

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

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Kimura R, Saiki A, Fujiwara-Tsukamoto Y, Ohkubo F, Kitamura K, Matsuzaki M, Sakai Y, Isomura Y. Reinforcing operandum: rapid and reliable learning of skilled forelimb movements by head-fixed rodents. *J Neurophysiol* 108: 1781–1792, 2012. First published June 27, 2012; doi:10.1152/jn.00356.2012.—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 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, which 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 verified the usefulness of spout-lever manipulation for behavioral experiments employing cutting-edge physiological techniques.

rat; operant learning; *in vivo* whole cell recording

IN OUR DAILY LIFE, we perform so-called skilled movements, e.g., reaching, grasping, pushing, and pulling, with our digits, hand, arm, and shoulder (Iwaniuk and Whishaw 2000). Skilled movements have been studied intensively in behaving monkeys that are operantly conditioned to repeat specific forelimb movements under head-fixed conditions for electrophysiology experiments (Evarts 1968; Evarts and Tanji 1974; Fetz and Cheney 1980; Georgopoulos et al. 1982). As skilled forelimb

movements are applicable to a wide variety of behavioral tasks, research using head-fixed monkeys performing operant forelimb movements has enhanced the knowledge of neural mechanisms underlying not only skilled movements (Kalaska and Crammond 1995; Mushiake et al. 1991; Okano and Tanji 1987) but also higher-order cognitive/motor functions (Isomura et al. 2003; Shima and Tanji 1998; Watanabe 1986a, 1986b).

Compared with primates, rodents in head-fixed conditions are more amenable to state-of-the-art physiological techniques such as intracellular/whole cell recordings (Brecht et al. 2004; Crochet and Petersen 2006; Fee 2000; Harvey et al. 2009), juxtacellular recording (de Kock and Sakmann 2009; Houweling and Brecht 2008; Isomura et al. 2009), multiphoton laser-scanning microscopy (Dombeck et al. 2007; Komiyama et al. 2010), and optogenetic manipulation (Hira et al. 2009; Matyas et al. 2010). Indeed, there are an 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; Stützgen et al. 2006; Stützgen and Schwarz 2008; Welsh et al. 1995) or whisking (Bermejo et al. 1996, 2004; Hentschke et al. 2006; O'Connor et al. 2010a, 2010b) 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 brain stem (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 application to various behavioral tasks, combined with 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, see 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.

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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 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 the Tama-gawa 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 AM and lights on at 9 PM). The rats were adapted to a stainless steel cylinder (hideaway) in their home cage and were briefly handled by an experimenter (10–15 min, 2 times). Under 2.0–2.5% isoflurane anesthesia (Univentor 400 anesthesia unit, Univentor), the rats had a sliding, light-weight 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; Panavia F2.0, Kuraray Medical; Unifast II, GC Corporation, Japan). In the present study, virtually no rats lost the head attachment by the end of the 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). 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, although 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 of water) was given to the rats, when necessary, to maintain >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; 30.0 cm × 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 h/day) in our automatic multirat task-training system (custom-made by O'hara, 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 its mouth (holding position, 0–3 mm; licking position, 6–9 mm from the front end) in response to the cue presentation, it 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 the spout-lever by a micropump with a 0.2- to 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 intertrial 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 intertrial 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 the external-trigger (Go) task in the task-training system, 2 days later some of them were transferred to a similar system for a single rat (O'hara) 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 pseudorandomly in a ratio of 1:1 (see Fig. 7A). 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 pseudorandomly in a ratio of 1:1 (see Fig. 8A). In the Go trials, the rats had to quickly pull the spout-lever <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 intertrial 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 the 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 multichannel amplifier (MEG-6116, Nihon Kohden; 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 3-day task training, the rats were subjected to a second surgery under isoflurane anesthesia to open a tiny hole in the skull and dura mater 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). The rats recovered from the anesthesia completely in several hours. Glass electrodes (GC150F-7.5, Harvard Apparatus) were prepared with a puller (PC-10, Narishige) and filled with an internal solution containing (in mM) 140 K-gluconate, 2 NaCl, 1 MgCl₂, 10 HEPES, 0.2 EGTA, 2.5'-ATP-Na₂, and 0.5 GTP-Na₂ (pH 7.4, 5–10 MΩ; Fujiwara-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-disk recorder (LX-120, TEAC or DataMax II, R.C. Electronics). These experimental data were further analyzed by MATLAB (MathWorks). The results are expressed as means \pm standard deviation (SD) and, when applicable, with statistical significance.

RESULTS

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

We then used the spout-lever device in a series of behavioral experiments with another group of rats in a head-fixed condition (Fig. 2A). The spout-lever device (Fig. 2B) was combined with our stereotaxic head-fixation device (Isomura et al. 2009), in which a head attachment (Fig. 2C) was slid smoothly forward into a head attachment clamp (Fig. 2D) in order to lessen the fear or stress under head-fixation conditions. A body restrainer and an armrest (Fig. 2E) 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 no. 6206, Fuchigami; 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°). Its position was always monitored by the angle encoder (MES-12-1000PC, Microtech Laboratory) attached with the pivot of the spout-lever. The stiffness and neutral position of the spout-lever were easily adjustable (0.02–0.2 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 the spout-lever was brought closer to the mouth of the rat by a three-dimensional manipulator, which held the whole spout-lever device. Adjustment of the spout-lever position (<5 mm) was required for individual rats in each experimental session. A tiny volume of reward water (5 μ l/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. 3A), and the head attachment was surgically attached to the skull in a stereotaxic manner under isoflurane anesthesia. After recovery from the surgery (Fig. 3B) 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 multirat task training system (Fig. 3C). This task training system, controlled by a computer program (Fig. 3D), 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 pretraining 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. 3E). 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 intertrial 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 its mouth immediately after the cue onset, then it could lick a drop of saccharin water on the tip of the 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. This is the very



Fig. 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 1 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 (center) than with a standard lever and spout (left) across the 1st and 2nd experimental days (right). Dots represent values for individual rats. Bars indicate means and standard deviation (SD). **Significantly different ($P < 0.005$) between the 2 groups.

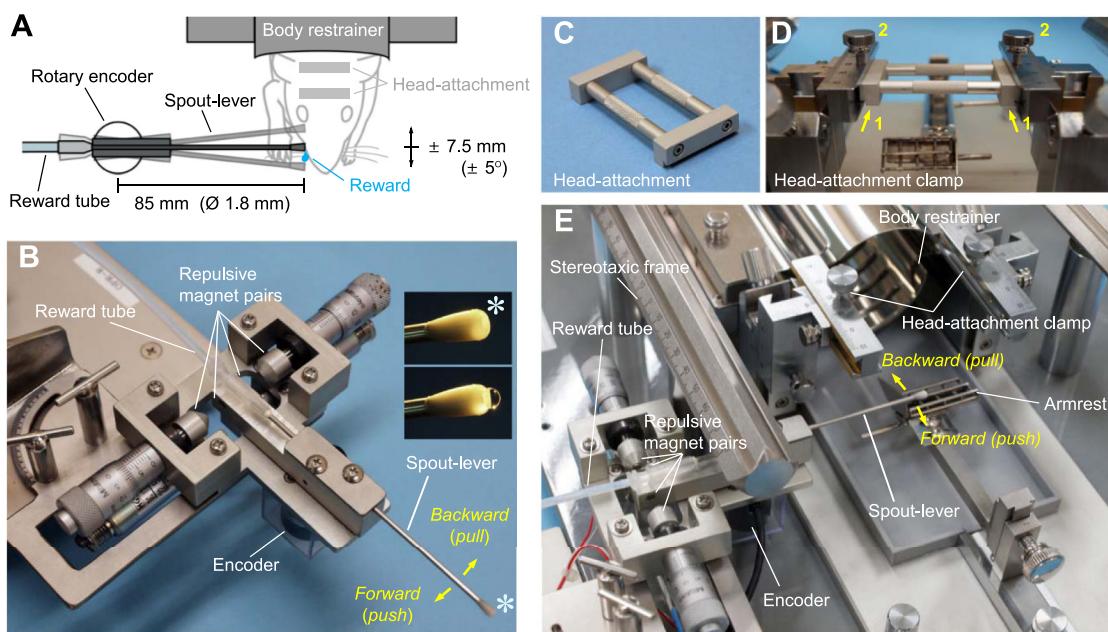


Fig. 2. Spout-lever device installed in a stereotaxic frame. *A*: schematic view of spout-lever manipulation by a head-fixed rat. *B*: spout-lever device. *Insets*, reward water is dispensed from the tip of the spout-lever (asterisk). *C*: a head attachment for head fixation (aluminum, 13 g, reusable). *D*: head-attachment clamp in a stereotaxic frame. Stereotaxic head-fixation was completed by just 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.

skilled forelimb movement consisting of a sequence of grasping, pushing, holding, and pulling motions. In particular, 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 3 consecutive days (Fig. 3*F*). We made out the step-up schedule for refresher training on all 3 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. 4*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 (asterisk, Fig. 4*C*), and this tendency became more pronounced over the next 2 days (Fig. 4*C*). A total of 25 rats were tested for the 3-day task training procedure, after we excluded 1 rat that died during the skull surgery and 1 rat that moved the forelimb restrictedly because of a badly implanted EMG electrode. All 25 rats successfully learned to consistently perform the external-trigger task according to schedule over the 3 days of testing [Fig. 4*D*; 571 ± 150 (1st day), 751 ± 214 (2nd day), and 814 ± 202 (3rd day; 487–1,136) 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 the 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 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 ex-

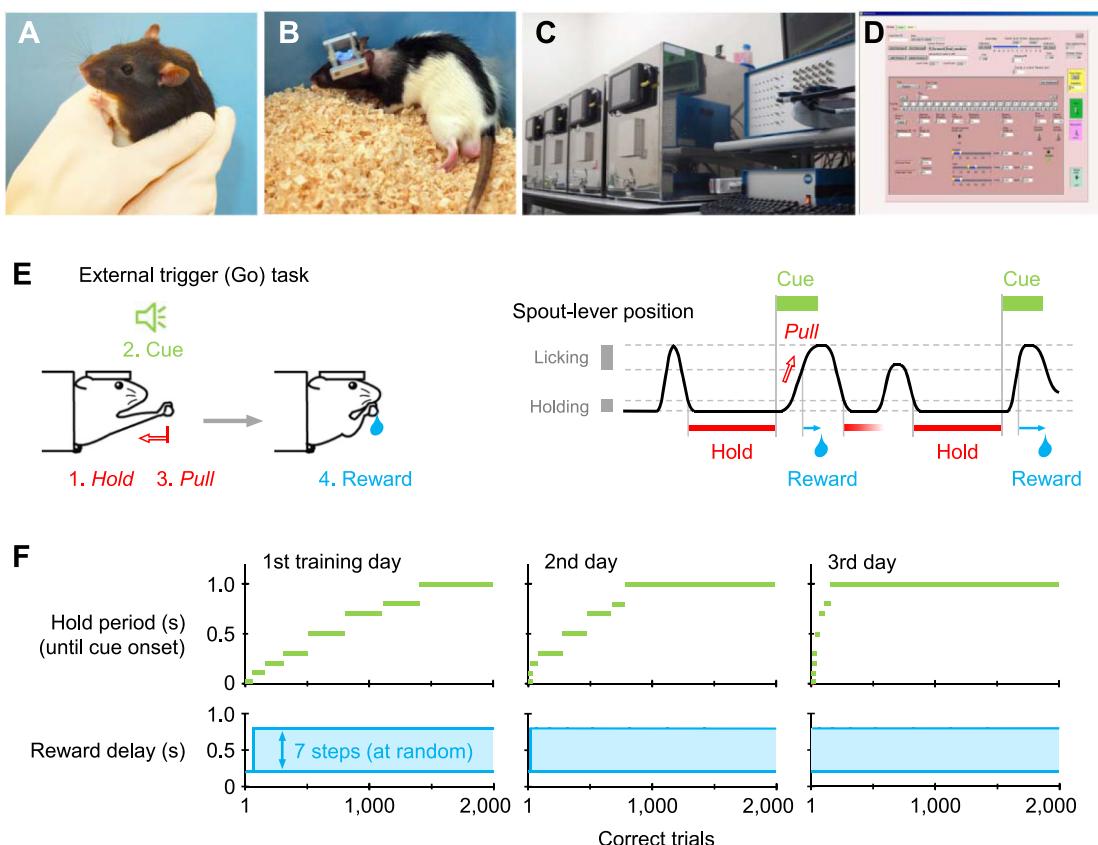


Fig. 3. Training procedure for basic spout-lever manipulation. *A*: presurgery handling to habituate a rat to the experimenter. *B*: recovery from the primary surgery to mount the head attachment on the skull. *C*: multirat task-training system that allowed 4 rats to learn an operant spout-lever manipulation 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 its right forelimb. If the rat held the spout-lever for a constant time (hold period) and pulled it quickly in response to auditory cue presentation, it was allowed to lick saccharin water on the tip of spout-lever as a reward. *F*: training schedule for operant learning of external-trigger (Go) task. The hold period (green) was extended step-by-step automatically up to 1 s (as a final form) across 3 training days. The reward delivery (blue) was randomly delayed (0.2–0.8 s). All rats were subjected to this schedule in the task-training system.

changes, 3) stable gigaseal formation against mechanical vibrations by their pulsation, respiration, and body movements, and 4) no contamination of electrical noises by the licking or touching of 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 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*; $N = 10$ rats, P50–P7). Sufficiently stable whole cell recordings (>5 min) were obtained from five neurons in the deep layer of the FL area (Fig. 6*B*; total recording time, 40.8 ± 58.3 min; stable whole-cell time, 19.6 ± 18.0 min; firing rate, 10.6 ± 12.6 Hz; resting membrane potential, -64.0 ± 13.3 mV; $n = 5$). In our experiment, we did not use temporary anesthesia with rapidly antagonizable anesthetics and antagonists to establish the whole cell configuration (e.g., Brecht et al. 2004). All of the recorded neurons included behavior time in which the rats were repeating the spout-lever manipulation (9.2 ± 5.1 min; total 67.8 ± 63.6 pulls, 50.2 ± 71.7 correct trials). After the stable whole cell time, three neurons were lost abruptly, whereas the others died gradually. A representative neuron

(neuron 1) displayed an increased occurrence of action potentials in correlation with the pull-to-push forelimb movements (Fig. 6, *C* and *D*; Mann-Whitney test, $P < 0.001$). Each licking response did not seem to be directly related to the membrane potential (Fig. 6*C*, inset). Two of the other neurons (neurons 3 and 4) also showed behavior-related spiking activity in their membrane potentials (Fig. 6*E*; $P < 0.05$ and $P < 0.001$, respectively). Electrical artifacts from licking and touching were negligible in all cases. Unlike anesthetized or deeply sleeping animals (Isomura et al. 2006), we never observed clear up-down fluctuations in the membrane potential during forelimb movements in any recorded neuron (data not shown). These trial experiments of whole cell recordings demonstrated the applicability of the spout-lever task system to advanced physiological techniques.

Application 1. Additional performance of external- and internal-trigger task. Voluntary movement can be initiated by a sensory signal from the outside (external trigger) 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

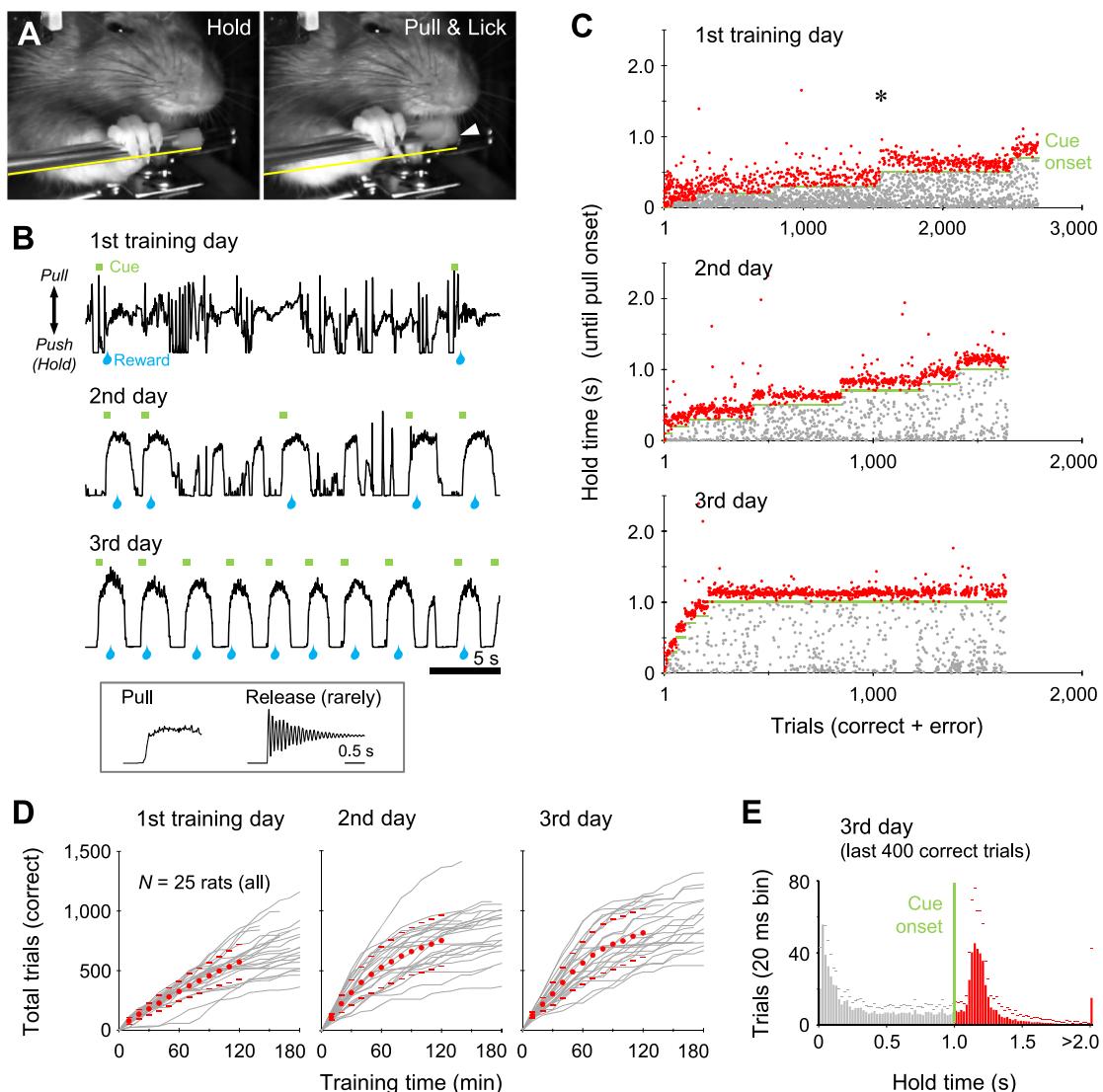


Fig. 4. Rapid operant learning of spout-lever manipulation. *A*: a head-fixed rat grasping the spout-lever with its right forelimb. It held the spout-lever (*left*) and then pulled to lick it with its tongue (*right*). *B*: lever trajectories in a trainee rat on the 1st, 2nd, and 3rd training days. A sequence of push, hold, pull, and lick was repeated stably on the last training day. *Inset*, pull movement with the forelimb (*left*) and damped oscillation of free (released) spout-lever (*right*). *C*: distribution of lever hold time (from the onset of hold period to the onset of pull movement) for individual trials on the 3 training days. Red and gray dots indicate correct (rewarded) and error (nonrewarded) trials, respectively. Green lines indicate the onset of cue presentation, which was automatically extended according to schedule (see Fig. 3*F*). Note that the pull response was rapidly adapted to the extension of cue presentation on the 1st day (asterisk). *D*: time course of training performance by 25 trainee rats on the 3 days. Gray lines indicate cumulative number of correct trials along training time for the individual rats, and red dots and error bars indicate the mean and SD among them, respectively. *E*: averaged histogram of the hold time (until the pull onset) in the last 400 correct trials (red) with error trials (gray) on the last day ($N = 25$ rats, means and SD).

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 that had already learned the external-trigger trials in the 3 training days (see Figs. 3–5). For this task, the rats must hold the spout-lever for at least for 1 s, 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).¹ The 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

¹ Supplemental Material for this article is available online at the Journal website.

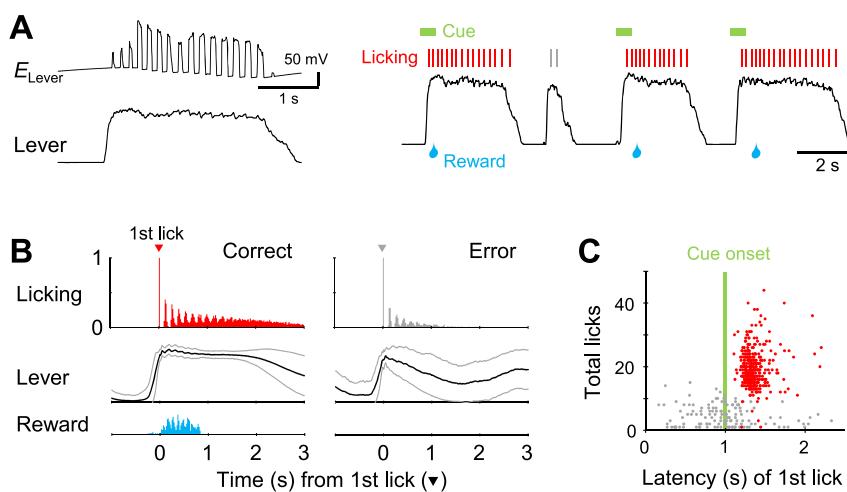


Fig. 5. Licking behavior for reward acquisition. *A*: Detection of licking response. *Left*: electropotential trace of spout-lever (E_{Lever} , *top*) and trajectory of spout-lever (*Lever*, *bottom*). *Right*: licking responses during correct (red) and error (gray) trials. *B*: licking activity histogram in a rat. Licking probability (*top*; 20-ms bins), lever trajectory (*middle*; mean and SD), and reward delivery (*bottom*) are aligned with first licks for correct (*left*) and error (*right*) trials, respectively. Note that first licks occurred prior to reward delivery in correct trials, and that licking activity persisted after the reward delivery. *C*: licking activity for individual trials with or without reward delivery in the same rat. The number of licking responses is plotted against the latency of first licks from the onset of hold period. Each dot represents a train of consecutive licks in 1 correct (red) or error (gray) trial.

new experimental session (Fig. 7, *D* and *E*); furthermore, they maintained the behavioral performance for several hours (Fig. 7*E*; $1,728 \pm 194$ 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*; $1,148 \pm 23$ ms (external) and $1,068 \pm 118$ ms (internal) from the onset of hold period, $N = 5$; paired *t*-test, $P = 0.18$], while the SD was much larger in the internal-trigger trials than in the external-trigger trials (*F*-test, $P < 0.004$). The rats appeared to judge the trial type instantaneously around the expected cue period.

Application 2. Extra-1-day learning of Go and No-go discrimination task. A behavioral task with a Go/No-go paradigm often provides valuable information on brain functionality of not only the intentional suppression of voluntary movements but also sensory discrimination, decision-making, motivation, and impulsivity. For instance, many studies using monkeys under head fixation have shown that several motor cortical areas (e.g., PMA and SMA) differentially contribute to the Go and No-go performance of skilled forelimb movements (Iso-mura et al. 2003; Kalaska and Crammond 1995; Kurata and Tanji 1985; Tanji and Kurata 1985; Watanabe 1986a, 1986b). It has recently been demonstrated that rodents under head fixation can also perform Go/No-go discrimination tasks, if they are allowed to respond by licking with the tongue (Gerdjikov et al. 2010; Komiyama et al. 2010; Narumi et al. 2007; O'Connor et al. 2010a, 2010b; Stützgen and Schwarz 2010). However, it is still extremely difficult to require that head-fixed rodents learn to make Go/No-go responses by skilled forelimb movements (cf. Harvey et al. 2001 as a rare success, though requiring several weeks for learning). Thus we used head-fixed rats that had learned the external-trigger (Go) forelimb response to examine how fast they could learn the corresponding No-go forelimb response.

In the Go/No-go discrimination task (Fig. 8*A*), the Go trials were similar to the 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 s 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 behav-

iors 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, although they were activated largely over all pull-to-push movements regardless of the Go or No-go trial type (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 1–6 h on the test session [Fig. 8*E*; $1,415 \pm 468$ 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 steplike 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), $76.7 \pm 14.8\%$, $N = 4$, $P < 0.04$ (*t*-test); last 100 trials, $75.7 \pm 4.6\%$, $P < 0.002$]. 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, $59.7 \pm 9.1\%$, $P > 0.1$; last, $83.5 \pm 13.3\%$, $P < 0.02$). We did not attempt to train the remaining three rats, because the 1-day learning of No-go trials by half of the 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 phys-

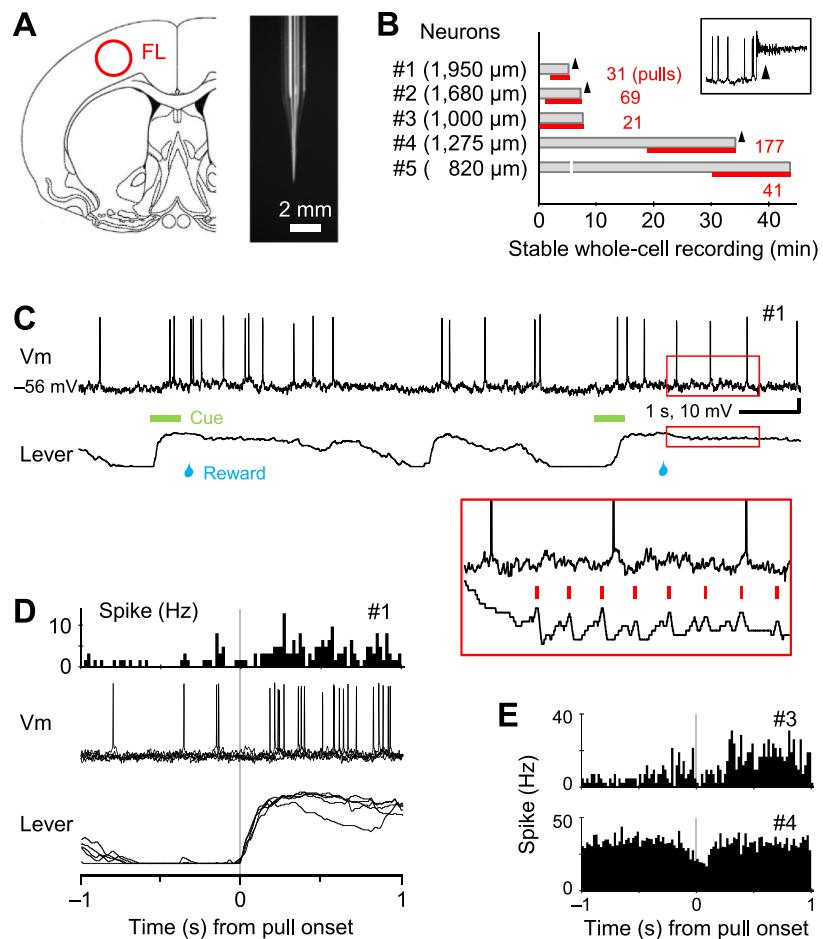


Fig. 6. Stable whole cell recording during spout-lever manipulation. *A*: whole cell recording from the forelimb area (FL) of motor cortex. Photograph shows shape of glass electrode. *B*: effective time for stable whole-cell recording in all recorded neurons (*neurons 1–5*, depth in parentheses). Gray bars represent stable whole cell recording time available for analysis. Red bars represent behaving time when the rat was performing the external-trigger trials (total number of pull movements on right). Triangles indicate that the recorded neuron was abruptly broken away (as illustrated by inset). *C*: membrane potential (V_m ; *neuron 1*) and lever position (Lever) during the task performance. Inset, membrane potential and lever position magnified from the original traces (red rectangles). Red ticks indicate licking responses that are presumed by slight shifts in the lever position. *D*: spike activity histogram, membrane potential, and lever position in relation to the onset of pull movements (0 s) in the same neuron (*neuron 1*). Five consecutive traces are overlaid for the membrane potential and lever position. The spike activity was correlated with the forelimb movements. *E*: spike activity histograms aligned with the pull onset in other neurons (*neurons 3 and 4*).

iological experiments used to elucidate a variety of brain functions.

Head-fixed vs. freely moving rodents. First of all, we 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 when using laboratory rats and mice compared with other experimental animals. There is also an enormous amount of knowledge on the anatomy, physiology, pharmacology, psychology, and molecular and cellular biology of the rodent central nervous system. Furthermore, rodents have a simple cortical structure, i.e., compact cortical areas with thin layers and no sulci, which is a prerequisite for two-photon microscopic calcium imaging and optical voltage-sensitive dye imaging. They are undoubtedly advantageous for various genetic modifications including gene-transgenic and knockin/knockout manipulations, fluorescent labeling and calcium indicator proteins, and recent optogenetics with channelrhodopsin-2. Nowadays, rodents have become the model mammal in the spotlight of experimental neuroscience.

Stereotaxic head fixation in itself is convenient for current physiological techniques needing behaving rodents because *1*) large physiological devices such as a laser-scanning microscope can be mounted on their head firmly; *2*) electrodes or optical fibers can be positioned into a target brain area precisely and stably; *3*) sensory (visual, tactile, or auditory)

stimuli can be presented to the subject in a spatially different manner; and *4*) voluntary movements (forelimbs, tongue, whiskers, or eyes) can be monitored wholly with a high-speed camera (see, e.g., Gentet et al. 2010 for factors *1, 2*, and *4*). In addition, operant learning can be enhanced if the subject is properly positioned close to an operandum and a reinforcer, as shown here.

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

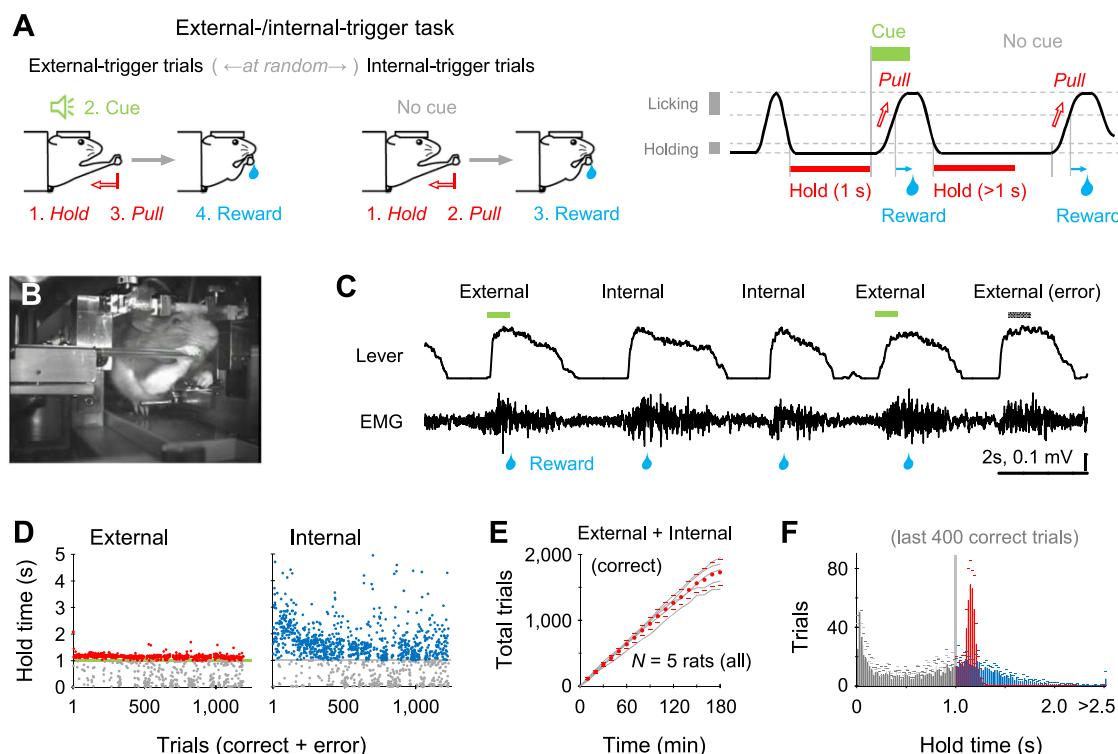


Fig. 7. Additional performance of external- and internal-trigger task. *A*: external-/internal-trigger task. The head-fixed rats that had learned the external-trigger trials in advance (see Figs. 3–5) were required to perform similar trials, but there was no cue presentation (internal trigger). The 2 trial types appeared at random. *B*: representative performance of the external-/internal-trigger task (see also Supplemental Video S1). *C*: electromyogram (EMG) activity in right forelimb during the task performance. Note that it was increased during the pulling, licking, and pushing motions in both trial types. *D*: distribution of lever hold time (until the pull onset) for the external-trigger (*left*) and internal-trigger (*right*) trials during the task performance. Colored (red, external; blue, internal) and gray dots indicate correct and error trials, respectively. A green line indicates the onset of cue presentation in the external-trigger trials. *E*: time course of task performance by 5 tested rats. Gray lines indicate cumulative number of correct trials (external and internal) along task performance time for the individual rats, and red dots and error bars indicate the mean and SD among them, respectively. *F*: averaged histogram of the hold time (until the pull onset) in the last 400 correct trials (red, external; blue, internal) with error trials (gray) ($N = 5$, means and SD).

ately. 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; Stütgen et al. 2006; Stütgen and Schwarz 2008; Welsh et al. 1995; whisking: Bermejo et al. 1996, 2004; Hentschke et al. 2006; O'Connor et al. 2010a, 2010b). 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 a 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 brain stem 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 spatiotemporal 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 a 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. Given these factors, 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

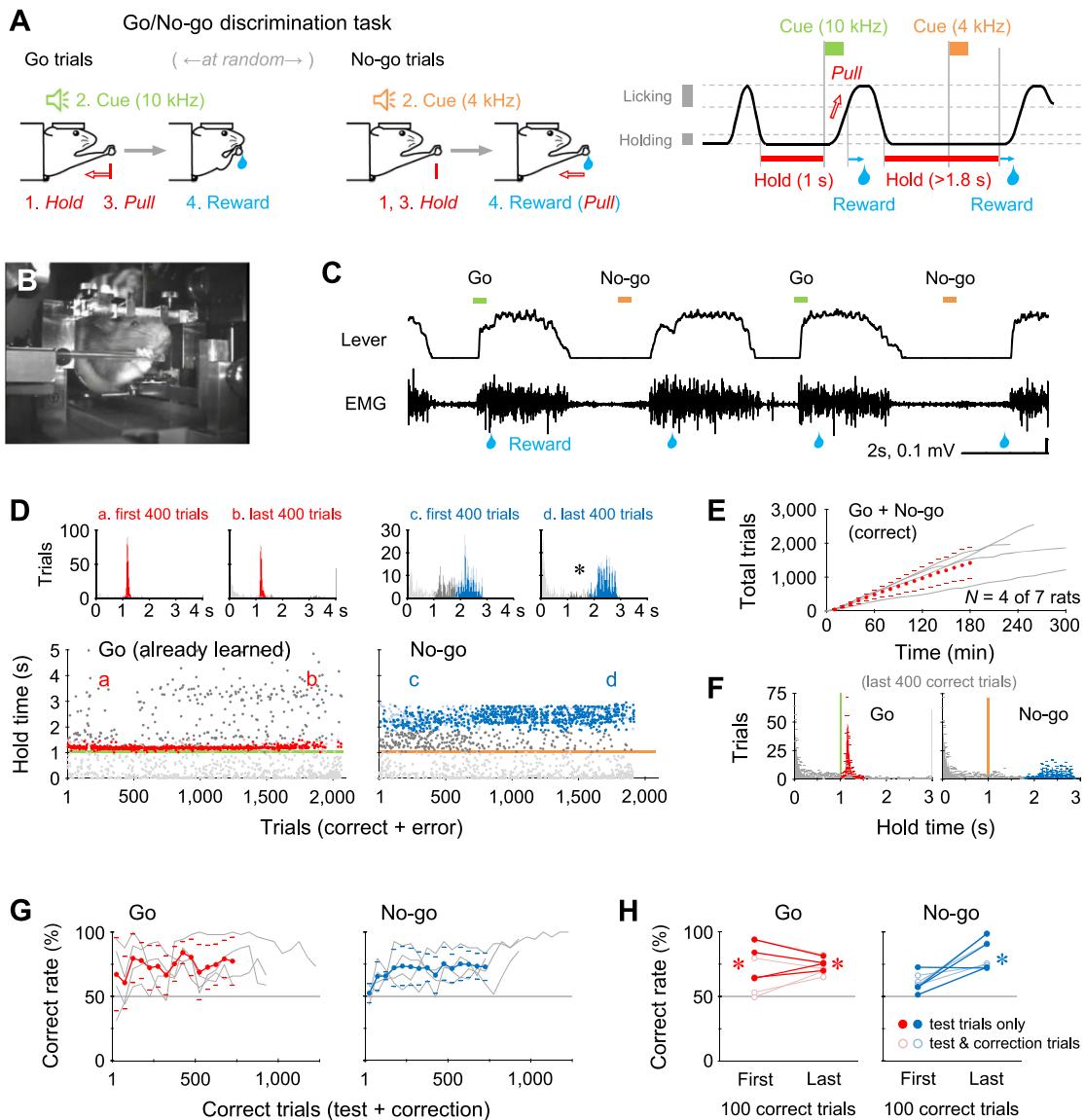


Fig. 8. Extra-1-day learning of Go and No-go discrimination task. **A:** Go/No-go discrimination task. The head-fixed rats that had learned the Go trials in advance (see Figs. 3–5) were required to learn to perform No-go trials in response to another cue presentation. They appeared at random. **B:** 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 (right) trials 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* and *c*) and last (*b* and *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 SD 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 SD). **G:** time course of correct rate for the Go (left) and No-go trials (right) in the 4 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 SD 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 4 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). *Significantly higher than chance level (50%).

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 rare success cases, it still took head-fixed rodents over weeks or months to learn a forelimb response using a standard lever. On the other hand, it

is known that freely moving rodents can learn an operant lever-press task effectively when the cue signal or operandum is placed spatially close to the reinforcer (e.g., Buzsáki et al. 1979). The head fixation may make it difficult to visually recognize spatially separated operandum and reinforcer. The spout-lever that we developed here, an integrated form of

operandum and reinforcer, has made it possible to learn a forelimb response in a head-fixed condition rapidly and reliably. It may be accomplished by immediate feedback from the tongue that felt the pull action directly. The remarkable enhancement of learning efficiency will be advantageous to physiological experiments over the standard lever. Using the spout-lever, we can quickly prepare the many task-performing animals necessary for whole cell or juxtagcellular recording experiments (see Fig. 6), which must be finished in several days once the dura mater is opened to insert a glass electrode. This allows us to obtain physiological data from undamaged neural tissue in a good physical condition before necrosis of the skull or a hypertrophy of the dura mater on account of the skull surgery. More importantly, 1-day learning of an operant response (see Figs. 7 and 8) enables us to continuously track the temporal change in spike activity of the same neurons all the way through the learning process in multineuronal recording experiments. These benefits will eventually lead to improved animal welfare.

The spout-lever has additional unique benefits compared with standard levers (cf. Isomura et al. 2009). For instance, we can always monitor the forelimb movement via the spout-lever position because the subjects keep their forelimb touching the spout-lever to lick it even after a correct response. As the spout-lever extends toward their mouth 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 the 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 with 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.K., A.S., Y.F.-T., F.O., and Y.I. performed experiments; R.K., A.S., Y.F.-T., Y.S., and Y.I. analyzed data; R.K., A.S., and Y.I. interpreted results of experiments; R.K., A.S., K.K., M.M., Y.S., and Y.I. edited and revised manuscript; R.K., A.S., Y.F.-T., F.O., K.K., M.M., Y.S., and Y.I. approved final version of manuscript; Y.I. conception and design of research; Y.I. prepared figures; Y.I. drafted manuscript.

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