Video Article

Study Motor Skill Learning by Single-pellet Reaching Tasks in Mice

Chia-Chien Chen[1], Anthony Gilmore[1], Yi Zuo[1]

[1]Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz

Correspondence to: Yi Zuo at yizuo@ucsc.edu

Abstract

Reaching for and retrieving objects require precise and coordinated motor movements in the forelimb. When mice are repeatedly trained to grasp and retrieve food rewards positioned at a specific location, their motor performance (defined as accuracy and speed) improves progressively over time, and plateaus after persistent training. Once such reaching skill is mastered, its further maintenance does not require constant practice. Here we introduce a single-pellet reaching task to study the acquisition and maintenance of skilled forelimb movements in mice. In this video, we first describe the behaviors of mice that are commonly encountered in this learning and memory paradigm, and then discuss how to categorize these behaviors and quantify the observed results. Combined with mouse genetics, this paradigm can be utilized as a behavioral platform to explore the anatomical underpinnings, physiological properties, and molecular mechanisms of learning and memory.

Video Link

The video component of this article can be found at https://www.jove.com/video/51238/

Introduction

Understanding the mechanisms underlying learning and memory is one of the biggest challenges in neuroscience. In the motor system, the acquisition of novel motor skills with practice is often referred as motor learning, whereas the retention of previously learned motor skills is regarded as motor memory[1]. Learning a new motor skill is usually reflected in improvement of desired motor performance over time, until a point when the motor skill is either perfected or satisfactorily consistent. For most cases, the acquired motor memory can persist for a long period of time, even in the absence of practice. In humans, neuroimaging studies using positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have shown that primary motor cortex (M1) activity changes during the acquisition phase of motor skill learning[2-4], and temporary interference of M1 activity by low frequency transcranial magnetic stimulation leads to significantly disrupted retention of motor behavioral improvement[5]. Similarly, forelimb-specific training in rats induces functional and anatomical plasticity in the M1, exemplified by the increase of both c-fos activity and synapse/neuron ratio in the M1 contralateral to the trained forelimb during the late phase of motor skill learning[6]. Furthermore, a similar training paradigm also strengthens layer 2/3 horizontal connections in the contralateral M1 corresponding to the trained forelimb, resulting in reduced long term potentiation (LTP) and enhanced long term depression (LTD) after rats acquire the tasks[7]. Such synaptic modification, however, is not observed in the M1 cortical regions corresponding to untrained forelimb or hindlimbs[8]. Alternatively, when the M1 is damaged through stroke, there are dramatic deficiencies in forelimb specific motor-skills[9]. While most of the motor behavioral studies have been conducted on humans, monkeys, and rats[2-8,10-17], mice become an attractive model system because of its powerful genetics and low cost.

Here we present a forelimb specific motor-skill learning paradigm: a single-pellet reaching task. In this paradigm, mice are trained to extend their forelimbs through a narrow slit to grasp and retrieve food pellets (millet seeds) positioned at a fixed location, a behavior analogous to learning archery, dart-throwing, and shooting basketballs in human. This reaching task has been modified from previous rat studies that have shown similar results between mice and rats[18]. Using two-photon transcranial imaging, our previous work has followed the dynamics of dendritic spines (postsynaptic structures for majority excitatory synapses) over time during this training. We found that a single training session led to rapid emergence of new dendritic spines on pyramidal neurons in the motor cortex contralateral to the trained forelimb. Subsequent training of the same reaching task preferentially stabilized these learning-induced spines, which persisted long after training terminated[19]. Furthermore, spines that emerged during repetitions of reaching task tended to cluster along dendrites, whereas spines formed during tandem execution of reaching task and another forelimb-specific motor task (*i.e.* the pasta handling task) did not cluster[20].

In the present video, we describe step-by-step the setup of this behavioral paradigm, from the initial food deprivation to shaping, and to motor training. We also describe the common behaviors of mice during the process of executing this behavioral paradigm, and how these behaviors are categorized and analyzed. Finally, we discuss the precautionary measures needed to practice such a learning paradigm and the issues that may be encountered during data analyses.

Experiments described in this manuscript were performed in accordance with the guidelines and regulations set forth by the University of California, Santa Cruz Institutional Animal Care and Use Committee.

1. Setup (Also See Materials List)

1. Use millet seeds as food pellets.
2. Use a custom-made clear Plexiglas training chamber (20 cm tall, 15 cm deep, and 8.5 cm wide, measured from outside, with the thickness of the Plexiglas 0.5 cm) that contains three vertical slits (one slit on 'shaping' edge, and two slits on the opposite 'training' edge). The vertical slits should be 0.5 cm wide and 13 cm tall and be located on the front wall of the box: in the center, on the left side, and on the right side (**Figure 1A**).
3. Use a tilted tray to hold the seeds used during shaping sessions. The tray can be custom-made from three glass slides (**Figure 1B**).
4. Prepare a food platform (8.5 cm long, 4.4 cm wide, and 0.9 cm tall). This food platform is placed in the front side (facing the trainer) of the training chamber during training sessions. There are two divot slots on the food platform for positioning seeds, one slot on the left, and the other slot on the right side. The divots are 0.3 cm from the long edge, and 2.4 cm from the width edge (**Figure 1C**). The left and right divot slots correspond to the left and right slit in the mouse training chamber and are used for training of dominant forelimbs. The purpose of having these divot slots is to ensure that the seed is placed consistently at the same place for each reaching attempt.
5. During sessions have in hand a pair of forceps, weighing scale, and stopwatch.

2. Food Deprivation (2 Days)

1. Weigh each mouse to obtain a baseline bodyweight before food deprivation.
2. Food-restrict mice for 2 days to initiate bodyweight loss. As a general starting point, mice are given 0.1 g of food per 1 g bodyweight per day (*e.g.* a mouse weighing 15 g, we usually start with 1.5 g of food). Adjust the amount of food based on the baseline bodyweight, the rate of weight loss, sex, and age of mice. While the bodyweight may continue to decrease a little more during the shaping phase, such reduced body weight (*i.e.* ~90% of the original baseline weight) should be maintained throughout training (**Figures 2A** and **2B**). The amount of food necessary to maintain the mouse's body weight is typically the same as the amount used in restriction.

3. Shaping (3-7 Days)

1. Group habitat acclimatization (Day 1): Put two mice into the training chamber at the same time. Place approximately 20 seeds/mouse inside the chamber for their consumption. Allow the mice to stay in the chamber for 20 min and then put them back into their home cage.
2. Individual habitat acclimatization (Day 2): The same setup as in step 3.1, but place mice into the training chamber individually. The purpose of group and individual habitat acclimatization is to get the mice familiar with both the training chamber and the millet seeds.
3. Determination of forelimb dominance (Day 3 and later): Place the single slit side of the training chamber facing downwards (**Figure 1D**). Fill the food tray with seeds. Press the food tray up against the front wall of the training chamber to allow the seeds accessible to the mouse. Place mice into the cage individually. If they are sufficiently interested in the seeds located on the food tray, they would plow through the slit to get the seed inside. They will then pick up the seeds and consume them. 'Shaping' is considered finished when both of the following criteria are met: 1) the mouse conducts 20 reaching attempts within 20 min, and 2) more than 70% reaches are performed with one forelimb.

Notes:

1. If the mouse uses its tongue to get the seeds into the chamber, move the tray back from the slit slightly. The increase in reaching distance discourages the mouse to use its tongue to acquire the seed and therefore facilitates its forelimb reaching.
2. If the mouse cannot finish shaping within a week, drop it from the experiment.

4. Training (8+ Days)

1. Place the double-slit side of the training chamber facing downwards (**Figure 1E**).
2. Place the mice in the cage individually. Put individual seeds on the food platform in the divot corresponding to the preferred paw (*i.e.* for the right-handed mouse, use the slit on the mouse's right side).
3. Observe mouse reaching behavior and score according to the following categories:
   1. **Success:** The mouse reaches with preferred paw, grasps and retrieves the seed, and feeds it into its mouth.
   2. **Drop:** The mouse reaches with preferred paw, grasps the seed, but drops it before putting it into its mouth.
   3. **Fail:** The mouse reaches with preferred paw towards the seed, but it either misses the seed or knocks it off from the holding plate.
4. Train the mice for 30 reaching attempts on the preferred limb or 20 min (whichever comes first) per day.
5. Place the mice back to their home cage after training and provide daily food quantum.

Notes:

1. In some cases, mice reach even when there is no seed placed on the food platform. Such reaches are considered "in-vain reaches" and are not counted towards the total number of reaching attempts. To discourage "in-vain reaches", train mice to walk back to the other end of the training chamber before placing the next seed. A similar strategy has been used in rats for a similar behavioral task[21]. Mice sometimes also

reach with nonpreferred paw in the presence of the seed. These reaches are considered 'contralateral reaches' and do not count towards the total number of reaches either.

2. To limit behavioral variations due to the fluctuations in circadian rhythm, perform all shaping and training sessions at the same time of the day, during normal waking hours for mice.
3. To avoid behavioral variation due to different trainers, make sure the same person trains the same mice throughout the experiment.
4. Attention of mice is critical for this behavioral test. Train mice in a separate and quiet room to minimize environmental perturbation.
5. Mice can be trained with more than 30 reaches daily (*e.g.* 50 reaches). Increasing the number of reaching attempts makes it possible to examine behavioral improvement within the same training session.

5. Data Quantification

There are many ways to quantify mouse behavior following training. Two most straight-forward analyses are:

1. Success rate = successful reaches over total reaching attempts, presented as percentages. Drop and failure rates can be plotted the same way.
2. Speed of success = number of successful attempts divided by time, presented as successful reaches per minute. In most cases, the speed of success continues to increase, even when the success rate attains the plateau.

Representative Results

Learning curve:

Mastery of a motor skill often requires persistent practice over time. A typical averaged learning curve is composed of two phases: an initial acquisition phase during which the success rate improves progressively, and a later consolidation phase when the success rate reaches the plateau (**Figure 2C**). It should be noted the learning curves of individual mice vary; different mice take different numbers of days to reach the plateau level, and the individual learning curves are usually not as smooth as the average one. Another way to present the mouse's improvement of reaching skills is the speed of success, which reflects the overall motor performance by taking accounts of the reaching speed as well as the accuracy. In general, the speed of success keeps improving after the mouse reaches its plateau success rate (**Figure 2D**).

In addition to individual variations of performance, some mice fail to learn the task (**Figure 2E**). These "nonlearners" usually make initial attempts to reach for the seeds, but for unknown reasons perform poorly despite repetitive training. They generally lose interest in reaching for the seeds after continuously failed attempts and stop reaching after 6-8 days. By contrast, some mice are over-shaped (**Figure 2E**). These over-shaped mice usually start with a very high success rate (>40%), and do not make significant improvement (*i.e.* 15% increase in success rate compared to first day of training) in motor performance with continued training. Their learning curves stay relatively flat, or may even decrease with continued training.

Motor memory:

Our previous data have suggested that once a motor skilled movement is mastered through repetitive practice, it can be stored as a form of motor memory and further practices are not required for its maintenance[19]. This motor memory can be measured by stopping the training after mice have reached plateau level of success rates, and retest their performance after an extended resting period (*e.g.* a few months). Retrained mice usually start with comparable success rates as have observed at the end of earlier training, and maintain high success rate in subsequent days. By contrast, age-matched naïve mice usually start with significantly lower success rates and progressively improve their performance with practice[19] (**Figure 2F**). While food deprivation (2 days) before commencement of retraining is required, reshaping the previously trained mice is not necessary.
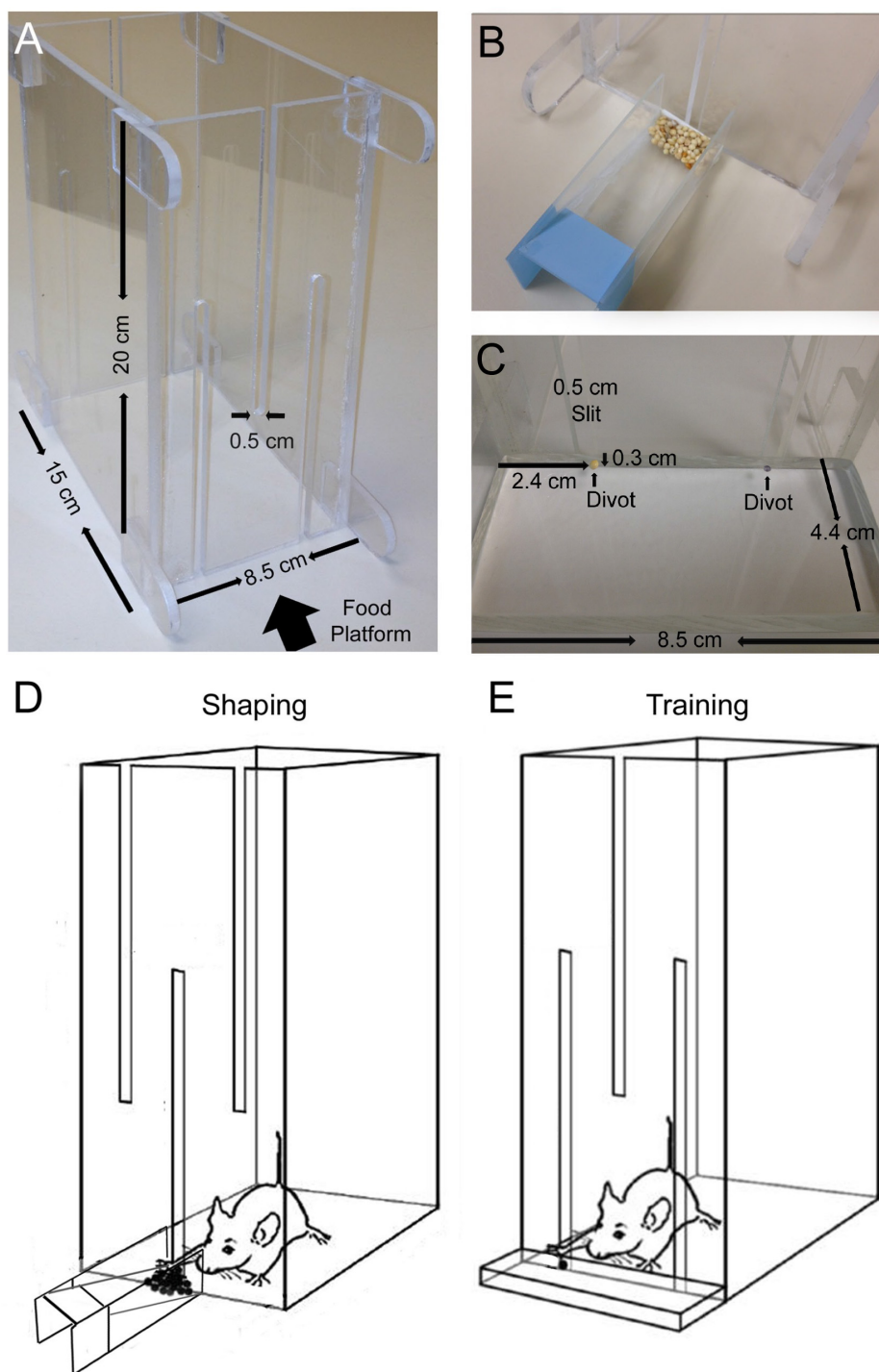
Figure 1. Design of the mouse training chamber. A. A photograph of the training chamber, with dimensions indicated. **B.** A photograph of the shaping seed tray, made from three slides glued together and placed in front of the shaping chamber. A pile of millet seeds are placed at the trough of the seed tray. **C.** A photograph of the food tray for training, placed in front of the training chamber. One millet seed is placed on the divot (pointed by arrows) that corresponds to right forelimb training. Dimensions and the location of the divot are indicated in the photograph. **D.** A cartoon drawing of the shaping chamber. The single-slit side of the training chamber is placed facing downwards. Seeds are placed in front of the middle slit and mice can use both paws to plow the seeds. **E.** A cartoon drawing of the training chamber. The double-slit side of the training chamber is placed facing downwards. One seed is placed on the food tray in front of the chamber slit corresponding to the preferred limb (in this case, the right forelimb). (**D** and **E**, modified from Xu et al.[19]). Click here to view larger image.
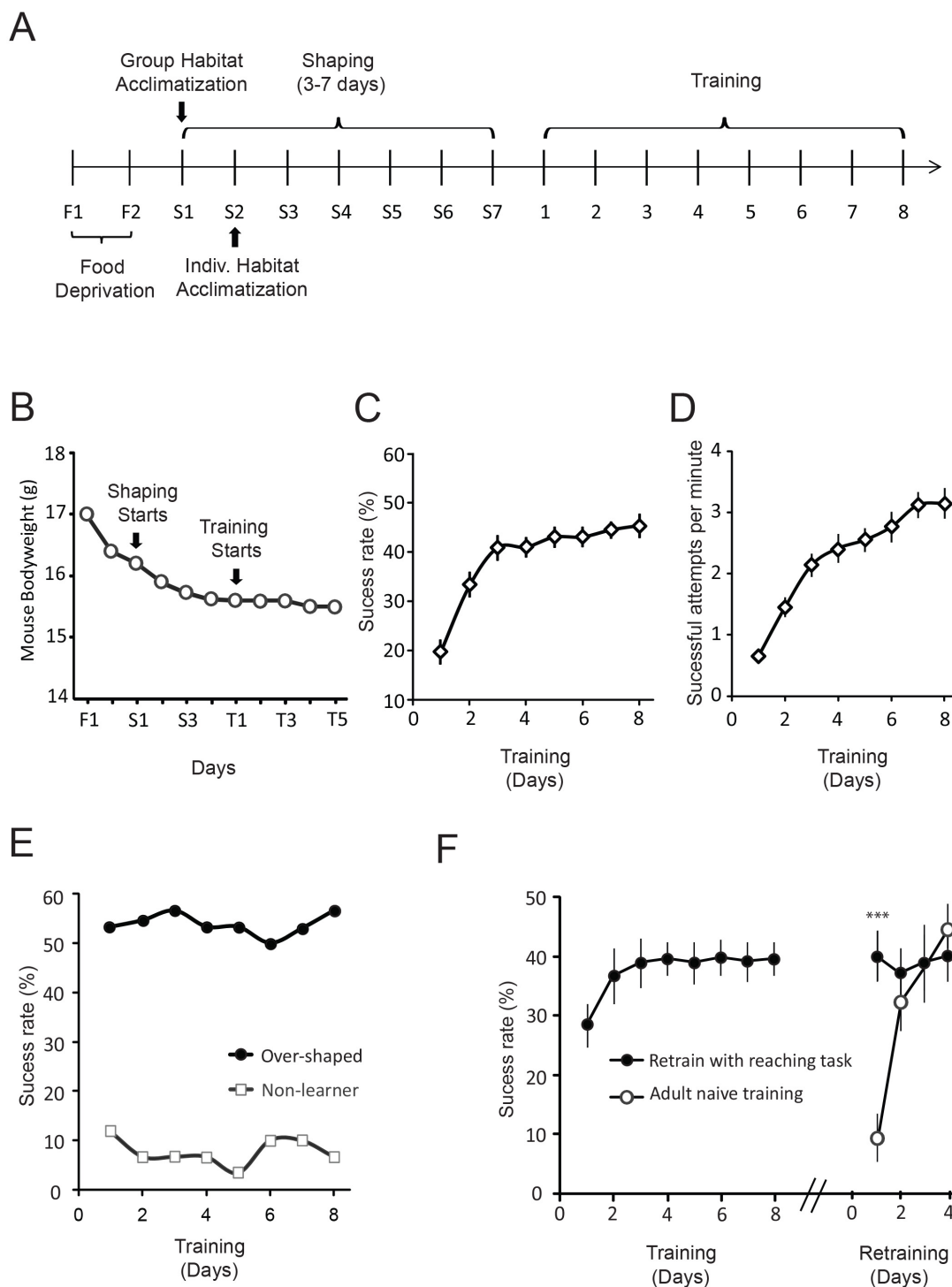
Figure 2. Representative results of mouse single-pellet reaching tasks. A. A general timeline of the experimental design. **B.** An example of bodyweight loss for a single mouse during food deprivation (F), shaping (S), and training (T). **C.** Average success rates improve over time during training (n=39). **D.** Average speed of success from the same group of mice presented in **C**. **E.** Success rates during training for an over-shaped mouse and a nonlearning mouse. **F.** Motor performance of pretrained (n=14) and naïve (n=10) adult mice during 4 day training (modified from Xu *et al.*[19]). All data are presented as mean ± s.e.m., ***, *P*<0.001. Click here to view larger image.

Discussion

Importance of the shaping phase:

Because of increased anxiety from being in an unknown environment, it is usually difficult for mice to be trained in a novel environment[21,22]. Therefore, the goal of shaping is to familiarize mice with the training chamber, the trainer (*i.e.* reduce their anxiety levels), and the task requirements (*i.e.* to identify seed as food source). Another goal of the shaping is to determine the preferred limbs of individual mice for future training. During shaping, it is critical that the mice are not over-shaped, as it may provide unwanted "reaching practice sessions", which would falsely inflate the success rate in the initial learning phase, resulting in no or little subsequent improvement of success rate in later training (**Figure 2E**). In these cases, rather than simply plowing the seed into the cage and then picking it up to eat, the over-shaped mice already started developing reaching skills during the shaping phase. The only way to limit over-shaping is keen observation during the shaping sessions. Over-shaped mice should be excluded from the data analysis. On the other hand, it is equally important that mice are not under-shaped. If the mouse does not recognize the millet seeds as the food source, if the mouse is unfamiliar with the task requirement, and/or if the mouse is too anxious, it will experience difficulties during the training sessions and will likely end up as a nonlearner (**Figure 2E**, also see "nonlearners" below).

Implementing proper controls:

To determine whether or not the associated changes are due to reaching-specific motor learning, it is important to implement various forms of controls. Several controls can be used: 1) The general control group: in this group, mice do not experience training or shaping, but food restriction, the food reward (seeds), and handling. 2) The shaping control group: mice in this group experience the shaping period as described above (see protocols), however they are not trained subsequently. Instead, they are placed in the training cage for 20 min, and provided with approximately 20 seeds/mouse. The shaping control group is useful for determining whether experience of the shaping period caused any plasticity in the interested brain areas. 3) The activity control group: in this group, mice experience exactly the same conditions as the trained mice except during the training period, the seed is always placed outside of the reaching range or held by the trainer. Hence, the activity of the forelimb muscles is similar to the trained mice, but the mice in the activity control group, unlike the trained mice, do not acquire the reaching skills. To promote continuous reaching attempts, seeds are periodically dropped in the training chamber from the reaching slit, and mice would pick up the seeds for consumption. Most of activity controls give up reaching after 6-8 days of training.

Nonlearners:

It is uncertain why some mice learn while others do not. Speculating from our experiences, some of these nonlearners may result from wrong judgment of paw preference, thus the acquisition of skilled movement is hampered by training the nondominant limb. It is also possible that under-shaping is responsible for these nonlearners, for these mice are not clear on the task requirement, and/or not yet comfortable with the testing environment. Other possible reasons include losing weight too rapidly and/or too much weight loss, while others might have lost not enough weight, in which either scenario will result in decreased hunger level and impeded motivation to reach for the food, thereby making the learning process difficult. Regardless of the reasons, these nonlearners do not acquire nor master the reaching skills, and may be treated as an additional type of control group, complementing the various types of controls mentioned above.

Circuitry for motor learning and memory:

Many brain regions have been identified to be involved in motor learning. In addition to the primary motor cortex[6,7,19,23], many other brain regions such as the substantial nigra and ventral tegmental area[16,24], striatum[25], and hippocampus[26] have been suggested to also play important roles in the single seed reaching task introduced here. Therefore, the single seed reaching task may be useful to study many discriminatory regions of the rodent brain associated with motor learning. Moreover, there is a plethora of other motor tasks, each with its own temporospatial pattern of motor execution, as well as brain macro/microstructures involved. For example, the accelerated rotarod has been used to study long term motor memory in both striatum and hippocampus[27], the delay eye-blink conditioning response engages implicit procedural motor learning which is predominantly mediated by the cerebellum[28,29], while the running-wheel task depends on the proper functioning of dorsal striatum[30]. Even for the motor tasks that induce synapse reorganization in the cortex (*i.e.* the Capellini handling task and single-pellet reaching task), different sets of synapses are likely to be involved in different tasks[19]. Such data suggest that each motor task could have its own specific neural coding, by recruiting different brain regions, neuronal populations and synapse sets. Proper experiments should be used to study different brain structures and motor circuits.

In summary, we have introduced a detailed protocol on how to perform the single-pellet reaching task in mice. This reliable and valid protocol will be useful for future researchers who are interested in studying biochemical, structural, physiological, and genetic changes in many discriminatory regions of the mouse brain associated with motor learning and memory formation.

Disclosures

The authors declare no conflict of interest.

Acknowledgements

References

1. Schmidt, R. A. Motor Learning Principles for Physical Therapy. *In: Foundations for Physical Therapy. Contemporary Management of Motor Control Problems, Proceedings of the II STEP Conference.* Alexandria, VA: Foundations for Physical Therapy. 49-63 (1991).
2. Honda, M., Deiber, M.P., Ibanez, V, Pascual-Leone, A., Zhuang, P. & Hallett, M. Dynamic cortical involvement in implicit and explicit motor sequence learning. A PET study. *Brain.* **121**, 2159-2173, doi:10.1093/brain/121.11.2159 (1998).
3. Karni, A., Meyer, G., Jezzard, P., Adams, M.M., Turner, R. & Ungerleider, L.G. Functional MRI evidence for adult motor cortex plasticity during motor skill learning. *Nature.* **377**, 155-158, doi:10.1038/377155a0 (1995).
4. Karni, A. *et al.* The acquisition of skilled motor performance: fast and slow experience-driven changes in primary motor cortex. *Proc. Natl Acad. Sci. U.S.A.* **95**, 861-868, doi:10.1073/pnas.95.3.861 (1998).
5. Muellbacher, W. *et al.* Early consolidation in human primary motor cortex. *Nature.* **415**, 640-644, doi:10.1038/nature712 (2002).
6. Kleim, J. A., Hogg, T.M., VandenBerg, P.M., Cooper, N.R., Bruneau, R. & Remple, M. Cortical synaptogenesis and motor map reorganization occur during late, but not early, phase of motor skill learning. *J. Neurosci.* **24**, 628-633, doi:10.1523/jneurosci (2004).
7. Rioult-Pedotti, M. S., Friedman, D. & Donoghue, J. P. Learning-induced LTP in neocortex. *Science.* **290**, 533-536, doi:10.1126/science.290.5491.533 (2000).
8. Rioult-Pedotti, M. S., Friedman, D., Hess, G. & Donoghue, J. P. Strengthening of horizontal cortical connections following skill learning. *Nat. Neurosci.* **1**, 230-234, doi:10.1038/678 (1998).
9. Farr, T. D. & Whishaw, I. Q. Quantitative and qualitative impairments in skilled reaching in the mouse (Mus musculus) after a focal motor cortex stroke. *Stroke J. Cereb. Circ.* **33**, 1869-1875, doi:10.1161/01.STR.0000020714.48349.4E (2002).
10. Huang, V. S., Haith, A., Mazzoni, P. & Krakauer, J. W. Rethinking motor learning and savings in adaptation paradigms: model-free memory for successful actions combines with internal models. *Neuron.* **70**, 787-801, doi:10.1016/j.neuron.2011.04.012 (2011).
11. Smith, M. A., Ghazizadeh, A. & Shadmehr, R. Interacting adaptive processes with different timescales underlie short-term motor learning. *PLoS Biol.* **4**, e179, doi:10.1371/journal.pbio.0040179 (2006).
12. Pavlides, C., Miyashita, E. & Asanuma, H. Projection from the sensory to the motor cortex is important in learning motor skills in the monkey. *J. Neurophysiol.* **70**, 733-741 (1993).
13. Paz, R., Boraud, T., Natan, C., Bergman, H. & Vaadia, E. Preparatory activity in motor cortex reflects learning of local visuomotor skills. *Nat. Neurosci.* **6**, 882-890, doi:10.1038/nn1097 (2003).
14. Paz, R. & Vaadia, E. Learning-induced improvement in encoding and decoding of specific movement directions by neurons in the primary motor cortex. *PLoS Biol.* **2**, E45, doi:10.1371/journal.pbio.0020045 (2004).
15. Plautz, E. J., Milliken, G. W. & Nudo, R. J. Effects of repetitive motor training on movement representations in adult squirrel monkeys: role of use versus learning. *Neurobiol. Learn. Mem.* **74**, 27-55, doi:10.1006/nlme.1999.3934 (2000).
16. Hosp, J. A., Pekanovic, A., Rioult-Pedotti, M. S. & Luft, A. R. Dopaminergic projections from midbrain to primary motor cortex mediate motor skill learning. *J. Neurosci.* **31**, 2481-2487, doi:10.1523/jneurosci.5411-10.2011 (2011).
17. Adkins, D. L., Boychuk, J., Remple, M. S. & Kleim, J. A. Motor training induces experience-specific patterns of plasticity across motor cortex and spinal cord. *J. Appl. Physiol.* **101**, 1776-1782, doi:10.1152/japplphysiol.00515.2006 (2006).
18. Whishaw, I. Q. An endpoint, descriptive, and kinematic comparison of skilled reaching in mice (Mus musculus) with rats (Rattus norvegicus). *Behav. Brain Res.* **78**, 101-111, doi:10.1016/0166-4328(95)00236-7 (1996).
19. Xu, T. *et al.* Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature.* **462**, 915-919, doi:10.1038/nature08389 (2009).
20. Fu, M., Yu, X., Lu, J. & Zuo, Y. Repetitive motor learning induces coordinated formation of clustered dendritic spines *in vivo*. *Nature.* **483**, 92-95, doi:10.1038/nature10844 (2012).
21. Whishaw, I. Q., Whishaw, P. & Gorny, B. The structure of skilled forelimb reaching in the rat: a movement rating scale. *J .Vis. Exp.* doi:10.3791/81 (2008).
22. Bailey, K. R. & Crawley, J. N. Anxiety-Related Behavior in Mice. *In Buccafusco JJ (Ed.) Methods of Behavior Analysis in Neuroscience.* Chapter 5, 2nd ed. Boca Raton, FL: CRC Press (2009).
23. Yu, X. & Zuo, Y. Spine plasticity in the motor cortex. *Curr. Opin. Neurobiol.* **21**, 169-174, doi:10.1016/j.conb.2010.07.010 (2011).
24. Qian, Y., Chen, M., Forssberg, H. & Diaz Heijtz, R. Genetic variation in dopamine-related gene expression influences motor skill learning in mice. *Genes Brain Behav.* **12**, 604-614, doi:10.1111/gbb.12062 (2013).
25. MacLellan, C. L., Gyawali, S. & Colbourne, F. Skilled reaching impairments follow intrastriatal hemorrhagic stroke in rats. *Behav. Brain Res.* **175**, 82-89, doi:10.1016/j.bbr.2006.08.001 (2006).
26. Hong, S. M. *et al.* Reduced hippocampal neurogenesis and skill reaching performance in adult Emx1 mutant mice. *Exp. Neurol.* **206**, 24-32, doi:10.1016/j.expneurol.2007.03.028 (2007).
27. Bureau, G., Carrier, M., Lebel, M. & Cyr, M. Intrastriatal inhibition of extracellular signal-regulated kinases impaired the consolidation phase of motor skill learning. *Neurobiol. Learn. Mem.* **94**, 107-115, doi:10.1016/j.nlm.2010.04.008, (2010).
28. McCormick, D. A., Lavond, D. G. & Thompson, R. F. Neuronal responses of the rabbit brainstem during performance of the classically conditioned nictitating membrane (NM)/eyelid response. *Brain Res.* **271**, 73-88, doi:10.1016/0006-8993(83)91366-5 (1983).
29. Molinari, M. *et al.* Cerebellum and procedural learning: evidence from focal cerebellar lesions. *Brain.* **120**, 1753-1762, doi:10.1093/brain/120.10.1753 (1997).
30. Willuhn, I. & Steiner, H. Motor-skill learning in a novel running-wheel task is dependent on D1 dopamine receptors in the striatum. *Neuroscience.* **153**, 249-258, doi:10.1016/j.neuroscience.2008.01.041 (2008).