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Overview
Behavioral Phenotyping of Rodents

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Established methods for analyzing behavioral traits in mutant lines of mice allow researchers to understand the outcomes of genetic manipulations in the nervous system. A rigorous six-tiered behavioral phenotyping strategy is described. Recommendations are offered for the design of mouse behavioral testing suites in animal housing facilities.

Fascinating new genetic technologies have emerged in the era of the Human Genome Project. We are now better able to understand the role of genes in normal biological processes and disease states. Genetic techniques used in rodent research include classic pedigree analysis of natural mutants, selective breeding, quantitative trait loci linkage analysis, whole-genome scans, chemical mutagenesis, targeted gene mutation, DNA microarrays, protein microarrays, and pharmacogenetics (1-9). Mice have become the species of choice for much of the basic research and many of the disease models (10-12). Rigorous, accurate, comprehensive phenotyping is the necessary partner to the molecular gene manipulations. For genes expressed in the brain, behavioral phenotyping is often the key discipline to explicate the functional outcome of a mutation. The commentary presented here will focus on strategies for behavioral phenotyping in mice. The concepts described herein can be equally applied to other phenotyping disciplines, including pathology, anatomy, physiology, biochemistry, and clinical chemistry (13-16).

Behavioral neuroscience has a long and illustrious history (17-23). A large number of rodent behavioral tests are available in domains such as social behaviors, reproduction, feeding, motor functions, sensory abilities, emotional responsivity, learning, and memory, as described (10, 23-30). We define behavioral phenotyping as "the complete characterization of the mutant mouse line on behavioral tests designed to address the hypothesized functions of the product of the targeted gene." This commentary describes some of the well characterized and standardized tests for behavioral phenotyping in mice. Further issues related to laboratory animal science and veterinary medicine are discussed within the phenotyping context. All methods described herein were approved by the National Institute of Mental Health Animal Care and Use Committee, and conform with the NIH Guidelines, "Using Animals in Intramural Research."

Six-tiered Strategy for Behavioral Phenotyping

This commentary will be focused on mice with a targeted gene mutation designed to generate a transgenic or knockout line. Transgenics have extra copies of a normal gene inserted into the genome, or a new gene, such as a human disease gene, inserted into the mouse genome. Knockouts, also called null mutants, have a mutation that disrupts the cDNA sequence of a gene, so that its protein product is not synthesized. Molecular methods for generating a new transgenic or knockout are extensively described elsewhere (3, 10).

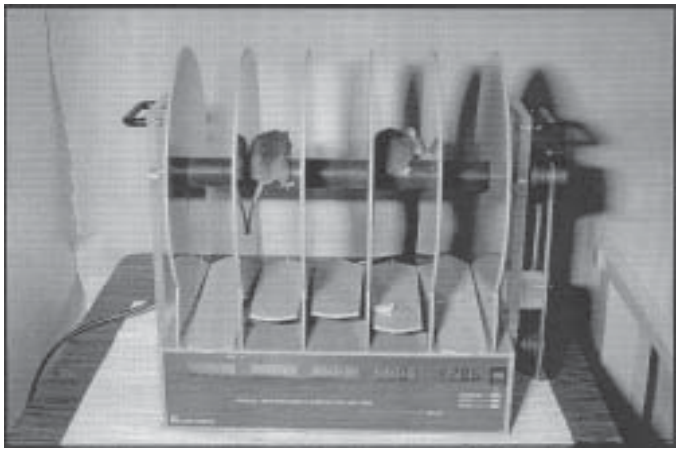

Figure 1. Ugo Basile mouse accelerating rotarod. The mouse must walk forward to balance on the rotating center bar. Revolutions per minute increase from 4 to 40 over a 5-min test session. Latency to fall is the independent variable. Normal mice are able to stay on the rotarod for most of the 300-sec session. Rotarod performance evaluates motor coordination and balance. Improvement in rotarod performance across repeated daily sessions provides a measure of motor learning. Mice with mutations in genes expressed in the cerebellum, and mouse models of motor diseases, such as Parkinson's, Huntington's, amyotrophic lateral sclerosis, ataxia telangiectasia, and Tay-Sachs, display poor rotarod performance (33).

To begin the behavioral phenotyping of a new transgenic or knockout line of mice, our laboratory and others have designed strategies involving sequential testing across a constellation of baseline behavioral tasks (10, 25, 31, 32). The goal is to enhance detection of the phenotype(s) specific to the gene, while avoiding artifactual interpretations.

One scheme (Appendix) is commonly used in our laboratory (25, 33-35). Specific tests are listed under each heading and are illustrated in Fig. 1-3. The first stage of evaluation of a new mutant mouse line is to look for obvious health problems that would impair performance on any behavioral task. A sick mouse

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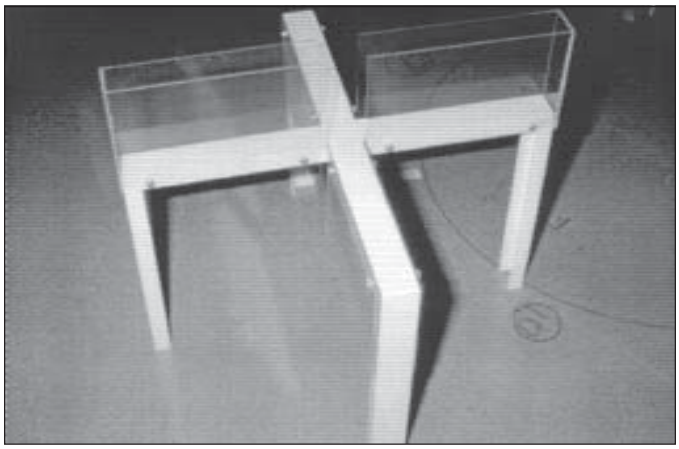

Figure 2. Elevated plus maze for analyzing anxiety-related behaviors. Naturalistic approach-avoidance conflict tests are widely used to model anxiety-related behaviors and the effects of anxiolytic drugs. This plus-shaped platform is raised one meter above the floor. Mice tend to explore novel environments, but prefer the arms with enclosed walls versus the open arms without walls. Anxiolytics increase the number of entries into the open arms and the amount of time spent on the open arms. Mice with mutations in neurotransmitter systems relevant to anxiety, such as GABA receptor subunits, substance P, galanin, corticotropin-releasing factor receptors, serotonin receptors, and the serotonin transporter, display unusual scores on the elevated plus maze (35, 38, 45).

is often poorly groomed, lethargic, and hyperreactive to handling. The second stage includes testing of a set of simple neurologic reflexes. A mutant mouse showing deficits on a neurologic reflex test is likely to be too impaired to perform many complex behavioral tasks. The third stage is testing of a series of sensory tasks. Sensory deficits may confirm the predicted hypothesis for genes involved in deafness, blindness, olfaction, taste, and analgesia. In the fourth stage, motor abilities are analyzed. Motor deficits may be specifically hypothesized for the targeted gene mutation (e.g., for genes expressed in muscle, spinal cord, striatum, or cerebellum). In such instances, these four stages of testing complete the phenotyping sequence.

The fifth stage addresses more complex hypotheses about functions of genes in the brain, usually relevant to neuropsychiatric disorders. Sets of complex tasks are chosen to analyze functions in each predicted behavioral domain. To choose the most relevant set of tasks, animal models of mental retardation, obesity, Huntington's disease, or schizophrenia require insights into the etiopathogenesis and clinical signs of the disease state, and thorough review of the mouse behavioral literature (10, 31). Genes mediating learning and memory, feeding, sexual behaviors, aggression, propensity to self-administer drugs of abuse, anxiety-like behaviors, and depression-related behaviors require carefully designed tests with appropriate controls and statistical evaluation. The novice is well advised to choose tests that have been previously well validated in the behavioral neuroscience literature by manipulations, such as drug treatments or lesions. Ideally, three or more tests within each behavioral domain are conducted. The constellation of tests is chosen for diversity of concept and modality. For example, if a mutation impairs feeding during four very different types of feeding tasks, strong conclusions can be drawn about the role of that gene in reducing ingestion. If a mutation is found to impair only carbohydrate consumption, hypotheses about macro-nutrient

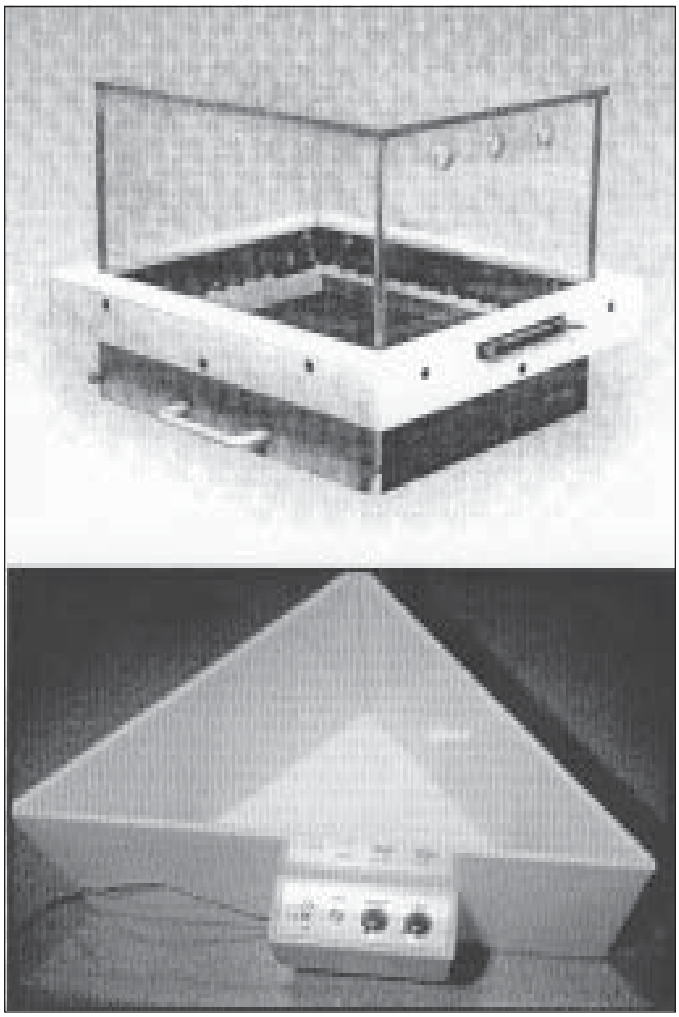

Figure 3. Cued and contextual fear conditioning apparatus for quantitating emotional learning and memory. Mice display freezing in response to an aversive stimulus. Freezing, defined as complete immobility except for breathing, is a species-specific response to fear-inducing environmental stimuli. Freezing in mice is quantitated by a human observer or automated videotracking system. High levels of freezing are seen immediately after a foot shock, delivered through the floor grid along with an auditory cue, in the foot shock chamber illustrated in the top panel. One day later, a mouse placed back in this chamber, in the identical room environment, shows high levels of freezing (contextual conditioning). Placing the mouse in a different environment, as illustrated in the bottom panel, induces low levels of freezing. If the auditory cue is sounded in the novel environment, freezing increases (cued fear conditioning). This fear conditioning task has detected memory deficits in mice with mutations in signal transduction genes, thought to be involved in cognitive processes (41).

regulation by the gene are explored in further macro-nutrient choice tests.

Stages 1-4 allow the investigator to avoid false-positive interpretations. For example, most learning and memory tests rely on a sensory cue, and require a motor response. A deaf mouse will do poorly on an auditory-cued operant task. A blind mouse will do poorly on the Morris swim task for spatial navigation that is based on visual room cues. Altered pain sensitivity will interfere with performance on a footshock avoidance task. Motor ataxia will interfere with radial maze running. Thus, an artifact, such as blindness or muscle weakness, could be misinterpreted as a

memory deficit, if sensory and motor abilities of the mutant line were not also evaluated. If a sensory or motor dysfunction is detected, the researcher can design tests that do not require that particular sensory or motor ability. For example, artifacts due to blindness could be avoided by choosing an olfactory memory task. A side effect of poor motor coordination and balance, detected in the accelerating rotarod task (Fig. 1), would be a problem for studies of circadian rhythm genes using a wheel-running cage, but not a problem for studies addressing the circadian rhythm of body temperature.

Stage 5, testing specific hypotheses within each behavioral domain of interest, addresses the original goal of the research. We recommend conducting three or four complementary tests within each behavioral domain of interest, to avoid false-negative interpretations. An interesting phenotype could easily be missed if only one quick task is run. For example, if the mutation is predicted to affect social behaviors, a five-minute test of social interaction between unfamiliar juvenile males is likely to detect a different type of social deficit than is a mating test between an adult male and female, or parental retrieval of pups removed from the nest. Anxiety-like behaviors on the elevated plus maze (Fig. 2) may be more relevant to human generalized anxiety, whereas anxiety-like behaviors in fear-conditioned startle may be more relevant to posttraumatic stress disorder.

In-depth descriptions of commonly used tasks for studying complex behaviors in mice, (Appendix and Fig. 1-3) are available (10, 36, 37). Cognitive tasks include spatial navigation in the Morris water task, Barnes maze, radial maze, T-maze, and Y-maze; schedule-induced reinforcement in an operant chamber; and aversive tasks, such as passive avoidance, cued and contextual fear conditioning (Fig. 3), and taste aversion. Attention is measured in a five-choice serial reaction time chamber or on operant timing schedules of reinforcement. Feeding assessment may involve 24-h consumption, limited daily access, macronutrient sources, taste discrimination, and sham feeding. Parental behaviors are quantitated as latency to retrieve pups to the nest, crouching over the pups, nursing the pups, grooming the pups, and time spent in the nest. Good models of anxiety-related behaviors include the elevated plus maze, the elevated zero maze, light/dark exploratory transitions, emergence from a small, dark, enclosed start box into an open field, and the Vogel thirsty-lick conflict test. Candidate genes for the propensity to abuse drugs, such as cocaine and alcohol, can be evaluated, using intravenous self-administration, conditioned place preference, and two-bottle choice tests. Step-by-step protocols for many of these rodent behavioral tests are published (36).

The sixth stage represents the translational value of the phenotyping process. The aberrant phenotype detected in a mutant mouse model of a human genetic disease is used to evaluate treatments for the disease. For example, rotarod deficits are a sensitive measure of functional improvement following stem cell transplantation in mouse models of Tay-Sachs and Sandhoff diseases (33). Anxiety-related behavioral phenotypes for corticotropin-releasing factor (CRF) receptor for knockout mice (38, 39) enable the search for CRF-1 selective receptor antagonists as treatments for stress-related diseases. Cyclic AMP response element binding protein (CREB) knockout mice with memory deficits provide a model system for testing memory enhancers working through postsynaptic signaling cascades (40, 41). Learning deficits in galanin-overexpressing transgenic mice can be used to evaluate the ability of galanin receptor antagonists to improve cognition (34). Amyloid-overexpressing mouse models of Alzheimer's disease are being used to screen immunization treatments designed to slow or reverse the accumulation of β-amyloid senile plaques (42).

Appendix: Multi-tiered mouse behavioral strategy for comprehensive behavioral phenotyping of mutant mice.

A new mutant mouse line is first "given a physical examination." Simple behavioral measurement of general health, home cage behaviors, and neurologic reflexes reveals any severe disabilities that will prohibit further behavioral testing. Sensory and motor tasks evaluate physical abilities. To avoid over-interpretation of artifacts, a deficit in one sensory or motor modality can be taken into account when designing more complex behavioral tests. Hypothesis-driven analyses are designed to include constellations of three or more tasks within each specific behavioral domain of interest. A robust behavioral phenotype becomes a useful surrogate marker for quantitating the efficacy of pharmacologic and gene therapies (adapted from reference 62).

I. General Health
Body weight, body temperature, appearance of fur and whiskers, home cage activity, reproductive success, aggression, nesting patterns

II. Neurologic Reflexes
Righting reflex, eye blink, ear twitch, whisker orientation, sensorimotor gating

III. Sensory Abilities
Gross measures: Preyer reflex, acoustic startle, visual cliff, visual placing (forepaw reach) response, sniffing a novel object, locating buried food, hot plate, tail flick

Sensory acuity: Reinforced behavioral discrimination tasks using olfactory, taste, visual, or auditory stimuli; Auditory brainstem response, neurophysiological recording from the sensory cortex during presentation of sensory stimuli; Von Frey hairs; taste aversion

IV. Motor Functions
Open field locomotion, home cage activity, circadian running wheel, rotarod, wire hang, grip gauge, footprint analysis, balance beam

V. Specific Behavioral Domains
Feeding: 24-h consumption, two-hour restricted access, refeeding after withholding of food overnight, two-bottle choice test, macronutrient selection, lickometer 24-h circadian meal pattern analysis

Learning and memory: Morris water maze, Barnes maze, radial maze, T-maze, Y-maze, contextual and cued fear conditioning, social recognition, object recognition, social transmission of food preference, passive avoidance, active avoidance, operant nose-poke reinforcement schedule, five-choice serial reaction time, rotarod motor learning, eyeblink conditioning, conditioned taste preference, conditioned taste aversion

Sexual: Male mounting, intromissions, ejaculation; female lordosis

Parental: Nest building, pup retrieval, crouching over pups, lactation, litter yield

Social: Social grooming, nesting, social dominance, aggression, juvenile play

Anxiety-like: Elevated plus maze, zero maze, light↔dark transitions, emergence test, center time in open field, staircase test, defensive burying, Vogel conflict test, Geller-Seifter conflict test

Depression-related signs: Porsolt swim test, tail suspension test

Schizophrenia-related signs: Prepulse inhibition, sensitization to psychostimulants, latent inhibition

Drug abuse models: Self-administration, conditioned place preference, two-bottle choice

VI. Evaluating Therapies
Optimized parameters of the behavioral phenotype are employed as the independent variable to evaluate pharmacological and gene therapies in mutant mouse models of human genetic diseases

Commitments and Caveats

Recent advances in mouse genetics translate into increasingly huge numbers of mice. Modern animal facilities have been built or retooled to handle these new research needs. Behavioral phenotyping, in particular, requires large amounts of dedicated

procedure room space, specialized equipment in long-term use, and large experimental group sizes. Ten mice per genotype is a bare minimum for an interpretable behavioral experiment. If sex differences are detected for the phenotype of interest, the researchers may need to test separate groups of males and females, thus doubling the required numbers. The reason for the large samples sizes is that behavior, like many biological processes, is characterized by innate variability among animals, and by variable and unpredictable environmental influences. To reduce biological variability, we design experiments with 15 or 20 mice per genotype and per sex. Averaging across large numbers of animals will minimize the effects of random environmental factors as much as possible. Further, these large numbers are necessary to satisfy the requirements of proper statistical analyses of the data (e.g., repeated measures analysis of variance followed by Newman-Keuls or Tukey's post-hoc tests). A second cohort of mice of similar numbers is needed to repeat the experiment, to confirm an initial finding.

These high numbers are dismaying to many molecular geneticists and veterinary staff. Large numbers of cages must be committed for months or years. Housing rooms must be maintained under constant conditions for the duration of the experiment. In particular, housing must be quiet. Construction noise, such as hammering and drilling, induces seriously deleterious effects on mouse behavior. Building construction projects, or even repairs in the vivarium will often shut down a behavioral experiment. Malfunctions in the circadian light timer, temperature and humidity controls, automatic watering system, water bottle spouts, or food hoppers have severe effects on behavior. Loud music may help the animal caretakers get through their difficult and much appreciated work, but are likely to adversely affect behavioral experiments. Cage or cage litter needs to be changed at the end of the day, since a new cage or fresh litter usually increases home cage activity, so that scores in behavioral tasks conducted immediately after a cage change become more variable.

Procedure rooms must be dedicated to long-term behavioral equipment. The equipment is often so delicate, that it cannot go through a standard cage wash. Video cameras and computers are routinely located within or adjacent to the behavioral test rooms. Special standard operating procedures for cleaning behavioral test equipment are conducted by the investigators. For experiments in which the animals live in the equipment for more than 24 h, such as long-term diet studies, the procedure room is, in effect, a housing room. However, due to the delicate equipment, the procedure room cannot be hosed down, but again must be cleaned by the caretakers or investigators following specialized protocols.

Mouse behaviors in many tasks are highly sensitive to interruptions, door openings, and the sights and smells of people. Facility inspections need to be scheduled to avoid interruptions of ongoing experiments. Compromises between the AAALAC standards and the research requirements have been worked out to address most of these issues.

Mouse behavioral phenotyping facilities are now being designed to minimize the difficulties inherent in behavioral phenotyping research. Closed facilities, in which animals taken out of the housing facility cannot be brought back in, are built with suites of behavioral procedure rooms located inside the facility barrier. Facilities maintained at high levels of cleanliness require considerable protective clothing, including jumpsuit, booties, hairnet, mask, and gloves. Behavioral neuroscientists spend most of their workdays running animals through behavioral tasks. This can translate into eight to 10 h per day, every day, for years. Procedure rooms are often small, 80 to 150 ft^2, closed, and windowless. Even the most dedicated investigator may suffer from impaired performance in conducting long, complex experiments under working conditions that involve claustrophobic rooms, hot and constricting protective clothing, and isolation from other researchers.

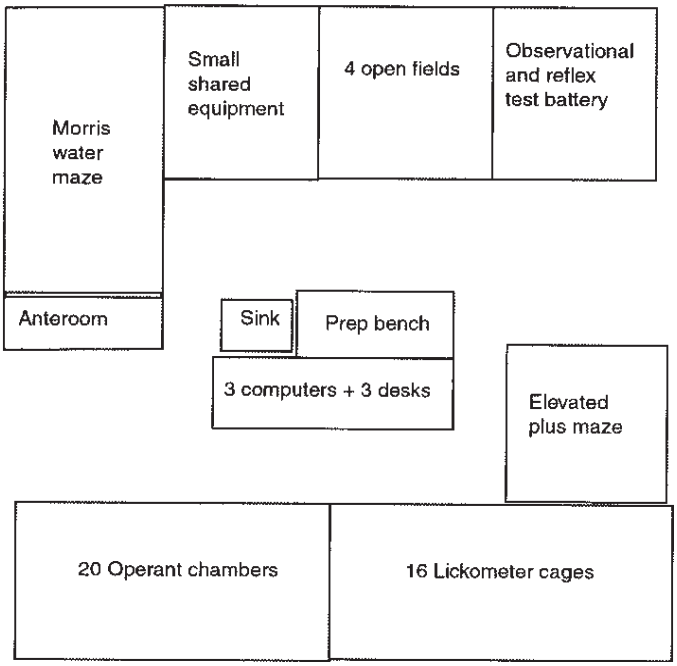

Figure 4. Idealized design for a mouse behavioral phenotyping suite. Dedicated behavioral procedure rooms are arranged around the perimeter. Investigator stations are located in the central space. Open entrance/exit areas are located at the left and right sides of the suite. See text for description of room usage and design advantages.

Better behavioral phenotyping suites are being designed to improve working conditions. One useful design is shown in Fig. 4. Test rooms are arranged around the perimeter. Investigator areas are clustered in the center. Test rooms vary in size. Four small test rooms (e.g., 100 ft^2 [approx. 10 m^2]) accommodate occasional use with small pieces of equipment. For example, one room could accommodate a rotarod (Fig. 1) a hot plate (analgesia test), two light/dark anxiety boxes, and two conditioned place preference boxes. Shelves, tables, and storage cabinets in these rooms allow storage of these several pieces of equipment until needed. When an investigator is ready to run an analgesia test, the hot plate is taken down from the shelf, 60 mice are tested over a three-hour period, and the hot plate is then returned to the shelf. These minimalist rooms could be made available to multiple investigators on a sign-up basis. Time-sharing and space-sharing are most feasible when a trained behavioral neuroscientist supervises the suite.

Three large test rooms (e.g., 200 ft^2 [approx. 20 m^2]) are dedicated to larger, long-term equipment. One large room contains a Morris water maze, with a video camera mounted from the ceiling. Visual cues are taped to the walls. This room has an antechamber, with space for a cart that holds several cages of mice

that are rotated through the daily training sessions. Computer controls are wired from the camera through the ceiling to the central control area. Another large room contains 20 operant chambers, similarly wired to a second computer in the central area. A third large room houses 16 cages fitted with lickometers to analyze 24-h consumption of a high-fat diet and microstructural analyses of meal pattern. The 16 cables exit to a third computer in the central area. Each test room is soundproofed and equipped with variable light intensities and a video camera. Doors to each room have a small window with a metal cover. The window can be uncovered to allow investigators and guests to watch an ongoing experiment without disturbing the mice.

The central control area is about 400 ft^2 (approx. 40 m^2). Computers and desks are grouped for convenient use by the investigators using the facility. This arrangement allows the investigators to program, initiate, and terminate a test session, to watch the ongoing behaviors through the video cameras, and to subsequently download and analyze the data. A laboratory bench is included for weighing mice and preparing special diets and drug treatments. A deep sink is conveniently placed for specialized cleaning of test equipment. The Morris swimming pool is filled and emptied at this sink. The suite is open at both ends, allowing easy access from two directions. An investigator is, thus, able to sit comfortably in the central area, interact with other investigators during the course of a long experiment, and take frequent breaks outside of the behavioral suite, without disturbing the experiments.

Alternatively, open facilities allow design of even better behavioral phenotyping suites. Ideally, mice can be tested in small procedure rooms, adjacent to small holding rooms, arrayed within the laboratory environment. Behavioral testing can thus be conducted within the investigator's own research space. Computer banks, desks, preparation benches, and sinks are conveniently located within the laboratory. Investigators work more efficiently because they move freely between their experiments and all other locations of their daily work. For example, an investigator is running an operant experiment that requires him to put a new set of mice into the operant chambers once per hour for 10 h. In an open facility near his laboratory and office, he can write a manuscript at his desk with the aid of reprints from office file cabinets, take a coffee break to talk with the laboratory director and coworkers about data interpretations, and attend part of a lecture in the departmental seminar room down the hall. In a closed facility located in the basement, three floors away from his laboratory and office, the investigator is considerably more limited in the use of his down time between hourly changes of mice in the operant chambers.

Generation and breeding of a targeted gene mutation usually takes up to two years. Behavioral phenotyping can take anywhere from a few weeks to several years, which is a tremendous investment. Further, order effects have been described (43, 44), in which one test procedure influences performance on another test procedure, so that a series of tests must be conducted in the same order across multiple batches of mice for the duration of the study. Researchers reap the benefits of this major investment by conducting multiple experiments, using a new line of transgenic or knockout mice. Lines of mutant mice are shared among investigators, laboratories, universities, and countries. Phenotyping often becomes an intense collaboration among researchers and their veterinary colleagues. The facility veterinarian and staff are essential members of the team that ensures large numbers of healthy mice of all genotypes and maintains environmental constancy in the housing rooms and procedural test rooms. At present, the biggest limitations to these collaborations are the barriers to importation and quarantine. The lifespan of a mouse is in the range of two years; mice older than one year are considered aged. Delays of several months for import approval, waiting for quarantine space to become available, quarantine of two months or longer, followed by serologic and pathologic evaluation, often result in mice becoming too old for behavioral testing, or even for breeding. Efficient design of a phenotyping facility includes large numbers of small quarantine rooms. Imported mice can, thus, be rapidly quarantined, isolated, and treated if necessary, and quickly released for research.

Cooperation between investigators and animal facility staff extends to breeding and housing issues. External factors that impact the interpretation of behavioral analyses include breeding strategy, background genes, group size and composition, housing environment, testing environment, and order of testing. To control individual cage factors, such as dominance hierarchies within a cage, parental behaviors that affect the offspring, and seasonal effects, mice from multiple breeding pairs are grouped for each behavioral experiment (37, 45). Groups include each genotype (+/+, +/-, and -/-) within each experiment. Another breeding issue is that background genes in the breeding strain can greatly influence the phenotypes expressed by the targeted gene mutation. For example, different inbred strains of mice are routinely used to generate: the embryonic stem cells, the blastocysts used for implantation, and the parental strain chosen for backcrossing. Strains vary enormously on all sorts of phenotypes. For example, learning and memory abilities are good in some strains of mice and abysmal in others (46-49). Therefore, phenotypes of a gene mutation will vary when the mutation is bred onto one background versus another. For example, insertion of a transgene to model Alzheimer's disease will result in production of different amounts of amyloid plaques and different degrees of cognitive impairment when bred onto FVB/N, C57BL, C57BLxSJL hybrids, or 129/SvxC57BL/6 hybrids (50, 51). Similarly, some inbred strains are highly susceptible to developing seizure activity (52, 53), which could prevent detection of an epilepsy-like phenotype in a single-gene knockout expected to make mice more seizure-prone. Strain differences in pain threshold (54) could bias detection of hyperalgesia in opiate receptor mutant mice. Generally, a background strain that is moderate for the behaviors of interest is chosen for breeding, allowing detection of increases and decreases (47). At least seven backcrosses into the chosen inbred strain are necessary for the mutation to be maintained on a pure background (11).

In conclusion, comprehensive behavioral phenotyping is labor intensive, requires dedicated procedure rooms, large quantities of specialized test equipment, and constant environmental conditions over a long period. Thus, experimental design must be tightly controlled over extended periods for successful behavioral phenotyping experiments.

Standardization of equipment, housing conditions, laboratory environment, and exact experimental methods for behavioral phenotyping is ongoing (45, 55-61). Understanding of these issues by veterinary researchers, facility managers, and animal care staff will greatly enhance the quality and success of mouse behavioral genetics.

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