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Innovative Methodology

Reinforcing operandum: rapid and reliable learning of skilled forelimb movements by head-fixed rodents

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Running head: A novel operant task system under head-fixation

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Abstract

Stereotaxic head-fixation plays a necessary role in current physiological techniques, such as *in vivo* whole-cell recording and two-photon laser-scanning microscopy, that are designed to elucidate the cortical involvement in animal behaviors. In rodents, however, head-fixation often inhibits their learning and performance of behavioral tasks. In particular, it has been considered inappropriate for head-fixed rodents to be operantly conditioned to perform skilled movements with their forelimb (e.g., lever-press task), despite the potential applicability of the task. Here, we have solved this problem conceptually by integrating a lever (operandum) and a rewarding spout (reinforcer) into one ″spout-lever″ device for efficient operant learning. With this device, head-fixed rats reliably learned to perform a pull manipulation of the spout-lever with their right forelimb in response to an auditory cue signal (external-trigger trial, namely Go trial) within several days. We also demonstrated stable whole-cell recordings from motor cortex neurons while the rats were performing forelimb movements in external-trigger trials. We observed a behavior-related increase in the number of action potentials in membrane potential. In the next session, the rats, who had already learned the external-trigger trial, effortlessly performed similar spout-lever manipulation with no cue presentation (internal-trigger trial) additionally. Likewise, some of the rats learned to keep holding the spout-lever in response to another cue signal (No-go trial) in the following session, so that they mastered the Go/No-go discrimination task in one extra day. Our results

52 **verified the usefulness of spout-lever manipulation for behavioral experiments**
53 **employing cutting-edge physiological techniques.**
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56 **Introduction**
57 In our daily life, we perform so-called *skilled movements*, e.g., reaching, grasping, pushing
58 and pulling, with our digits, hand, arm, and shoulder (Iwaniuk and Whishaw 2000). Skilled
59 movements have been studied intensively in behaving monkeys who are operantly
60 conditioned to repeat specific forelimb movements under head-fixed conditions for
61 electrophysiology experiments (Evarts 1968; Evarts and Tanji 1974; Fetz and Cheney 1980;
62 Georgopoulos et al. 1982). As skilled forelimb movements are applicable to a wide variety
63 of behavioral tasks, research using head-fixed monkeys performing operant forelimb
64 movements have enhanced the knowledge of neural mechanisms underlying not only
65 skilled movements (Kalaska and Crammond 1995; Mushiake et al. 1991; Okano and Tanji
66 1987) but also higher-order cognitive/motor functions (Isomura et al. 2003; Shima and
67 Tanji 1998; Watanabe 1986a,b).
68 Compared with primates, rodents in head-fixed conditions are more amenable to
69 state-of-the-art physiological techniques such as intracellular/whole-cell recordings (Brecht
70 et al. 2004; Crochet and Petersen 2006; Fee 2000; Harvey et al. 2009), juxtacellular
71 recording (de Kock and Sakmann 2009; Houweling and Brecht 2008; Isomura et al. 2009),
72 multi-photon laser-scanning microscopy (Dombeck et al. 2007; Komiyama et al. 2010), and
73 optogenetic manipulation (Hira et al. 2009; Matyas et al. 2010). Indeed, there are an
74 increasing number of studies employing rats or mice under head-fixation, many of which

introduce licking with the tongue (Gerdjikov et al. 2010; Houweling and Brecht 2008; Komiyama et al. 2010; Narumi et al. 2007; Ono et al. 1985; Stüttgen et al. 2006; Stüttgen and Schwarz 2008; Welsh et al. 1995) or whisking (Bermejo et al. 1996, 2004; Hentschke et al. 2006; O'Connor et al. 2010a,b) as motor responses in conditional behavior tasks. However, licking and whisking movements are different from skilled movements in that they are characterized by automatic repetition of stereotyped motion driven by central pattern generator(s) in the brainstem (Gao et al. 2003; Travers et al. 1997).

Because rodents have a natural ability to perform skilled movements with their forelimbs (Iwaniuk and Whishaw 2000; Whishaw 2005; Whishaw and Pellis 1990), forelimb movements may be optimal for applying to various behavioral tasks, combined with the advanced physiological techniques, under head-fixation. Despite the potential for wide-ranging applications, surprisingly few research groups have reported operant learning of a skilled forelimb response (reaching or lever-pressing) in rodents under head-fixed conditions (Harvey et al. 2001; Heck et al. 2007; Isomura et al. 2009; for review, Schwarz et al. 2010). The development of an innovative behavioral task system in which head-fixed rodents can learn more skillful forelimb movements rapidly and reliably is long overdue.

Our methodological solution to this problem was to integrate a lever (*operandum*) and a rewarding spout (*reinforcer*) into one ″spout-lever″ device. This device dramatically improved the efficiency in learning and performance of a skilled forelimb movement by naive rats under head-fixation. Subsequently, these animals quickly learned to perform a discriminative forelimb movement task (external-/internal-trigger task or Go/No-go discrimination task). The practical usefulness of this methodology for cutting-edge measurement techniques is evident in our stable whole-cell recordings from adult rats

conducting the skilled movement.

Materials and Methods

Animal preparation

All experiments were carried out in accordance with the animal experiment protocol approved by Tamagawa University Animal Care and Use Committee. Adult male Long-Evans rats (150–250 g; Institute for Animal Reproduction) were kept under an inverted light schedule (lights off at 9 a.m., and lights on at 9 p.m.). The rats were adapted to a stainless steel cylinder (hideaway) in their home cage and were briefly handled by an experimenter (10–15 min, two times). Under 2.0–2.5% isoflurane anesthesia (Univentor 400 anesthesia unit, Univentor), the rats had a sliding, lightweight, aluminum head-attachment (custom-made by Narishige, Japan; cf., Isomura et al. 2009) surgically attached to the skull with tiny anchor screws (stainless steel, M1, 2 mm long) and dental resin cements (Super-Bond C & B, Sun Medical, Japan; Panavia F2.0, Kuraray Medical, Japan; Unifast II, GC Corporation, Japan). In the present study, there were virtually no rats that lost the head-attachment by the end of behavioral experiments (9-13 days later). During the isoflurane anesthesia, body temperature was maintained at 37°C by an animal warmer (BWT-100, Bio Research Center, Japan). In some experiments, twisted Teflon-coated silver wire electrodes (A-M systems; 180 μm in diameter each) were implanted into the right upper forelimb (near the biceps brachii) to measure its electromyogram (EMG) activity during task performance. For whole-cell recording, two silver wire electrodes were implanted above the cerebellum as a reference and a ground. After recovery from the

surgery (2–3 days later), the rats were deprived of drinking water in the home cage, though food was available *ad libitum*. A sufficient amount of water was provided as a reward for their daily task performance in the laboratory. An agar block (containing 15 ml water) was given to the rats, when necessary, to maintain over 80% of their body weight. The surgical procedure was omitted for a preliminary experiment using intact (freely-moving) animals.

Behavioral tasks

In a preliminary experiment, intact rats were put into a Skinner box (ENV-007 modular test chamber, MED Associates, VT; 30.0 cm x 24.5 cm) with a standard lever and spout (separated by 5.5 cm) or with a spout-lever (see Fig. 1 for details). If the freely-moving rats touched the standard lever or spout-lever, they were allowed to drink a drop of 0.1% saccharin water (5 μl) from the spout or spout-lever as a reward. The rats learned to touch the standard lever or spout-lever repeatedly to acquire the reward in an operant conditioning manner. We then evaluated their learning efficiency by counting the lever-touches for 60 min on two consecutive days. The freely-moving rats were not used for further experiments.

In the main behavioral experiments using head-fixed rats, we first trained the rats to perform an external-trigger (i.e., Go) task by manipulating the spout-lever with their right forelimb (see Figs. 2 and 3). They learned the external-trigger task under head-fixation over three consecutive days (2–5 hours a day) in our automatic multi-rat task-training system (custom-made by O'hara & Co., Ltd., Japan; www.ohara-time.co.jp, now available from there). The rats were able to start each trial spontaneously by pushing the spout-lever and holding it for a short period (″hold period″) with the right forelimb. After the hold

period was completed, a pure-tone cue sound was briefly presented to them (usually 8 kHz tone for 0.5 s; 10 kHz for 0.3 s for Go/No-go discrimination). If the rat pulled the spout-lever toward his mouth (holding position, 0–3 mm; licking position, 6–9 mm from the front end) in response to the cue presentation, then he was allowed to lick the spout-lever to drink saccharin water (5 or 10 μl) as a reward. The reward was accurately dispensed from the tip of spout-lever by a micropump with a 0.2–0.8 s delay (after its entry to the licking position; 0.1 s steps at random). The reward delivery period was followed by a short inter-trial interval (0.2–0.8 s). Unless they held the spout-lever throughout the hold period, or unless they pulled it correctly earlier than 5.5 s (or 0.8–1.3 s for Go/No-go discrimination) after the cue onset, the rats were not rewarded (error trial) and had another attempt after the inter-trial interval (0.2–0.8 s). The task-training system automatically extended the hold period from 0 s up to 1 s (final) in a step-by-step manner according to the total number of success trials.

Once the rats completed the operant learning of external-trigger (Go) task in the task-training system, two days later, some of them were transferred to a similar system for a single rat (O'hara & Co., Ltd.) for a final behavioral experiment (either external-/internal-trigger task or Go/No-go discrimination task). The external-/internal-trigger task consisted of external-trigger trials and internal-trigger trials presented pseudo-randomly in a 1:1 ratio (see Fig. 7*A*). The internal-trigger trials were similar to the final form of the external-trigger trials (described above), except there was no cue presentation. The Go/No-go discrimination task consisted of Go trials and No-go trials presented pseudo-randomly in a 1:1 ratio (see Fig. 8*A*). In the Go trials, the rats had to quickly pull the spout-lever less than 0.5 s after the onset of the Go cue (10 kHz for 0.3 s)

to acquire the reward. In the No-go trials, they had to keep holding the spout-lever for at least 0.8 s after the onset of the No-go cue (4 kHz for 0.3 s). The reward (5 μl) was delivered 0.2–0.8 s after a correct response for both the Go and No-go trials symmetrically. If they failed to respond correctly to the original (test) trial, they had to retry the same trial type after the inter-trial interval (correction trials) until they successfully cleared it. We performed these final behavioral experiments only once for each rat.

Physiological recordings

The position of the spout-lever was continuously tracked by an angle encoder throughout the behavioral experiments. To detect licking activity, an electropotential difference between the spout-lever and the head of the rat was measured by a general-purpose amplifier (EX4-400, Dagan; gain, 1 or 2; band-pass filter, 1 Hz to 10 kHz; Hayer et al. 2006). The shaft of spout-lever, which was ungrounded electrically, was insulated with silicone rubber tubing to avoid an electrical connection with the forelimb. We did not attempt to combine the detection of licking activity with other electrophysiological recordings. The tongue as well as forelimb movements were monitored with an infrared video camera. The EMG activity for the movement of the right forelimb was obtained by a multi-channel amplifier (MEG-6116, Nihon Kohden, Japan; final gain 2,000, band-pass filter, 0.3–5.0 Hz to 10.0 kHz).

We demonstrated *in vivo* whole-cell patch-clamp recordings (Margrie et al. 2002) from motor cortex neurons while the rats were performing the external-trigger task under head-fixation. After they finished the three-day task training, the rats were subjected to a second surgery under isoflurane anesthesia to open a tiny hole in the skull and dura matter

above the left forelimb (FL) area of the motor cortex (1.0 ± 0.5 mm anterior, 2.5 ± 0.5 mm lateral from bregma; Isomura et al. 2009). The hole was covered with 1.5–2.0% agarose (Agarose-HGT, Nacalai Tesque, Japan). The rats recovered from the anesthesia completely in several hours. Glass electrodes (GC150F-7.5, Harvard Apparatus) were prepared by a puller (PC-10, Narishige), and filled with an internal solution containing (in mM): 140 K-gluconate, 2 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 5'-ATP Na_2, and 0.5 GTP Na_2 (pH 7.4, 5–10 MΩ; F.-Tsukamoto et al. 2010). In an awake state, the electrode was inserted near vertically into the FL area with a water hydraulic microdrive manipulator (MWS-1B, Narishige) on a stereotaxic frame (SR-8N; Narishige) to blindly search for target neurons. The membrane potential was measured in I-clamp mode with a patch-clamp amplifier (Axopatch 1D, Axon Instruments, in combination with EX-1, Dagan; final gain, 25; high-cut filter, 5 kHz).

Data analysis

Data for task events were compiled from a trial log file that was produced automatically by the task-training system. All trace data (including task event signals, lever, licking, EMG, and membrane potential) were digitized at 20 kHz and recorded in a hard-disc recorder (LX-120, TEAC; or DataMax II, R.C. Electronics). These experimental data were further analyzed by MATLAB (MathWorks). The results are expressed as the mean ± standard deviation (s.d.), and when applicable, with statistical significance.

Results

213 **The concept of spout-lever manipulation**

214 Figure 1*A* shows the conceptual difference in our ″spout-lever″ device in contrast to the
215 standard-type lever and spout that are typically used in behavioral experiments. In
216 traditional operant (instrumental) conditioning, a subject learns to operate an instrument
217 (operandum; e.g., lever) appropriately in order to acquire a reward (reinforcer; food or
218 water); however, the reward delivered from a separate place. In other words, the subject
219 needs to associate the operandum with the reinforcer after a long period of trial and error. If
220 the reward is delivered directly from the instrument itself, then it may facilitate or even
221 eliminate the difficulty in the association process. A logical step would be to integrate the
222 spout and lever, so that rats only have to take the spout-lever with their forelimb and lick it,
223 a natural and skilled movement, to receive the reward water. In a preliminary experiment
224 using an operant chamber, we evaluated the effect of the spout-lever on operant learning of
225 a simple lever-touch task by freely-moving rats (Fig. 1*B*). As expected, the rats using
226 spout-lever learned to touch it to receive the reward much faster than those using standard
227 lever and nearby spout [standard lever, 17.0 ± 11.3 (1st day) and 38.8 ± 80.6 (2nd day)
228 touches, $N = 5$ rats; spout-lever, 643 ± 220 (1st day) and 1,865 ± 231 (2nd day) touches, N
229 $= 5$; *t*-test, $p < 0.004$ (1st day) and $p < 0.0001$ (2nd day)].

230 We then used the spout-lever device in a series of behavioral experiments with
231 another group of rats in a head-fixed condition (Fig. 2*A*). The spout-lever device (Fig. 2*B*)
232 was combined with our stereotaxic head-fixation device (Isomura et al. 2009), in which a
233 head-attachment (Fig. 2*C*) was slid smoothly forwards into a head-attachment clamp (Fig.
234 2*D*) in order to lessen the fear or stress under head-fixation conditions. A body restrainer
235 and an armrest (Fig. 2*E*) were also provided to relax the rats during head-fixation. The

spout-lever device consisted of a spout-lever, an angle encoder, stoppers, repulsive magnet pairs, and silicone rubber tubing from a micropump (Fig. 2*B* and *E*). The spout-lever (gastric tube #6206, Fuchigami, japan; stainless steel, 1.8 mm in outside diameter, 95 mm in length, 85 mm long from the pivot of angle encoder) could be moved horizontally in both forward (push) and backward (pull) directions (15 mm wide; roughly 10^{o}). Its position was always monitored by the angle encoder (MES-12-1000PC, Microtech Laboratory, Japan) attached with the pivot of spout-lever. The stiffness and neutral position of the spout-lever were easily adjustable (0.02 to 0.2 N, usually 0.1 N) by moving the repulsive magnet pairs. The spout-lever was covered with silicone rubber tubing (4 mm in diameter) for good grip and electrical insulation in most experiments. Prior to the beginning of the behavioral task, the tip of spout-lever was brought closer to the mouth of the rat by a three-dimensional manipulator, which held the whole spout-lever device. The adjustment of spout-lever position (less than 5 mm) was required for individual rats in each experimental session. A tiny volume of reward water (5 μl/shot) was accurately dispensed from the tip of the spout-lever each time it was manipulated correctly.

Rapid operant learning of basic spout-lever manipulation

Initially, we examined how efficiently adult rats could learn a simple spout-lever manipulation in a head-fixed condition. Rats were exposed to brief handling by the experimenter (Fig. 3*A*), and the head-attachment was surgically attached to the skull in a stereotaxic manner under isoflurane anesthesia. After recovery from the surgery (Fig. 3*B*), the rats were deprived of drinking water in their home cages, and then they were subjected to automatic task training under head-fixation in our multi-rat task training system (Fig. 3*C*).

This task training system, controlled by a computer program (Fig. 3*D*), trains several head-fixed rats simultaneously and independently to learn spout-lever manipulations in a step-by-step manner automatically as described below. The rats had never experienced pre-training habituation to the experimental devices until this task training system. Furthermore, they were free of sedation or any other medication throughout behavioral experiments.

Initially, the head-fixed rats worked on only an external-trigger (Go) task in the task training system (Fig. 3*E*). In this external-trigger task, a head-fixed rat was able to start each trial by spontaneously pushing the spout-lever with the right forelimb after an inter-trial interval. After the lever was held for a constant hold period, a pure-tone sound was presented briefly (Go cue signal). If the rat pulled the spout-lever toward his mouth immediately after the cue onset, then he could lick a drop of saccharin water on the tip of spout-lever as a reward. In short, the rat was simply required to take the spout-lever for licking the reward in response to the Go signal. It is the very skilled forelimb movement consisting of a sequence of grasping, pushing, holding, and pulling motions. Particularly, this procedure required a distinct holding motion unlike simple lever-press or reaching tasks. To make the task training more efficient, the hold period (until the cue onset) was automatically extended step-by-step according to a set schedule over three consecutive days (Fig. 3*F*). We made out the step-up schedule for refresher training on all the three days.

From the beginning of task training, the rats did not struggle, but rather quickly became accustomed to our head-fixation device; they often sniffed, whisked, or licked the spout-lever curiously, and even tried to push or pull it with the right forelimb (Fig. 3*A*). The rats moved the spout-lever awkwardly at first, but they became skillful in manipulating it

(i.e., push, hold, pull, and lick it sequentially) by the third training day (Fig. 4*A* and *B*). As the rats intended to lick the spout-lever, they rarely let go of it uselessly; a lever release resulted in distinctive damped oscillation in lever trajectory (Fig. 4*B*, inset). The distribution of actual hold time for individual trials reveals that the spout-lever tended to be pulled in response to the cue onset even on the first training day (indicated by an asterisk), and this tendency was becoming more pronounced over the next two days (Fig. 4*C*). Total 25 rats were tested for the three-day task training procedure, after we excluded one rat that died during the skull surgery and one that moved the forelimb restrictedly because of badly implanted EMG electrode. All the 25 rats successfully learned to consistently perform the external-trigger task according to schedule over the three days of testing [Fig. 4*D*; 571 ± 150 (1st day), 751 ± 214 (2nd day), and 814 ± 202 (3rd day; 487 to 1,136) correct trials for 120 min, $N = 25$]; thus, there were no dropouts in this behavioral task (see individual gray lines, Fig. 4*D*). We observed a single peak of pull onset just after the cue onset in the histogram of hold time distribution on the last day (Fig. 4*E*; 172 ± 63 ms from cue onset, $N = 25$). If the reward delivery was removed, the rats soon stopped the task performance, suggesting that their behavior was based on an operant learning process and was neither reflex nor habitual action (data not shown). Thus, we found that the head-fixed rats learned the external-trigger (Go) manipulation of spout-lever much more rapidly (1–3 days) and reliably (100%), compared with previous studies using standard levers (learning took at least several weeks to months, e.g., Isomura et al. 2009; see also Schwarz et al. 2010 for review).

We also analyzed licking behaviors for reward acquisition during the spout-lever manipulation ($N = 3$ rats). Each lick response was detected by measuring the

electropotential difference between the spout-lever and the rat (Fig. 5*A*). Licking usually started, regardless of reward delivery, just after the completion of pull movements (Fig. 5*B*). The licking responses were repeated at 6–7 Hz very regularly, and they lasted several seconds once the reward was delivered from the spout-lever in correct trials. The number of licking responses was significantly larger in correct (rewarded) trials than that in error (not rewarded) trials (Fig. 5*C*; correct, 19.4 ± 5.7 licks/trial; error, 4.6 ± 3.3 licks/trial; *t*-test, $p < 0.0001$). It seems unlikely that a longer hold time increased the number of licking responses (Fig. 5*C*). Our spout-lever task system may be uniquely useful in examining the spatial coordination between the forelimb(s) and tongue during eating or drinking.

Stable whole-cell recording during spout-lever manipulation

Next, we tested whether our behavioral task was applicable to *in vivo* whole-cell recording, a delicate but beneficial physiological technique. This technique requires 1) the efficient preparation of many animals repeating specific operantly conditioned behaviors, 2) no or little interruption of their performance by extraneous light and sounds in our electrode exchanges, 3) stable giga-seal formation against mechanical vibrations by their pulsation, respiration, and body movements, and 4) no contamination of electrical noises by the licking or touching the spout-lever device. To our knowledge, there are no studies on whole-cell recording experiments that meet these requirements. Furthermore, it is also difficult to obtain stable whole-cell recording from mature neurons in adult rats.

The experiment consisted of the following. A glass patch-clamp electrode was inserted into the forelimb (FL) area of the left primary motor cortex (Isomura et al. 2009) in head-fixed rats that had been trained to perform the external-trigger task (Fig. 6*A*; $N = 10$

328 rats, P50–57). Sufficiently stable whole-cell recordings (>5 min) were obtained from five
329 neurons in the deep layer of the FL area (Fig. 6*B*; total recording time, 40.8 ± 58.3 min;
330 stable whole-cell time, 19.6 ± 18.0 min; firing rate, 10.6 ± 12.6 Hz; resting membrane
331 potential, –64.0 ± 13.3 mV; $n = 5$). In our experiment, we did not use temporary anesthesia
332 with rapidly antagonizable anesthetics and antagonists to establish the whole-cell
333 configuration (e.g., Brecht et al. 2004). All of the recorded neurons included behaving time
334 in which the rats were repeating the spout-lever manipulation (9.2 ± 5.1 min; total 67.8 ±
335 63.6 pulls, 50.2 ± 71.7 correct trials). After the stable whole-cell time, three neurons were
336 lost abruptly, whereas the others died gradually. A representative neuron (#1) displayed an
337 increased occurrence of action potentials in correlation with the pull-to-push forelimb
338 movements (Fig. 6*C* and *D*; Mann-Whitney test, $p < 0.001$). Each licking response did not
339 seem to be directly related to the membrane potential (Fig. 6*C*, inset). Two of the other
340 neurons (#3, 4) also showed behavior-related spiking activity in their membrane potentials
341 (Fig. 6*E*; $p < 0.05$ and $p < 0.001$, respectively). Electrical artifacts from licking and
342 touching were negligible in all cases. Unlike anesthetized or deeply sleeping animals
343 (Isomura et al. 2006), we never observed clear up-down fluctuations in the membrane
344 potential during forelimb movements in any recorded neuron (data not shown). These trial
345 experiments of whole-cell recordings demonstrated the applicability of the spout-lever task
346 system to advanced physiological techniques.
347
348 **Application 1. Additional performance of external- and internal-trigger task**
349 Voluntary movement can be initiated by a sensory signal from the outside (external trigger)
350 or can spontaneously occur without it (internal trigger). An electrophysiological study using

monkeys under head-fixation has shown that the primate premotor area (PMA) and supplementary motor area (SMA) participate in the external and internal initiation of skilled forelimb movement, respectively (Okano and Tanji 1987). In contrast, it is unknown whether the rodent secondary motor cortex (putative PMA/SMA counterpart) plays a differential role in the external and internal initiation of voluntary movements, likely because there has been no useful behavioral task available for rats or mice under head-fixation. However, since we have shown that our head-fixed rats have efficiently learned to perform forelimb movements in response to an external Go signal, it is possible that they may also perform this movement at their own timing (i.e., without the Go signal).

For the external-/internal-trigger task (Fig. 7*A*), we used head-fixed rats who had already learned the external-trigger trials in the three training days (see Figs. 3–5). For this task, the rats must hold the spout-lever for at least for one second, and then pull it in response to the Go signal (external-trigger trials) or spontaneously without any cue presentation (internal-trigger trials) to acquire the reward. Indeed, we found that head-fixed rats were capable of performing both types of forelimb movement trials that appeared in random sequence (Fig. 7*B* and Supplemental Video S1). The electromyogram (EMG) activity for the right forelimb was increased during the pull-to-push movements in both the external- and internal-trigger trials (Fig. 7*C*). Unexpectedly, the rats effortlessly cleared the internal-trigger trials as well as the external-trigger trials from the beginning of the new experimental session (Fig. 7*D* and *E*); furthermore, they maintained the behavioral performance for several hours (Fig. 7*E*; 1,728 ± 194 correct trials for 180 min, $N = 5$). The histogram of hold time distribution showed that the peak latency of pull onset was not significantly different between the correct external- and internal-trigger trials [Fig. 7*F*;

374 1,148 ± 23 ms (external) and 1,068 ± 118 ms (internal) from the onset of hold period, N =
375 5; paired t-test, p = 0.18], while the standard deviation was much larger in the
376 internal-trigger trials than in the external-trigger trials (F-test, $p < 0.004$). The rats appeared
377 to judge the trial type instantaneously around the expected cue period.
378
379 **Application 2. Extra-one-day learning of Go and No-go discrimination task**
380 A behavioral task with a Go/No-go paradigm often provides valuable information on brain
381 functionality of not only the intentional suppression of voluntary movements but also
382 sensory discrimination, decision-making, motivation, and impulsivity. For instance, many
383 studies using monkeys under head-fixation have shown that several motor cortical areas
384 (e.g., PMA and SMA) differentially contribute to the Go and No-go performance of skilled
385 forelimb movements (Isomura et al. 2003; Kalaska and Crammond 1995; Kurata and Tanji
386 1985; Tanji and Kurata 1985; Watanabe 1986a,b). It has recently been demonstrated that
387 rodents under head-fixation can also perform Go/No-go discrimination tasks, if they are
388 allowed to respond by licking with the tongue (Gerdjikov et al. 2010; Komiyama et al.
389 2010; Narumi et al. 2007; O'Connor et al. 2010a,b; Stüttgen and Schwarz 2010). However,
390 it is still extremely difficult to require that head-fixed rodents learn to make Go/No-go
391 responses by skilled forelimb movements (cf., Harvey et al. 2001 as a rare success, though
392 requiring several weeks for learning). Thus, we used head-fixed rats that had learned the
393 external-trigger (Go) forelimb response to examine how fast they could learn the
394 corresponding No-go forelimb response.
395 In the Go/No-go discrimination task (Fig. 8*A*), the Go trials were similar to the
396 external-trigger trials that the trainee rats had learned very efficiently, as described above

(Figs. 3–5). The No-go trials required the rats to keep holding the spout-lever for at least 0.8 seconds after presentation of the lower-tone cue sound. The same amount of reward was delivered to them each time in the successful Go and No-go trials (symmetrical reinforcement), and no punishment was given for their failure. Thus, we would be able to compare the Go and No-go behaviors in the same operant condition. The Go and No-go trials appeared randomly (test trials), but if the rats failed to discriminate the trial type, then they had to retry the same trial type until they succeeded (correction trials).

In a representative Go/No-go task performance, a head-fixed rat pulled and licked the spout-lever immediately after the Go cue presentation in the Go trials and at the sound of reward-pumping in the No-go trials (Fig. 8*B* and Supplemental Video S2). We observed that the muscles of the right forelimb were not activated excessively during the extended hold period after the No-go cue presentation, though they were activated largely over all pull-to-push movements regardless of the Go or No-go trial types (Fig. 8*C*). Surprisingly, about half of the rats examined here (4 of 7 rats) successfully learned to perform the No-go trials in addition to the Go trials on the very first day of No-go learning. These four rats appeared to learn how to make a No-go response correctly after they repeated the correction trials for an error response (Fig. 8*D*), and they performed the Go/No-go discrimination task in one to six hours on the test session [Fig. 8*E*; 1,415 ± 468 correct (test and correction) trials for 180 min]. The latency of the pull response after the cue onset was 170 ± 22 ms in the Go trials, whereas the latency ranged from 810 to 1,960 ms, owing to the step-like delays of reward delivery, in the No-go trials (Fig. 8*F*). The rats maintained the correct rate for Go trials above chance level (50%) from the beginning of the test session [Fig. 8*G* and *H*; first 100 correct trials (test trials only), 76.7 ± 14.8%, $N = 4$, $p < 0.04$ (*t*-test); last 100

trials, 75.7 ± 4.6%, $p < 0.002$]. In contrast, the correct rate for No-go trials, at first around the chance level, was increased to a significantly selective level throughout the test session (Fig. 8*G* and *H*; first, 59.7 ± 9.1%, $p > 0.1$; last, 83.5 ± 13.3%, $p < 0.02$). We did not attempt to train the remaining three rats, because the one-day learning of No-go trials by a half of rats was sufficient for our future physiological experiments.

Discussion

In the present study, we have established a novel operant behavioral task that requires rodents to learn spout-lever manipulations with their forelimbs in a head-fixed condition. In light of our behavioral and electrophysiological observations, this technique is clearly advantageous to state-of-the-art physiological experiments used to elucidate a variety of brain functions.

Head-fixed vs. freely-moving rodents

First of all, we would like to summarize the advantages and disadvantages of physiological experiments using rodents in head-fixed and freely-moving conditions. Because laboratory rodents are genetically less variable, we can expect high reproducibility of behavioral experiments using laboratory rats and mice compared to other experimental animals. There is also an enormous amount of knowledge in the anatomy, physiology, pharmacology, psychology, and molecular and cellular biology of the rodent central nervous system. Furthermore, rodents have simple cortical structure, i.e., compact cortical areas with thin layers and no sulci, which is a prerequisite for two-photon microscopic calcium imaging

443 and optical voltage-sensitive-dye imaging. They are undoubtedly advantageous to various
444 genetic modifications including gene-transgenic and knock-in/out manipulations,
445 fluorescent labeling and calcium indicator proteins, and recent optogenetics with
446 channelrhodopsin-2. Nowadays, rodents have become the model mammal in the spotlight
447 of experimental neuroscience.

448 Stereotaxic head-fixation in itself is convenient for current physiological
449 techniques needing behaving rodents, because 1) large physiological devices such as a
450 laser-scanning microscope can be mounted on their head firmly; 2) electrodes or optical
451 fibers can be positioned into a target brain area precisely and stably; 3) sensory (visual,
452 tactile, or auditory) stimuli can be presented to the subject in a spatially different manner;
453 and 4) voluntary movements (forelimbs, tongue, whiskers, or eyes) can be monitored
454 wholly with a high-speed camera (e.g., Genet et al. 2010 for 1, 2, and 4). In addition,
455 operant learning can be enhanced if the subject is properly positioned close to an
456 operandum and a reinforcer, as shown here.

457 On the other hand, many behavioral experiments have been conducted with
458 rodents in freely-moving conditions. Standard psychological tests are directly available for
459 freely-moving rodents (e.g., T-maze learning) with little pain and stress; furthermore, it is
460 possible to measure neural activity continuously through their wake-sleep cycles up to
461 several days. Social interactions can also be easily observed in such an unrestricted
462 situation. Thus, freely-moving conditions are more desirable than head-fixed ones for
463 physiological measurements during various natural behaviors. However, there are still
464 severe technical limitations for several physiological techniques such as two-photon
465 laser-scanning microscopy and whole-cell recordings, although these techniques have been

improved partly (see Helmchen et al. 2001; Lee et al. 2006, but after anesthesia antagonization). In any case, the spout-lever manipulation is a powerful behavioral tool that is effective for head-fixed as well as freely-moving rodents (see Figs. 1 and 4).

Forelimb movements vs. other motor responses

In general, operant (instrumental) conditioning requires a specific motor response of manipulating an operandum (instrument) appropriately. Licking and whisking have often been utilized as the operant motor response in head-fixed rodents (licking: Gerdjikov et al. 2010; Houweling and Brecht 2008; Komiyama et al. 2010; Narumi et al. 2007; Ono et al. 1985; Stüttgen et al. 2006; Stüttgen and Schwarz 2008; Welsh et al. 1995; whisking: Bermejo et al. 1996, 2004; Hentschke et al. 2006; O'Connor et al. 2010a,b). Licking and whisking responses are so natural that the rodents are able to learn the operant behaviors efficiently, and thus, they are useful and effective for some types of behavioral experiments. For example, it takes only one to several days (excluding a habituation period) for head-fixed mice to learn a Go/No-go discrimination task with licking response (Komiyama et al. 2010; O'Connor et al. 2010a). However, these responses are not composed of discrete skilled movements, since central pattern generator(s) in the brainstem automatically drive a rhythmic train of uniform tongue or whisker motions (Gao et al. 2003; Travers et al. 1997). In addition, these motor responses may not be (strictly) considered the operant manipulation of a real (observable) instrument. Locomotion using the four limbs on a treadmill is also available for head-fixed rodents (Dombeck et al. 2007, 2009; Fee 2000; Harvey et al. 2009). Yet locomotion is basically achieved by a pattern generator in the spinal cord, and it is not a skilled movement (Hermer-Vazquez et al. 2004).

In rodents as well as primates, movement to manipulate a lever with the forelimb is regarded as a skilled movement, which is the spatio-temporal coordination of reaching, grasping, pushing, holding, and pulling components (Iwaniuk and Whishaw 2000; Whishaw 2005; Whishaw and Pellis 1990). Given that a skilled movement is acquired by adjusting to an objective action, skilled forelimb movement will be easily adjustable to a desired form of motor response in different operant behavioral tasks. Unlike licking and whisking, skilled forelimb movements in rodents are in fact comparable to those in primates (Iwaniuk and Whishaw 2000). Thus rodents would be an appropriate behavioral model for the pathophysiology of motor disturbances (as a result of cerebral infarction, tumor, Parkinson syndrome, and spinal cord injury, etc.) and for the development of brain-machine-interface (Chapin et al. 1999). Also, it is known that information on skilled forelimb movements is encoded preferentially by excitatory and inhibitory neurons in the primary motor cortex (Hermer-Vazquez et al. 2004; Isomura et al. 2009). The advanced physiological techniques to analyze signal interactions among cortical neurons during skilled movements will help elucidate the cortical circuit mechanism of motor behaviors in rodents. Taken together, skilled forelimb movements have great potential for a wide range of behavioral studies in head-fixed rodents.

Spout-lever vs. standard lever manipulations

In contrast to the plenitude of research using Skinner boxes, which allow intact rodents to operantly learn a lever-press response, there have been very few publications on operant learning of similar motor responses by head-fixed rodents (Fontanini et al. 2009; Harvey et al. 2001; Heck et al. 2007; Isomura et al. 2009; see Schwarz et al. 2010 for review). In the

512 rare success cases, it still took head-fixed rodents over weeks or months to learn a forelimb
513 response using a standard lever. On the other hand, it has been known that freely-moving
514 rodents can learn an operant lever-press task effectively by placing cue signal or operandum
515 spatially close to reinforcer (e.g., Buzsáki et al. 1979). The head-fixation may make it
516 difficult to visually recognize spatially separated operandum and reinforcer. The spout-lever
517 that we developed here, an integrated form of operandum and reinforcer, has made it
518 possible to learn a forelimb response in a head-fixed condition rapidly and reliably. It may
519 be accomplished by immediate feedback from the tongue that felt the pull action directly.
520 The remarkable enhancement of learning efficiency will be advantageous to physiological
521 experiments over the standard lever. Using the spout-lever, we can quickly prepare many
522 task-performing animals necessary for whole-cell or juxtacellular recording experiments
523 (see Fig. 6), which must be finished in several days once the dura matter is opened to insert
524 a glass electrode. This allows us to obtain physiological data from undamaged neural tissue
525 in a good physical condition before necrosis of the skull or a hypertrophy of the dura mater,
526 on account of the skull surgery. More importantly, one-day learning of an operant response
527 (see Figs 7 and 8) enables us to continuously track the temporal change in spike activity of
528 the same neurons all the way through the learning process in multi-neuronal recording
529 experiments. These benefits will eventually lead to improved animal welfare.

530 The spout-lever has additional unique benefits when compared to standard levers
531 (cf., Isomura et al. 2009). For instance, we can always monitor the forelimb movement via
532 the spout-lever position because the subjects keep their forelimb touching the spout-lever to
533 lick it even after a correct response. As the spout-lever extends toward their mouth
534 horizontally from one side, the rats rarely make a mistake and grasp it with the other

forelimb. The rats can naturally adjust the horizontal position of spout-lever for comfort by themselves, without the experimenter interfering in the experiment. Furthermore, examining the spatial coordination of skilled forelimb movements and natural licking with the tongue (see Fig. 5) will be possible with the spout-lever manipulation.

Future applications

We have demonstrated two simple and practical applications of spout-lever manipulation: the external-/internal-trigger task and the Go/No-go discrimination task (see Figs. 7 and 8). Indeed, the spout-lever manipulation has great capacity for use in a variety of behavioral experiments. For example, a perturbing force may be added to the spout-lever with electromagnetic repulsion during a specific aspect of forelimb movement. The force of forelimb movements may be measured using a force transducer attached to the spout-lever. One may design a behavioral choice task with multiple spout-levers (left and right, or more) or literally a ″spout-joystick″ to examine goal-directed sensory-motor processes such as sensory discrimination, decision-making, and response selection. In conclusion, our spout-lever manipulation is a promising behavioral method that will be suitable for cutting-edge physiological techniques designed to understand neural mechanisms underlying not only skilled movements but also higher-order cognitive/motor functions.

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Author contributions:

Y.I. conceived and designed research; R.K., A.S., Y.F.T., F.O., Y.S., and Y.I. performed experiments and analyzed data; R.K., A.S., K.K., M.M., Y.S., and Y.I. interpreted results of experiments; Y.I. prepared figures and drafted manuscript; R.K., A.S., K.K., M.M., Y.S., and Y.I. edited manuscript.

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Legends for Figures and Movies

Figure 1. Spout-lever device. ***A***: Conceptual difference between the standard lever (left) and our spout-lever (right). We integrated a lever (operandum; red) and a rewarding spout

(reinforcer; blue) into one spout-lever device. ***B***: Operant learning of a simple lever-touch task by freely-moving rats in a preliminary experiment. The task learning was much more efficient with our spout-lever (middle) than with a standard lever and spout (left) across the first and second experimental days (right). Dots represent values for individual rats. Bars indicate means and standard deviation (s.d.).

Figure 2. Spout-lever device installed in a stereotaxic frame. ***A***: Schematic view of spout-lever manipulation by a head-fixed rat. ***B***: Spout-lever device. Insets, reward water is dispensed from the tip of spout-lever (asterisk). ***C***: A head-attachment for head-fixation (aluminum, 13 g, reusable). ***D***: Head-attachment clamp in a stereotaxic frame. Stereotaxic head-fixation was completed just by sliding in (1) and fastening (2) the head-attachment. ***E***: Spatial arrangement of the spout-lever device and the stereotaxic head-fixation device. The spout-lever was swung horizontally by right forelimb movement forward (push) and backward (pull) from a neutral position.

Figure 3. Training procedure for basic spout-lever manipulation. ***A***: Pre-surgery handling to habituate a rat to the experimenter. ***B***: Recovery from the primary surgery to mount the head-attachment on the skull. ***C***: Multi-rat task-training system that allowed four rats to learn an operant spout-lever manipulation in the head-fixed condition. ***D***: A computer program to carry out the task-training schedule for rats simultaneously and independently. ***E***: External-trigger (Go) task. A head-fixed rat started each trial by pushing the spout-lever with his right forelimb. If he held it for constant time (hold period) and pulled it quickly in response to auditory cue presentation, he was allowed to lick saccharin water on the tip of

749 spout-lever as a reward. ***F***: Training schedule for operant learning of external-trigger (Go)
750 task. The hold period (green) was extended step-by-step automatically up to one second (as
751 a final form) across three training days. The reward delivery (blue) was randomly delayed
752 (0.2–0.8 sec). All rats were subjected to this schedule in the task-training system.
753
754 **Figure 4.** Rapid operant learning of spout-lever manipulation. ***A***: A head-fixed rat
755 grasping the spout-lever with his right forelimb. He held the spout-lever (left), and then
756 pulled to lick it with his tongue (arrowhead) very naturally (right). ***B***: Lever trajectories in a
757 trainee rat on the first, second, and third training days. A sequence of push, hold, pull, and
758 lick was repeated stably on the last training day. Inset, pull movement with the forelimb
759 (left), damped oscillation of free (released) spout-lever (right). ***C***: Distribution of lever-hold
760 time (from the onset of hold period to the onset of pull movement) for individual trials on
761 the three training days. Red and gray dots indicate correct (rewarded) and error
762 (non-rewarded) trials, respectively. Green lines indicate the onset of cue presentation,
763 which was automatically extended according to schedule (see Fig. 3*F*). Note that the pull
764 response was rapidly adapted to the extension of cue presentation on the first day (asterisk).
765 ***D***: Time course of training performance by 25 trainee rats on the three days. Gray lines
766 indicate cumulative number of correct trials along training time for the individual rats, and
767 red dots and error bars indicate the mean and standard deviation among them, respectively.
768 ***E***: Averaged histogram of the hold time (until the pull onset) in the last 400 correct trials
769 (red) with error trials (gray) on the last day ($N = 25$ rats, means and s.d.).
770
771 **Figure 5.** Licking behavior for reward acquisition. ***A***: Detection of licking response. Left,

772 electropotential trace of spout-lever (top, E_{Lever}) and trajectory of spout-lever (bottom,
773 Lever). Right, licking responses during correct (red) and error (gray) trials. ***B***: Licking
774 activity histogram in a rat. Licking probability (top; 20 ms bins), lever trajectory (middle;
775 mean and standard deviation), and reward delivery (bottom) are aligned with first licks for
776 correct (left) and error (right) trials, respectively. Note that first licks occurred prior to
777 reward delivery in correct trials, and that licking activity persisted following the reward
778 delivery. ***C***: Licking activity for individual trials with or without reward delivery in the
779 same rat. The number of licking responses is plotted against the latency of first licks from
780 the onset of hold period. Each dot represents a train of consecutive licks in one correct (red)
781 or error (gray) trial.
782
783 **Figure 6.** Stable whole-cell recording during spout-lever manipulation. ***A***: Whole-cell
784 recording from the forelimb area (FL) of motor cortex. Photograph shows the shape of
785 glass electrode. ***B***: Effective time for stable whole-cell recording in all recorded neurons
786 (#1 to 5, depth in parenthesis). Gray bars represent stable whole-cell recording time
787 available for analysis. Red bars represent behaving time when the rat was performing the
788 external-trigger trials (right side, total number of pull movements). Triangles indicated that
789 the recorded neuron was abruptly broken away (as illustrated by an inset). ***C***: Membrane
790 potential (Vm; neuron #1) and lever position (Lever) during the task performance. Inset,
791 membrane potential and lever position magnified from the original traces (red rectangles).
792 Red ticks indicate licking responses that are presumed by slight shifts in the lever position.
793 ***D***: Spike activity histogram, membrane potential, and lever position in relation to the onset
794 of pull movements (0 s) in the same neuron (#1). Five consecutive traces are overlaid for

795 the membrane potential and lever position. The spike activity was correlated with the
796 forelimb movements. ***E***: Spike activity histograms aligned with the pull onset in other
797 neurons (#3 and #4).
798
799
800 **Figure 7.** Additional performance of external- and internal-trigger task. ***A***:
801 External-/internal-trigger task. The head-fixed rats that had learned the external-trigger
802 trials in advance (see Figs. 3–5) were required to perform similar trials but there was no cue
803 presentation (internal trigger). The two trial types appeared at random. ***B***: Representative
804 performance of the external-/internal-trigger task (see also Supplemental Video S1). ***C***:
805 Electromyogram (EMG) activity in right forelimb during the task performance. Note that it
806 was increased during the pulling, licking, and pushing motions in both the trial types. ***D***:
807 Distribution of lever-hold time (until the pull onset) for the external-trigger (left) and
808 internal-trigger trials (right) during the task performance. Colored (red, external; blue,
809 internal) and gray dots indicate correct and error trials, respectively. A green line indicates
810 the onset of cue presentation in the external-trigger trials. ***E***: Time course of task
811 performance by 5 tested rats. Gray lines indicate cumulative number of correct trials
812 (external and internal) along task performance time for the individual rats, and red dots and
813 error bars indicate the mean and standard deviation among them, respectively. ***F***: Averaged
814 histogram of the hold time (until the pull onset) in the last 400 correct trials (red, external;
815 blue, internal) with error trials (gray) ($N = 5$, means and s.d.).
816
817 **Figure 8.** Extra-one-day learning of Go and No-go discrimination task. ***A***: Go/No-go

discrimination task. The head-fixed rats that had learned the Go trials in advance (see Figs. 3–5) were required to learn to perform No-go trials in response to another cue presentation. They appeared at random. ***B***: Representative performance scene of the Go/No-go discrimination task (see also Supplemental Video S2). ***C***: EMG activity in right forelimb during the task performance. Note that it was increased largely during the actual pull-lick-push movements in the Go and No-go trials. ***D***: Distribution of lever-hold time for the Go (left) and No-go trials (right) during the task performance. Colored (red, Go test trials; pink, Go correction trials; blue, No-go test trials; light blue, No-go correction trials) and gray (light and dark gray, before and after cue presentation, respectively) dots indicate correct and error trials, respectively. Green or orange line indicates the onset of cue presentation. Insets (a–d), histograms of the hold time in the first (a or c) and last (b or d) 400 correct trials (indicated by colors as described above) with error trials (indicated by gray as described above). Note that false Go responses (c, dark gray) were abolished after learning the No-go trials (d, asterisk). ***E***: Time course of task performance by 4 of 7 rats tested here. Gray lines indicate cumulative number of correct trials (Go and No-go) along task performance time for the individual rats, and red dots and error bars indicate the mean and standard deviation among them, respectively. ***F***: Averaged histogram of the hold time (until the pull onset) in the last 400 correct trials (red, Go; blue, No-go) with error trials (gray) (N = 4 rats, means and s.d.). ***G***: Time course of correct rate for the Go (left) and No-go trials (right) in the four rats. Gray lines indicate correct rates every 100 correct trials (including correction trials) for the individual rats. Colored dots and error bars indicate the mean and s.d. among them, respectively. Note that the correct rate was increased from the chance level (50%) in the No-go trials. ***H***: Summary of additional No-go learning by the

four rats. Circles represent their correct rates for the first and last 100 correct trials of the No-go learning session (filled, test trials only; open, test and correction trials). Asterisks indicate that they are significantly higher than the chance level (50%).

Supplemental Video S1. A rat performing the external-/internal-trigger task under head-fixation.

Supplemental Video S2. Another rat performing the Go-/No-go discrimination task under head-fixation.

Figure 1 (Kimura et al.)

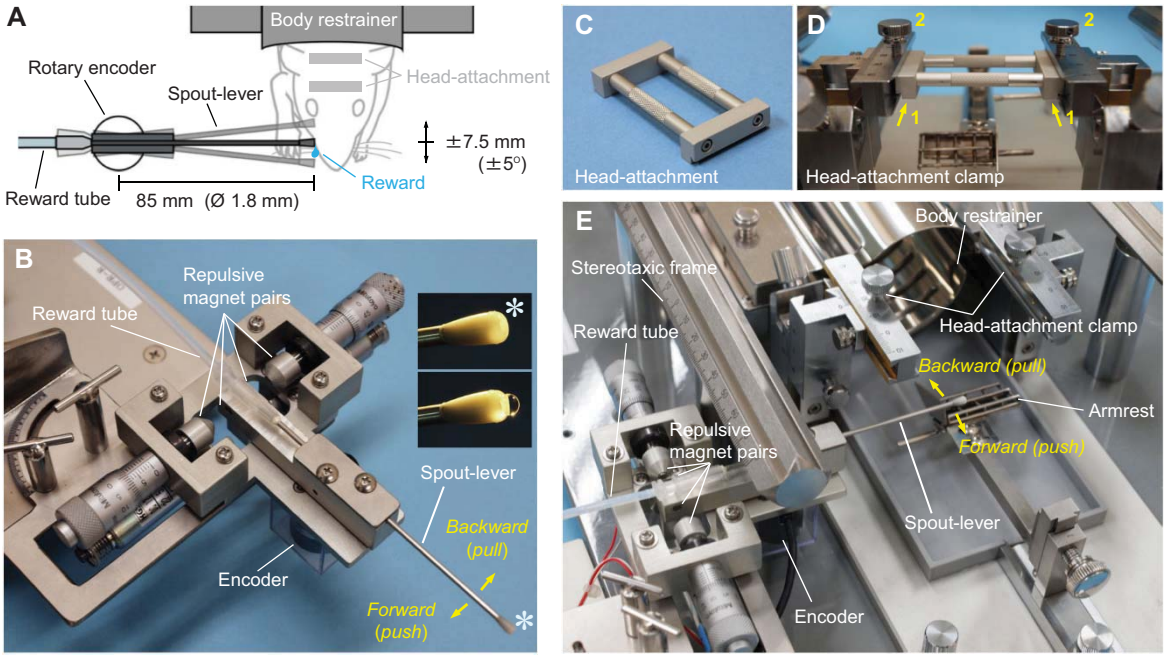


Figure 2 (Kimura et al.)

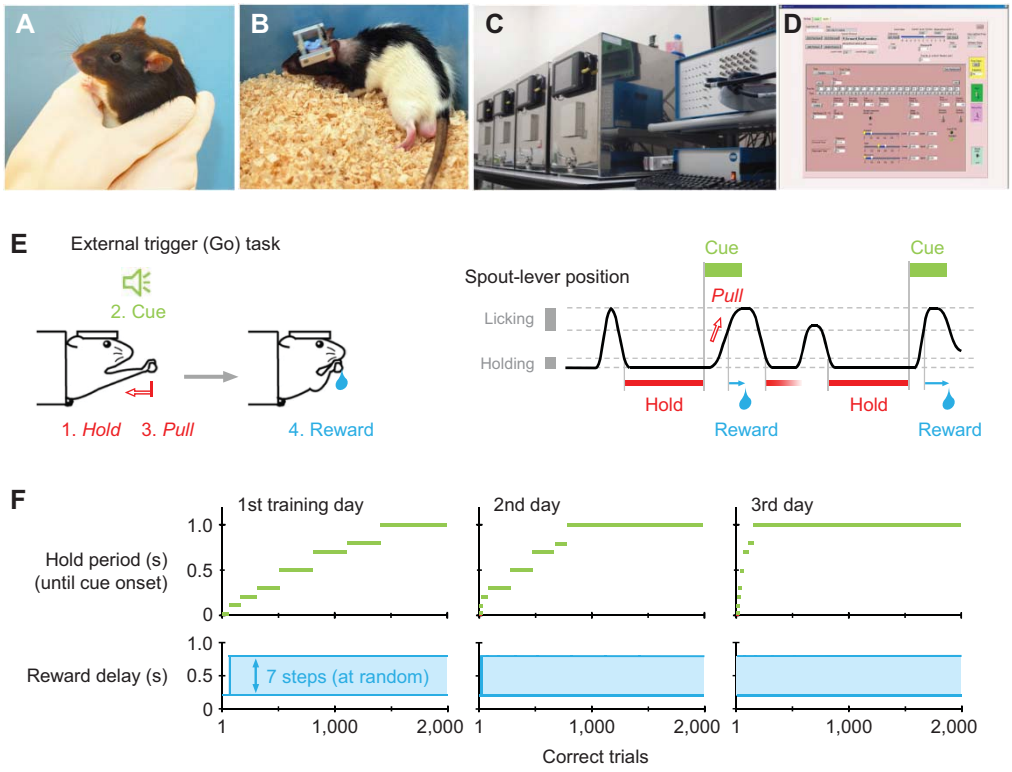


Figure 3 (Kimura et al.)

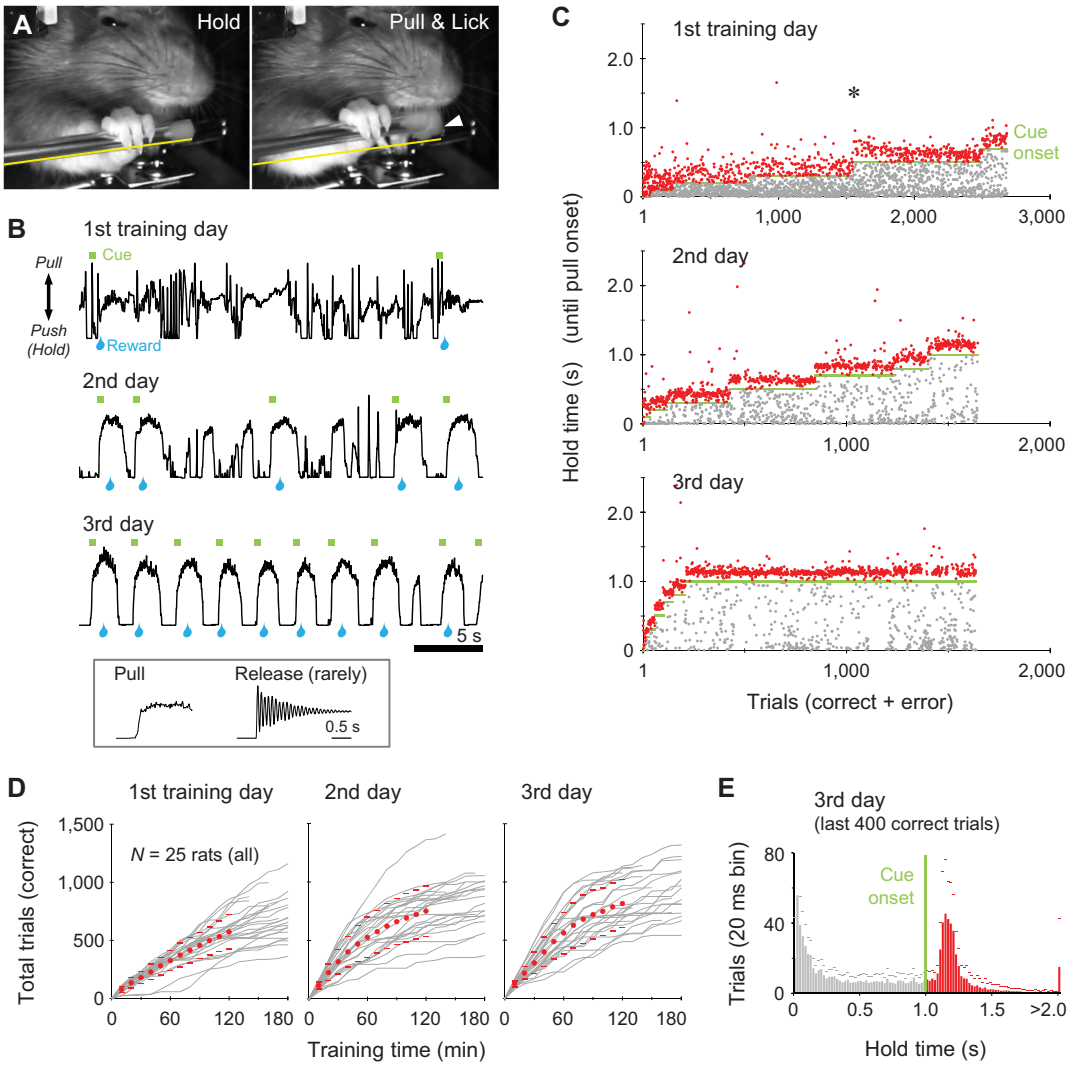


Figure 4 (Kimura et al.)

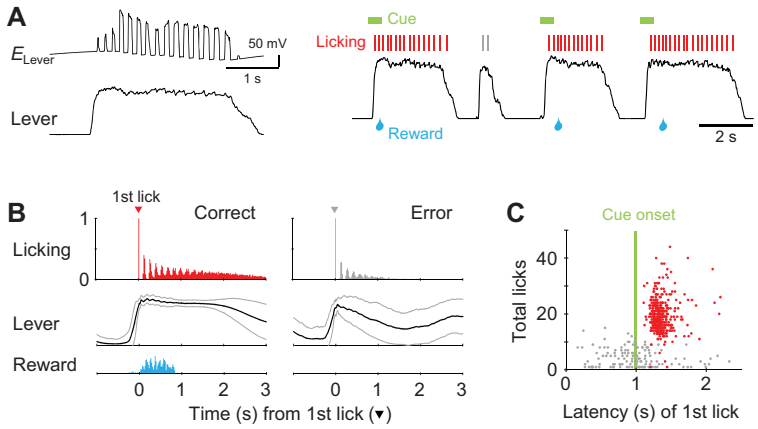


Figure 5 (Kimura et al.)

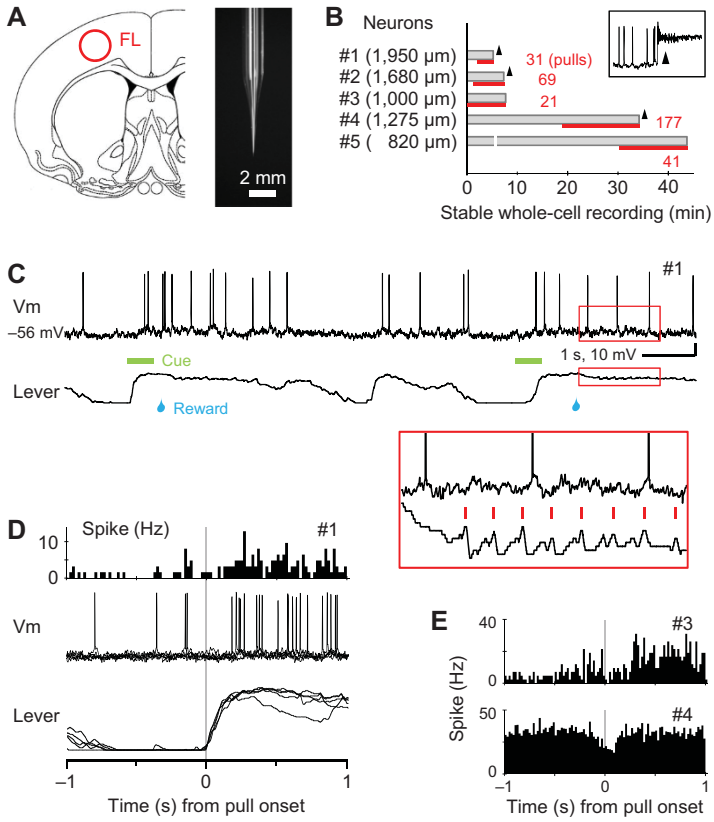


Figure 6 (Kimura et al.)

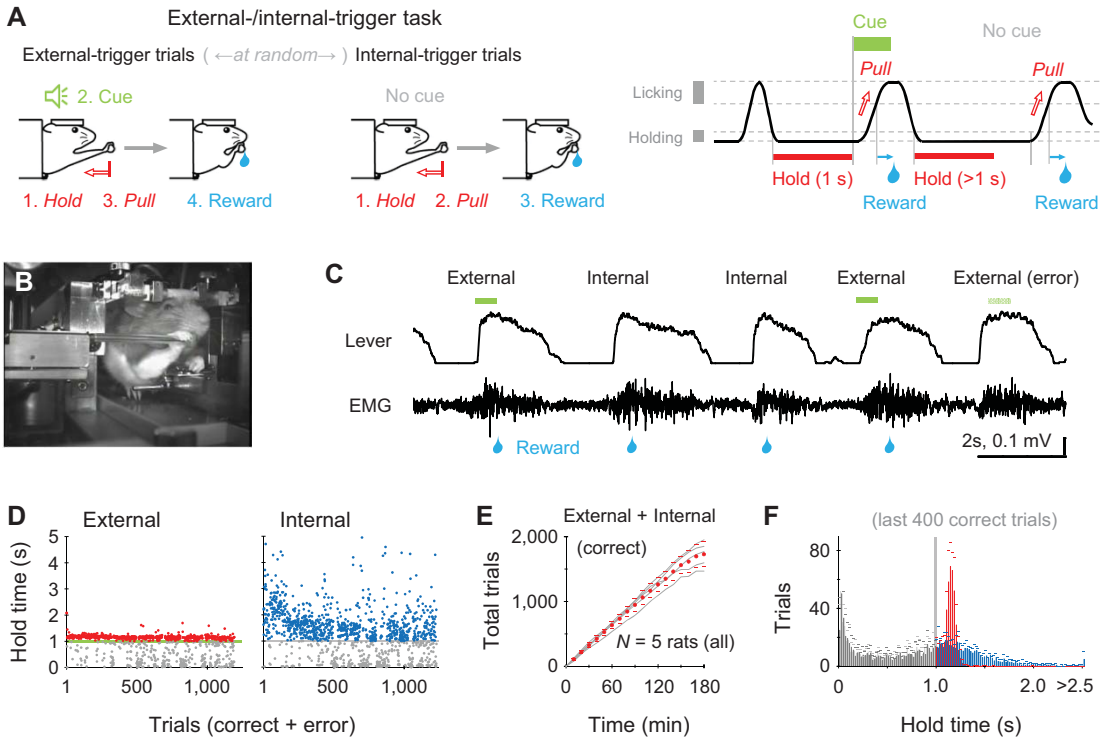


Figure 7 (Kimura et al.)

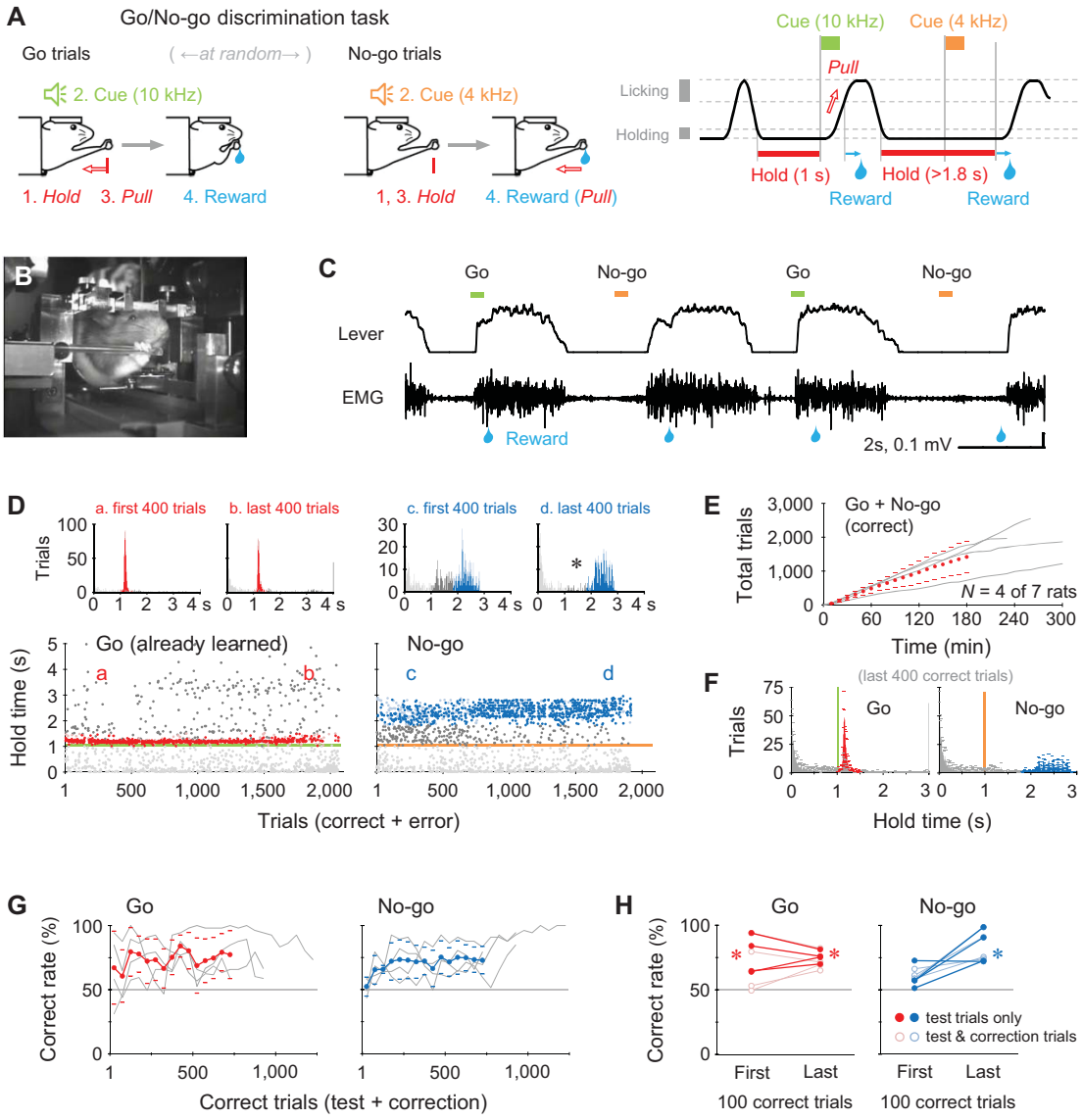
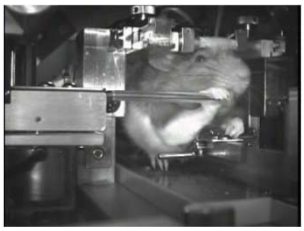
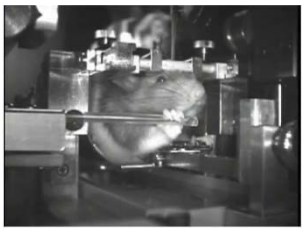


Figure 8 (Kimura et al.)



Supplemental Video 1 (Kimura et al.)



Supplemental Video 2 (Kimura et al.)