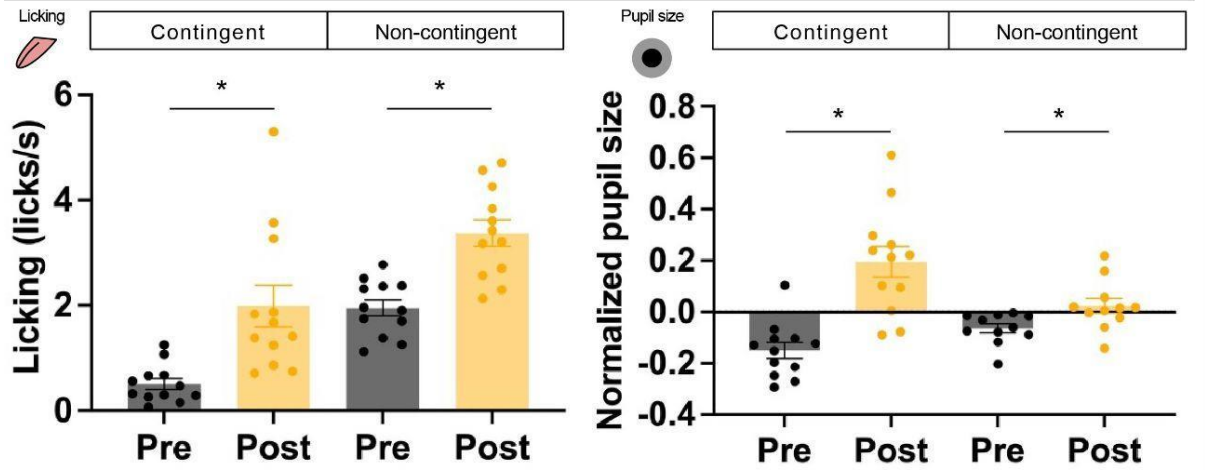


Figure 3. Temporal changes in licking responses and pupil size aligned with onsets of bouts.

(A) Schematic representation of the response bout analysis. Initiation of the bout was set as 0, and the 3s before and after bout initiation was used for the analysis. (B) Examples of raster plots before and after the start of the licking bout (top) and temporal changes in licking responses and pupil size (middle and low) in contingent and non-contingent groups (N = 1 for each group). (C) Average temporal changes in licking and pupil size in contingent and non-contingent groups (N = 4 for each group, 3 sessions each). (D) Mean number of licking (left) and pupil size (right) at 3s before and after the bout initiation (N = 4 for each group, 3 sessions each). In both (B) and (C), data including CS and US presentations within 3s before and after the initiation of the bout were excluded. Individual data are shown as colored lines.

To examine the effect of licking responses on pupil size, we analyzed temporal changes in licking and pupil responses around the onset of licking bout. When we aligned the licking and pupil responses with the licking bout onset, both groups' licking responses and pupil size increased with the bout onset (Figure 4). Licking responses were phasically increased at the bout onset, and the pupil size increased slightly after the bout onset. The pupil size slightly decreased before the bout onset and increased after the the bout onset. These results indicate that pupil size increased after the initiation of licking responses. We performed t-tests to examine whether the number of licking responses and pupil size increased after the bout onset compared to before that. As we conducted t-tests independently for each condition, we set the significance level at α = 0.025 according to Bonferroni's correction. Licking responses increased after the bout onset in both of the contingent and the non-contingent groups (Contingent group: Pre bout vs. Post bout, t (11) = -3.604, p = 0.003; Non-contingent group: Pre bout vs. P

ost bout, t (11) = -4.885, p < 0.001). Pupil size increased after the bout onset in both the contingent and the non-contingent groups (In the contingent group, Pre bout vs. Post bout, t (11) = -5.123, p < 0.001; In the non-contingent group, Pre bout vs. Post bout, t (11) = -2.549, p = 0.021).

In order to further investigate whether the increase of the pupil size resulted solely from licking responses or reward prediction independently of licking responses, we attempted to suppress licking responses in the same task. We thusly intraperitoneally injected haloperidol, a dopamine D2 receptor antagonist known to suppress licking responses and locomotor activity (Fowler and Mortell, 1992; Liao and Ko, 1995; Strömbom, 1977; Bernardi et al., 1981; Conceição and Frussa-Filho, 1996; Arruda et al., 2008). After saline administration, licking responses and pupil size increased after the auditory stimulus presentation in the contingent group, but remained unchanged in the non-contingent group (Figure 5A, B). We observed an increase of the licking frequency and pupil size at the reward delivery in both the contingent and non-contingent groups (Figure 5A, C). Systemic administration of haloperidol suppressed licking responses and pupil size in both the contingent and non-contingent groups (Figure 5A-D). In particular, the increase in pupil size that occurred after the reward delivery was eradicated in the contingent group (Figure 5A, C). We performed Dunnett's tests for each group to examine whether licking and pupil size increased during CS and US periods compared to the Pre CS period. As the tests were performed for the haloperidol condition in each group, we set the significance level at α = 0.00625 (0.05/8) according to the Bonferroni correction. After saline injection in the contingent group, licking responses increased after the auditory stimulus and the reward presentations compared to the period before the auditory stimulus presentation (Figure 5D; Pre CS vs. CS, t (23) = 24.47, p < 0.001, Pre CS vs. US, t (23) = 30.97, p < 0.001). These differences disappeared in all the haloperidol conditions (Figure 5D; 0.1 mg/kg: Pre CS vs. CS, t (7) =2.62 , p = 0.03, Pre CS vs. US, t (7) = 2.058, p = 0.093; 0.2 mg/kg: P

re CS vs. CS, t (7) = 2.831, p = 0.019, Pre CS vs. US, t (7) = 1.603, p = 0.212; 0.5 mg/kg: Pre CS vs. CS, t (7) = 2.934, p = 0.015, Pre CS vs. US, t (7) = 2.002, p = 0.103). In the non-contingent group, licking response increased after the reward delivery except in the 0.2 mg/kg condition (Figure 5D; saline: Pre CS vs. US, t (23) = 37.012, p < 0.001; 0.1 mg/kg Pre CS vs. US, t (7) = 4.641, p < 0.001; 0.2 mg/kg: Pre CS vs. US, t (7) = 2.421, p = 0.049; Pre CS vs. US, t (7) = 4.342, p < 0.001). Moreover it did not increase after the auditory stimulus presentation in all haloperidol conditions. (Figure 5D; saline: Pre CS vs. CS, t (23) = 0.479, p = 0.848; 0.1 mg/kg: Pre CS vs. CS, t (7) = 0.23, p = 0.962; 0.2 mg/kg: Pre CS vs. CS, t (6) = 0.256, p = 0.953; 0.5 mg/kg: Pre CS vs. CS, t (7) = 0.772, p = 0.663). After saline injection in the contingent group, the pupil size was increased during auditory stimulus presentation and after reward presentation compared to that of before the auditory stimulus (Figure 5D; Pre CS vs. CS, t (23) = 4.707, p < 0.001, Pre CS vs. US, t (23) = 8.852, p < 0.001). Pupil size increased after the reward delivery (Figure 5D; 0.1 mg/kg: Pre CS vs. US, t (7) = 6.943, p < 0.001; 0.2 mg/kg: Pre CS vs. US, t (7) = 9.464, p < 0.001; 0.5 mg/kg: Pre CS vs. US, t (7) = 7.674, p < 0.001), but not after the auditory stimulus presentation (Figure 5D; 0.1 mg/kg: Pre CS vs. CS, t (7) = 2.307, p = 0.057; 0.2 mg/kg: Pre CS vs. CS, t (7) = 1.915, p = 0.122; 0.5 mg/kg: Pre CS vs. CS, t (7) = 2.339, p = 0.053) in all haloperidol conditions. In the non-contingent group, the pupil size remained unchanged after the auditory stimulus and reward presentation in all conditions (Figure 5D; saline: Pre CS vs. CS, t (23) = 1.681, p = 0.17, Pre CS vs. US, t (23) = 2.928, p = 0.009; 0.1 mg/kg: Pre CS vs. CS, t (7) = 1.692, p = 0.182, Pre CS vs. US, t (7) = 2.127, p = 0.081; 0.2 mg/kg: Pre CS vs. CS, t (6) = 1.548, p = 0.236, Pre CS vs. US, t (6) = 1.751, p = 0.168; 0.5 mg/kg: Pre CS vs. CS, t (7) = 2.921, p = 0.015, Pre CS vs. US, t (7) = 1.920, p = 0.121). The increase in licking responses and pupil size after the auditory stimulus presentation was examined by calculating the difference between the mean values of lickin

g responses and pupil size for 3s before and after the auditory stimulus presentation. We performed two-tailed one-sample t-tests to examine whether the increase was greater than zero. As the tests were performed for each group and haloperidol conditions, we set the significance level at α = 0.00625 (0.05/8) according to the Bonferroni correction. Licking responses increased after the saline injection (Figure 5E; t (23) = 26.243, p < 0.001). However, the difference disappeared after the haloperidol injection (Figure 5E; 0.1 mg/kg: t (7) = 2.714, p = 0.030; 0.2 mg/kg: t (7) = 2.845, p = 0.025; 0.5 mg/kg: t (7) = 3.209, p = 0.015) in the contingent group. Licking responses did no increase in the non-contingent group in all haloperidol conditions (Figure 5E; aaline: t (23) = -0.056, p = 0.956; 0.1 mg/kg: t (7) = 0.218, p = 0.834; 0.2 mg/kg: t (6) = 1.364, p = 0.222; 0.5 mg/kg: t (7) = 1.079, p = 0.316). Although the increase of licking responses was eliminated by haloperidol injection, the pupil size increased in the contingent group in all haloperidol conditions (Figure 5E; t (23) = 6.797, p < 0.001; 0.1 mg/kg: t (7) = 5.194, p = 0.001; 0.2 mg/kg: t (7) = 8.806, p < 0.001; 0.5 mg/kg: t (7) = 4.926, p = 0.002). Meanwhile, the pupil size did not increase in the non-contingent group in all haloperidol conditions. (Figure 5E; Saline: t (23) = 1.908, p = 0.069; 0.1 mg/kg: t (7) = 2.206, p = 0.063; 0.2 mg/kg: t (6) = 2.450, p = 0.05; 0.5 mg/kg: t (7) = 2.084, p = 0.076).

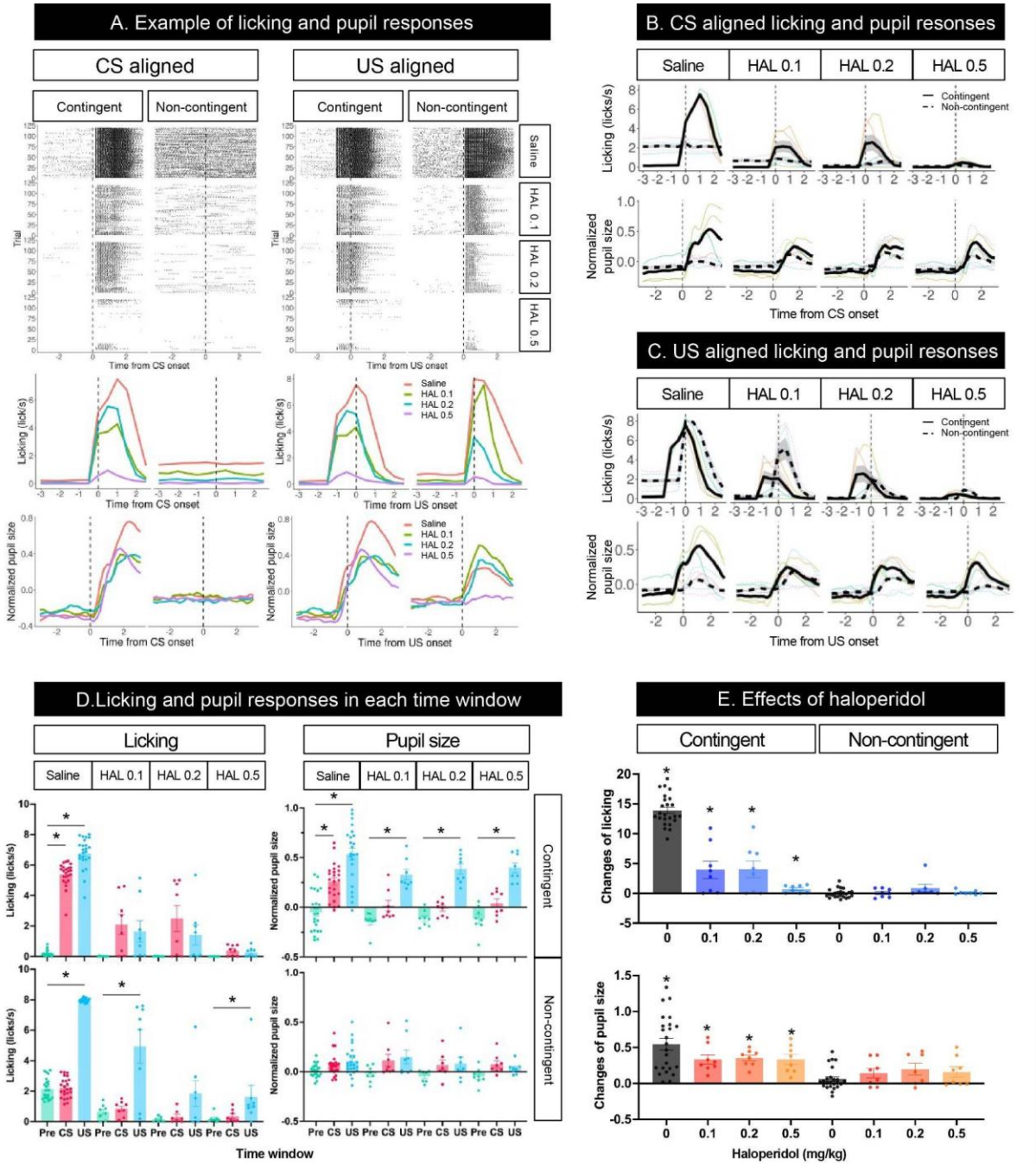


Figure 4. Effects of haloperidol injection on the licking and pupil respon ses after Pavlovian conditioning training.

(A) Representative raster plot (top), temporal change in licking and pupil responses (middle and low) of individuals in contingent and non-contingent groups. Periods of 1s before the auditory stimulus presentation (Pre CS), d uring the auditory stimulus presentation (CS), and after the reward present ation (US) are shown in green, red, and blue, respectively (N = 1 for each group, 120 trials each). (B) Mean temporal changes in licking and pupil siz

e before and after CS presentations (N = 4, 6 sessions for saline condition and 2 sessions for the all haloperidol conditions). The upper panel indicat es licking responses. The horizontal axis indicates the time from the rewar d onset. The vertical axis indicates frequencies of licking responses. The lower panel indicates the data of the pupil size. The horizontal axis indic ates the time from the reward onset. The vertical axis indicates the normal ized pupil size. (C) Mean temporal changes in licking and pupil responses b efore and after US presentations. (D) Licking responses at Pre CS, CS, and US periods (N = 4, 6 sessions for saline condition and 2 sessions for the a ll haloperidol conditions). (E) The pupil size at Pre CS, CS, and US perio ds. (F) Difference between the mean values of licking responses and the nor malized pupil size during a 3s before and after CS presentation. HAL indica rtes haloperidol.

Discussion

This study explored the dynamics of the licking response and pupil size while mice performed a Pavlovian delay conditioning task to investigate the relationship between reward prediction and pupil size. The head-fixed experimental setup combined with deep learning based image analysis enabled us to reduce mice' s spontaneous locomotor activity and to track the precise dynamics of licking responses and pupil size of the behaving mice. By manipulating the predictability of the reward in the Pavlovian delay conditioning task, we demonstrated that the pupil size of mice is modulated by reward prediction, consumption of the reward, and body movements associated with reward processing. Additionally, we clarified that the pupil size is modulated by reward prediction even after the disruption of body movements by intraperitoneal injection of haloperidol, a dopamine D2 receptor antagonist.

In Experiment 1, we trained head-fixed mice on the Pavlovian delay conditioning task while recording licking and pupil responses. In this task, we designed contingent and non-contingent conditions to manipulate the predictability of the delivery of the sucrose solution by the auditory stimulus. In the contingent group, the auditory stimulus signaled the sucrose solution delivery. The mice showed increased licking responses and pupil size after the auditory stimulus presentation, suggesting that they could predict the outcome in this group. In the non-contingent group, the auditory stimulus did not signal the reward delivery. Licking responses and the pupil size of mice remained unchanged by the auditory stimulus presentation, suggesting that they did not associate the auditory stimulus to the reward in this group. In addition, the behavioral results obtained from the non-contingent group demonstrated that the sensory stimulus itself did not affect changes in licking responses and pupil size. The frequencies of the auditory stimulus presentation and reward delivery were identical between the contingent and non-contingent groups, with the only difference being the predictability

of the outcome following the auditory stimulus. This well-controlled rigid behavioral design allowed us to investigate modulation of behavioral states induced by reward prediction with the same sensory signals.

Detailed bout analysis of licking responses revealed that pupil size increased after the licking bout initiation in both the contingent and non-contingent groups, suggesting that licking responses modulate pupil size. Bout-aligned pupil size also showed a clear decrease before the increase in pupil size. Before the bout initiation, there was no licking response for approximately 0.5s (Fig 4, top panel). This result also confirms the close relationship between pupil size and licking responses. Many kinds of anticipatory behaviors occur when the stimulus signals a future outcome. Thus, whether changes in pupil size reflect signals related to reward prediction, or are simply modulated by the motor-related signals accompanied by the predictive movement, is unclear.

To examine whether the changes of the pupil size reflect the modulations by the prediction irrespective of motor related signals, we examined the effects of intraperitoneal injection of haloperidol, a dopamine D2 receptor antagonist, on the dynamics of the pupil size of mice performing the Pavlovian delay conditioning task in Experiment 2. Intraperitoneal injection of haloperidol suppressed licking responses in a dose-dependent manner, supporting previous findings (Fowler and Mortell, 1992; Liao and Ko, 1995). Although haloperidol administration decreased pupil size, the effect was not as drastic as that of licking responses (Fig 5). The highest dose of haloperidol injection almost completely disrupted licking responses; however, we still observed pupil dilation after the auditory stimulus presentation in the contingent group. This result implies that changes in pupil size reflect reward predictive signals irrespective of movement-related modulations.

The results demonstrated that changes in pupil size were not modulated by the reward prediction error. In the non-contingent group, the relationship between the auditory stimulus and reward was random. Licking responses

and pupil size were not modulated after the auditory stimulus presentation (Figures 3 and 5), suggesting that the mice could not predict the timing of the reward delivery in this group. Because the mice did not predict the timing of the reward, the reward delivery should be unpredictable to them. Existing literature on neurophysiological findings from primates and rodents consistently shows that neuronal activities of midbrain dopamine neurons are phasically increased by unpredictable rewards (Schultz et al., 1997; Hollerman and Schultz, 1998; Bayer and Glimcher, 2005; Cohen et al., 2012; Eshel et al., 2016; Satoh et al., 2003). Despite the unpredictable nature of the reward in the non-contingent group, we found no remarkable increase in pupil size after unpredictable reward delivery (Fig 3 A and B; Figure 5 A and C). This result shows that changes in the pupil size do not reflect a reward prediction error signal.

In this study, we explored the dynamics of pupil size of mice performing the Pavlovian delay conditioning task and found that pupil dynamics reflected reward prediction signals, irrespective of modulations by body movements. Pupil size is modulated by the autonomic nervous system activity. Sympathetic and parasympathetic activation lead to pupil expansion and contraction, respectively. The sympathetic control of the pupil is mediated by neuronal activity in the intermediolateral cell column (IML) of the cervical and thoracic regions of the spinal cord. Cholinergic neurons mediate the parasympathetic control in the Edinger-Westphal nucleus (EWN). Most neurons in the locus coeruleus (LC) are noradrenergic, and their direct projections to the IML stimulate sympathetic activation via noradrenergic $\alpha 1$ receptors. Most LC neurons are noradrenergic and stimulate sympathetic activation via $\alpha 1$ receptors by direct projection to the IML. Direct projections to the EWN are thought to suppress the parasympathetic nervous system by acting in an inhibitory manner via $\alpha 2$ receptors (Joshi and Gold, 2020). Simultaneous measurements of LC neuronal activity and pupil size in monkeys and rats have been reported to correlate (Joshi et al., 2016; Liu et al., 2017). Therefor

e, pupil size measurement can be interpreted as an indirect measure of LC a ctivities.

Considering the neurobiological circuit mechanisms underlying the pup illary control system, the present findings that changes in pupil size refl ect reward prediction signals and do not reflect reward prediction error si gnals. Our findings suggest the necessity of reconsidering the neuronal cir cuits computing reward prediction error signals. Cohen et al. (2012) report ed that neuronal activities of GABAergic neurons in the rodent' s ventral t egmental area (VTA) reflect the prediction of the upcoming reward values, a nd these activities are considered the source of the prediction for computi ng reward prediction errors encoded in dopamine neurons in the VTA. In this study, the authors recorded neuronal activities while the mice performed a Pavlovian trace conditioning task, in which each odor cue was associated wi th different upcoming outcomes, for example, small and large amounts of liq uid rewards and air puffs. GABAergic neurons in the VTA showed persistent r amping activity during the delay period between the presentation of cues an d reward. However, conditioned responses, such as licking reward spout, occ urred during the delay between the cue and the reward delivery. In such cas es, it is difficult to assess whether neuronal activity reflects the reward value or behavioral expression, for example, motor activity involved in lic king responses modulated by the reward value. In the present study, we att empted to overcome this problem by suppressing body movements with haloperi dol and found that the changes in pupil size reflected reward prediction si gnals independent of licking movements. The integrative approach of behavio ral analysis, image analysis, pupillometry, and pharmacological manipulatio ns employed in the present study will pave the way for understanding the ps ychological and neurobiological mechanisms involved in the computation of r eward prediction and reward prediction errors, which are essential features of learning and behavior.

We identified two limitations in this study: (1) the influence of body movements other than licking responses and (2) the pharmacological selectivity of haloperidol. In appetitive Pavlovian conditioning, the presentation of the cue that predicts the outcome leads to the observation of approach behavior to the cue or to the location where the reward is presented (Hearst and Jenkins, 1974; Boakes, 1977). Locomotor activity also occurs in mice under a head-fixed situation and has been reported to affect pupil size (Cazettes et al., 2021). Intraperitoneal injection of haloperidol has been known to dose-dependently decrease spontaneous activities, including locomotor activities. Therefore, in the open-field task, hypothesized that the effect of locomotion on pupil size would be low. However, we can not exclude this possibility because we could only measure licking responses and no other motor expressions in our head-fixed setup. Second, we used haloperidol to suppress mice' s body movements, but haloperidol' s non-selective nature might affect pupil size. Haloperidol is a non-selective dopamine D2 antagonist that binds to D2-like receptors, including D3 and D4 receptors, and others such as adrenergic $\alpha 1$ receptors. Adrenergic $\alpha 1$ receptors are involved in pupil dilation, and haloperidol has been reported to suppress pupil dilation produced by adrenaline administration in mice (Korczyn and Keren, 1980). Although haloperidol does not increase pupil size, we might obtain cleaner results if a more selective antagonist is used. In future investigations, the use of selective dopamine D2 antagonists, such as eticlopride, may refine our understanding of the neurobiological mechanisms underlying the relationship between the pupil size and reward prediction.

To verify that organisms predict future outcomes, behavioral evidence of preparatory or anticipatory responses is needed. Commonly, anticipatory responses are accompanied by motor expressions; thus, it is difficult to discern whether the physiological changes related to reward prediction encode the signal of the prediction itself or are simply modulated by motor-related signals. Here, we successfully measured changes in pupil size in mice per

forming the Pavlovian delay conditioning task in the head-fixed situation using image processing. We revealed that dynamic changes in pupil size reflect reward predictive signals. Pharmacological intervention experiments using haloperidol demonstrated that pupil size increased even when licking responses were suppressed, supporting that the changes in the pupil size reflect reward prediction. Considering the brain circuits involved in controlling pupil size, the predictive feature of pupil size suggests that reward prediction is encoded in regions other than those reported by Cohen et al. (2012) and Tian et al (2016). These results pave the way for our understanding of reward prediction signals in the brain by neutralizing the factor of motor expression and suggest a different hypothesis for the neuronal circuits of predictive learning. Future studies are expected to identify the neuronal circuit that computes the reward prediction and reward prediction error by eliminating the modulation of motor expressions.

Data Availability Statement

Data supporting the findings of this study are available from the correspon ding author upon reasonable request. The original codes written for the ana lysis are available from the corresponding author upon reasonable request.

Ethics Statement

The experimental and housing protocols adhered to the Japanese National Reg ulations for Animal Welfare and were approved by the Animal Care and Use Co mmittee of the Keio University.

Author Contributions

YK and KT designed the experiments. YK conducted stereotaxic surgery and ph armacological manipulations for mice and collected all the data from the he ad-fixed Pavlovian conditioning experiment with the help of KT. YK and KT a nalyzed the data and created all figures. YK and KT wrote the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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