2007 SHORT COURSE I

What's Wrong with My Mouse? Strategies for Rodent Behavior Phenotyping

Organized by Jacqueline N. Crawley, PhD

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Introduction

Robust, well-validated mouse behavioral tasks are central to investigations of targeted mutations of genes expressed in the brain. Which are the best methods for testing your hypotheses? What is the optimal experimental design, to yield replicable results and avoid overinterpretation of artifacts? What are the unique concerns that must be addressed when conducting behavioral research? This short course will provide an overview of phenotyping assays for mutant mice in several behavioral domains, including motor functions, sensory abilities, learning and memory, drug abuse, social behaviors, epigenetic influences on stress-related behaviors, depression-related behaviors, and schizophrenia-related behaviors. Multidisciplinary combinations of behavioral, neuroanatomical, electrophysiological, and neuropharmacological phenotyping will be presented, to illustrate comprehensive analyses of mouse models of neuropsychiatric disorders. Breakout group discussions with the speakers will offer opportunities for participants to obtain specific advice from experts in each behavioral domain.

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Motor Behaviors and Neuroanatomical Phenotypes in Mouse Models of Huntington's and Parkinson's Diseases

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Introduction

Huntington's disease (HD) and Parkinson's disease (PD) are neurodegenerative disorders that affect different neuronal populations; the most extensive cell loss occurs within the basal ganglia. In HD, the main pathological feature is a loss of medium-sized striatal efferent neurons, which are GABA-ergic (Gusella and Macdonald, 2006). In PD, the primary lesion affects dopaminergic neurons that project from the substantia nigra pars compacta to the striatum (nigrostriatal neurons) (Langston, 2006). Thus, the two diseases are mirror images of each other, as also suggested by their clinical phenotype: HD patients exhibit chorea, or involuntary dancelike movements of the limbs and the trunk (Gusella and Macdonald, 2006), whereas PD patients suffer from bradykinesia (slowness of movement) and akinesia (difficulty in initiating movement) (Langston, 2006). In addition, PD patients exhibit tremor, rigidity, and postural instability, whereas in HD, cognitive dysfunction leading to dementia is prominent.

It is important, however, to realize the limitations of these simplistic definitions. In both diseases, the pathological lesions extend beyond the basal ganglia. In HD, cell loss occurs in the cerebral cortex, and white matter thinning appears to precede diagnosis (Rosas et al., 2006). Furthermore, in juvenile cases, cell loss extends to the hippocampus and cerebellum (Vonsattel and DiFiglia, 1998). In PD, cell loss occurs in the locus coeruleus, raphe, and numerous other brain regions; the presence of the pathological hallmark of PD, the Lewy body, is even more widespread (Braak et al., 2003). Therefore, the classical "selectivity" of neuronal degeneration in both diseases is relative rather than absolute. This distinction has implications for the analysis of behavioral and pathological aspects of models of these disorders. In addition, despite the obvious differences in presentation of the two diseases, they share some symptoms. For example, dystonia (co-contraction of antagonistic muscles) can complicate both disorders; akinesia occurs in patients with HD; cognitive disruption is present in a subset of PD patients; both disorders have a high rate of affective disorder and depression; and sleep disturbances are present in both HD and PD (Glosser, 2001; Petit et al., 2004; Langston, 2006). Thus, despite the presence of different pathologies, one should not be surprised to find similar behavioral deficits in models of these disorders.

Before the discovery of genetic mutations, the only models available for HD and PD had been based on the use of neurotoxins that kill, more or less selectively, the neurons that are lost in patients. These models have been very helpful and, in the case of PD, remain widely used in order to reproduce the loss of nigrostriatal dopaminergic neurons (Bové et al., 2005). Toxin-based rodent models for HD were based primarily on local injections of quinolinic acid into the striatum or peripheral 3-nitroproprionic acid (3-NP) (Brouillet et al., 1999). Because HD is caused by a single genetic mutation with high penetrance, expression of the mutated gene has high construct validity; thus, little justification remains for the use of toxin models.

The situation is more complex for PD. The large majority of cases are sporadic, and only about 5% constitute familial forms, which are the result of a variety of genetic mutations, 6 of which have been identified so far (Klein and Lohmann-Hedrich, 2007). A clear link with sporadic PD has been established only for α-synuclein, a protein that accumulates in Lewy bodies and neurites in sporadic PD and that causes the disease when mutated or overexpressed as a result of gene duplication (Litvan et al., 2007). Other mutations occur in proteins involved in mechanisms that also appear defective in sporadic PD, such as proteasomal (parkin, UCHL1) or mitochondrial (DJ1, Pink1) function. No direct link, however, has been established between sporadic PD and proteins mutated in these familial forms. Therefore, the construct validity of genetic models of PD appears good for α-synuclein overexpression but less so for the other mutations identified so far. Nevertheless, these models can lead to a better understanding of the mechanisms underlying PD pathophysiology.

One key consideration regarding the relationship between motor and anatomical phenotypes of mouse models of HD and PD is that in most models, the neuronal cell loss that "defines" the disease in patients occurs late, or is even absent, in the lifetime of the mouse (Fleming et al., 2005; Chesselet, 2007). However, profound behavioral anomalies can be detected by using sensitive behavioral tests, indicating that the mutations induce neuronal dysfunction before they cause cell death (Levine et al., 2004). One caveat is that such tests as open field and rotarod (most commonly used to assess motor skills) are not always sensitive enough to detect the earliest deficits in models of basal ganglia dysfunction. Indeed, these tests are sensitive primarily to cerebellar or motor neuron deficits. We will review the tests that we found most useful in our own experience for assessing early motor deficits in genetic mouse models of HD and PD while emphasizing considerations to keep in mind in their choice and interpretation.

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Motor Tests for Genetic Mouse Models of HD

Most mouse models of HD do not develop obvious behavioral anomalies in their home cage until late in the disease. These obvious behaviors are sometimes referred to as "symptomatic," which in our view is misleading because it implies that they correspond to the appearance of clinical symptoms in patients. In fact, motor deficits in behavioral tasks are observed much earlier than the onset of these obvious deficits; thus, it is not possible to know which behavioral phase corresponds to the early stages of the human disease. This difference has become an issue for clinical studies as well. As the examination of individuals testing positive for the disease-causing mutation reveals more and more subtle deficits before the onset of clear clinical symptoms, the words "premanifest" and "manifest" HD are replacing "presymptomatic" and "symptomatic" in the clinical literature. When present, spontaneous home-cage behavioral anomalies can be scored on a nonparametric scale for the following: presence or absence of tremor, presence or absence of an unsteady or uncoordinated gait, in addition to turning ability and the width of the hindlimb base, the extent of piloerection (indicative of poor coat maintenance), and clasping.

Another important consideration is that, as in humans, the disease in these animals is progressive. Many models show hyperactivity followed by hypoactivity (Menalled et al., 2003). Therefore, one needs to test the animals at different times, carefully match groups for age, and take this possibility into account when choosing statistical analysis of longitudinal data. Finally, one should remember that mice are diurnal animals and, therefore, many motor tests need to be conducted during the dark phase. If overlooked, this detail can lead to confusion as to the reproducibility of the data. It is useful to have access to a reversed-phase animal room or, in lieu of that, to choose tests that can be performed during the light phase.

The rotarod is one of the most frequently used tests to assess motor deficits in mouse models of HD. It is easy to use, relatively automated, and reproducible from lab to lab, as long as the conditions of use are clearly defined. This consistency allows for a comparison of models generated by different laboratories. Indeed, there is a broad range of models of HD, from transgenics expressing a fragment of the gene, to mice expressing a full-length human mutated gene, such as *YAC* or *BAC* (Menalled et al., 2002; Menalled and Chesselet, 2002; Hickey and Chesselet, 2003b). The availability of multiple models of HD is important for tailoring the choice of the model to the experiment. For example, studies of proteolysis or phosphorylation (Borrell-Pages et al., 2006) require a mouse that expresses a full-length human *huntingtin* gene. In contrast, if one wishes to study a rapidly progressive disease model, it is better to use mice that express a short transgene, for example, *exon 1*. Indeed, truncated mutated *huntingtin* is far more toxic than the full-length protein (Hickey and Chesselet, 2003a). Even in fast-progressing models, such as the widely used R6/2 mice, classical tests such as clasping and rotarod do not reveal deficits until after synaptic, molecular, and even pathological anomalies (nuclear inclusions) have been detected (Levine et al. 2004; Hickey et al., 2005). Several laboratories have developed more-sensitive tests that detect anomalies at an earlier age. We have focused on a battery of tests that are inexpensive and easy to use and that detect very early deficits with sufficient power to be useful for preclinical drug testing in HD mice.

Motor Tests for Preclinical Drug Testing in R6/2 HD Transgenic Mice

R6/2 mice express *exon 1* of the human *HD* gene with approximately 150 CAG repeats (Mangiarini et al., 1996). Genetic drift occurs in these mice; therefore, it is important to test for repeat length in each colony and in the animals actually used for the experiment. They have a rapid disease course and die at between 12 and 16 weeks (Li et al., 2005) of age, although other labs report longer survival times. Good husbandry, including easy access to food, prolongs their life (Carter et al., 2000).

Behavioral tests that can be performed during the phase of low activity (day) include **climbing behavior**, a test that does not require complex or expensive equipment and is very sensitive. We found deficits as early as 4.5 weeks in R6/2 mice (Hickey et al., 2005). Mice are placed at the bottom of wire cylinder cages (diameter, 10.5 cm, height, 15.5 cm), and spontaneous activity is videotaped for five minutes to quantify the number of climbs (defined as all 4 paws on the side of the cage). The same tapes can be used to quantify the number of rears made by the mouse. There is always a concern with such tests that an impaired performance could be the result of decreased motivation, and it is therefore advantageous to use other tests in parallel. **Rotarod analysis** can be performed in either the light or the dark phase. Despite its low sensitivity to basal ganglia dysfunction, HD mice tend to show anomalies at advanced stages of the disease. The standard apparatus is purchased from Ugo Basile, Varese, Italy, and is used to measure the latency period until mice fall from the rotating rod. Usually, a few mice "cling" to the axle,

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so to prevent bias, these are removed after 3 consecutive rotations; the latency at that time is used as the score for that animal. Sometimes the axle of the rotarod is covered with smooth rubber ("smooth axle") to reduce clinging and to make the task more difficult than it was on the grooved axle. The duration and frequency of trials, as well as their speed, can differ, so it is important to document all parameters used in the published reports. In our experiments, mice were trained to run on the smooth axle that is accelerated from 4 rpm to 40 rpm over 10 minutes (3 trials per day for 4 days). Even with this protocol, however, deficits in rotarod performance of R6/2 mice were not detected until after initial climbing and open-field rearing deficits appeared (Hickey et al., 2005).

We have developed the use of **running wheels** to assess motor deficits in HD mice. Running wheels are widely used in research on circadian rhythms and are fully automated. They represent a significant initial investment but are valuable for preclinical drug testing because of their high power for detecting differences among groups and because of the minimal investigator time involved. Mice were placed in individual cages equipped with a running wheel (23 cm diameter, Mini Mitter Company Inc., Bend OR) for up to 8 days. Each rotation of the wheel was detected by a magnet and recorded using VitalView Data Acquisition Software V 4.0 (Mini Mitter) in 3-minute bins. It is important to house running-wheel cages in cabinets (8 cages/cabinet) to minimize light and sound disturbance. Cabinets must be equipped with fans to allow air circulation, and illumination should follow the same pattern of light-dark phases as in the vivarium. Running activity is recorded continuously, and activity is recorded in light and dark phases. It is critical to use only mice of the same sex for the calculations because the activity of females differs from that of males and changes during the estrus cycle. Wheel-running activity (speed) during dark and light phases can be calculated using ActiView V 1.2 (Mini Mitter). We found that during the dark phase, R6/2 transgenic mice showed a profound reduction in running, from 4.5 weeks of age; 8 mice were required to see an improvement of 50% using 80% power (α = 0.05) (Hickey et al., 2005).

Motor Tests for Preclinical Drug Testing in a Knock-In Mouse Model of HD With 140 CAG Repeats

We have used a model developed by the laboratory of S. Zeitlin (University of Virginia) with a human *exon 1* containing approximately 140 CAG repeats inserted in the mouse gene (Menalled et al., 2003). Remarkably, many of the same tests used in R6/2 detected deficits in CAG140 mice at a very early age, despite earlier observations that behavioral deficits in HD knock-in (KI) mice were absent, limited in scope, or did not occur until 8–12 months of age (Menalled, 2005; but see Woodman et al., 2007). In addition to anomalies in open-field testing (Menalled et al 2003), in our recent unpublished studies we have found deficits in climbing as early as 1.5 months and profound running-wheel deficits at 6 months (males) or 8 (females) months. Power analysis indicates that as few as 5 male KI mice would be required to detect 50% rescue of running-wheel performance at 6 months, using 80% power and α = 0.05.

In addition, we have used the **pole task** in testing these mice. For this test, mice are placed on a vertical pole (1 cm in diameter, 60 cm high). The spontaneous behavior for mice is to turn downwards and descend the pole. After habituating the mice to the task in 2 trials per day for 2 days, on the third day mice are given 5 trials. The time taken to turn to face downwards (t.turn) is measured, in addition to the total time to complete the task (t.total) and the time taken to descend (t.descend), following the turn. In our KI mice, at 4 months we found a marked increase in the total time to complete the task. It is important to note that some tests need to be adapted to increase their sensitivity for use in KI mice. For example, we added fixed-speed trials to the rotarod paradigm to detect subtle deficits in rotarod performance in the KI mice.

In conclusion, both simple but labor-intensive (climbing cage, pole test) and almost or fully automated tests (open field, running wheel) can be used to detect early behavioral deficits in mouse models of HD. These tests do not require extensive prior training of the investigator, but they do require careful planning in terms of phase of day, age of the animals, and gender matching. Although we have preliminary data indicating that drug effects can be detected using these tests, a systematic evaluation of different lines of mice using the same tests under the same conditions is lacking. In the absence of this information, it is recommended that one use a battery of tests and to choose tests that have demonstrated the greatest sensitivity for detecting motor anomalies in HD models.

Rodent Models of PD: General Considerations

Modeling PD in rodents presents very different challenges than modeling HD. Indeed, the majority of cases of PD are sporadic, and their cause is unknown. Evidence implies environmental causes: In particu-

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lar, exposure to agricultural pesticides (Brown et al., 2006). Neurotoxins (especially agents affecting mitochondrial complex 1 such as MPTP and rotenone) and agents inducing oxidative stress (such as 6-hydroxydopamine [6-OHDA] and paraquat) can be used to somewhat selectively kill dopaminergic neurons in animals, thereby reproducing the major pathological feature of PD (Bové et al., 2005; Chesselet et al., 2005). These toxins are given by either local stereotaxic (6-OHDA) or peripheral injections. Local injections are usually given unilaterally in order to avoid the severe feeding deficits associated with bilateral dopamine cell loss in the ventral mesencephalon, and the behavioral tests used in these models take advantage of the resulting limb asymmetry (Schallert and Tillerson, 2000). Paradoxically, peripheral injections of neurotoxins do not always result in easily detectable motor deficits (Sedelis et al., 2000), probably because a lower level of dopamine depletion is achieved in these models. Nevertheless, recent studies have shown that behavioral deficits can be observed after MPTP administration in mice and rotenone administration in rats (Sedelis et al., 2000; Tillerson et al., 2002; Fleming et al., 2004). Paraquat induces the loss of only ~25% of nigrostriatal dopaminergic cell bodies, and even sensitive tests do not detect motor deficits, possibly because of compensatory mechanisms (Fernagut et al., in press). Unfortunately, a promising model of nigrostriatal degeneration induced by peripheral injections of proteasome inhibitors has not yet been widely reproduced (McNaught et al., 2004; Bové et al., 2006; Manning-Bog et al., 2006).

New models of nigrostriatal degeneration have been created by ablating genes involved in the differentiation or maintenance of dopaminergic neurons. Mice that lack the homeobox transcription factor Pitx3 lose nigrostriatal dopaminergic neurons during early postnatal development, and we have shown that they exhibit profound behavioral deficits that are reversed by administering levodopa (L-dopa), the standard symptomatic treatment for PD (Hwang et al., 2005). Postnatal degeneration of dopaminergic neurons of the ventral mesencephalon in mice heterozygous for the loss of the *Engrailed 1* gene leads to impaired motor skills, anhedonia-like behavior, an enhanced resignation phenotype, and poor performance in social interactions (Sonnier et al., 2007). Under some experimental conditions, region-specific loss of neurotrophic factor receptors TrK and Ret also causes progressive loss of nigrostriatal dopaminergic neurons, though the effects vary from study to study, and behavioral assessment of these models has been minimal (Li et al., 2006). Overall, a major limitation of these models has been the tenuous relationship between the experimental manipulations used to induce the loss of dopaminergic neurons and the pathophysiological mechanisms leading to PD in humans.

As discussed in the introduction, among mutations known to cause rare familial forms of PD, alpha-synuclein overexpression has so far the best construct validity, and many lines of α-synuclein–overexpressing mice have been generated (Fernagut and Chesselet, 2004; Fleming et al., 2005; Chesselet, 2007). Earlier reports raised doubts about the usefulness of these mice for modeling PD because of the general lack of nigrostriatal degeneration. More recently, nigrostriatal cell loss and/or loss of striatal dopamine have been reported in lines with double-mutated or truncated α-synuclein (Chesselet, 2007). In one line, however, the cell loss occurs embryologically and does not mimic the progressive degeneration of PD (Wakamatsu et al., 2006). Another caveat is that most lines have not been examined at a very old age, and age is the main risk factor for PD in humans. Even lines of mice without neuronal degeneration within their life span, however, can provide valuable information. Indeed, some of these mice present evidence of neuronal dysfunction that may shed light on the early events that precede neuronal loss in PD (Fleming et al., 2005; Chesselet, 2007). We have extensively characterized the behavioral deficits presented by mice overexpressing α-synuclein under the Thy1 promoter, which confers broad neural expression of the transgene (Thy1-aSYN mice) (Rockenstein et al., 2002). This model confers a distinct advantage because pathology in PD is not confined to nigrostriatal neurons but affects other brain and peripheral neurons as well (Braak et al., 2003).

Testing Motor Function in α-Synuclein–Overexpressing Mice

In most studies, investigators have used standard tests such as the rotarod, gait, or spontaneous activity in the open field. In general, these are not ideal for detecting deficits of nigrostriatal function, as shown by the inconsistency of deficits using this measure in MPTP-treated mice (Sedelis et al., 2000). This may be one reason why deficits are observed only at a late age in most models of α-synuclein overexpression (Fleming and Chesselet, 2006). The battery of sensorimotor tests developed by our laboratory in order to characterize genetic mouse models of PD is based on novel as well as established tests (more details are provided in Fleming and Chesselet, 2005, and Fleming et al., 2004). Although we focus here on the results obtained from Thy1-aSYN mice, it should be noted that we have observed qualitatively similar but milder

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deficits in mice expressing another PD-causing mutation: a deficit in parkin (Goldberg et al., 2003). All our behavioral analyses were done in male mice, and all data were compared with wild-type littermates.

One of the simplest tests we use is the measurement of spontaneous movement in a small transparent **cylinder** (height, 15.5 cm, diameter, 12.7 cm), which is placed on a piece of glass. A mirror is positioned at an angle beneath the cylinder to allow a clear view of movements along the floor and walls of the cylinder. Spontaneous activity is videotaped for 3 minutes, and the number of rears, forelimb and hindlimb steps, as well as time spent grooming, are measured. In Thy1-aSYN mice, we found significantly decreased spontaneous activity in all measures at 2 months compared with wild-type littermates, as well as reduced hindlimb stepping, which worsens at up to 8 months of age (Fleming et al., 2004).

We have also observed profound deficits in Thy1-aSYN mice on the pole test, described above, indicating that this test can detect motor deficits in models of both HD and PD. In contrast, HD mice did not show any deficits in the **challenging beam** test, which has proved to be one of the most sensitive and reliable measures of sensorimotor deficits in the PD mice we have examined.

The challenging beam traversal test is conducted on a custom-made Plexiglas beam consisting of four sections (25 cm each, 1 meter total length) of different widths. The first section has a width of 3.5 cm, and the beam gradually narrows to 0.5 cm by 1 cm increments. Ledges (1 cm in width) are placed 1.0 cm below the upper surface of the beam. Animals are trained to traverse the length of the beam toward the narrowest section, which leads directly into the animal's home cage, to increase motivation. After two days of testing, a mesh grid (composed of 1-cm squares) of corresponding width is placed approximately 1 cm above the beam surface. Animals are videotaped, and the videotapes are analyzed in slow motion to determine time to traverse, number of steps, and number of errors per step. These motor performance and coordination parameters are altered as early as 2 months of age in Thy1-aSYN mice and worsen with age (Fleming et al., 2004).

To measure **motor response to sensory stimuli**, we used a stimulation test in which small adhesive labels are placed on the snout of each mouse. To remove the stimulus, animals must raise both forelimbs and swipe off the stimulus with both forepaws, and the time to make contact and remove the stimulus are recorded. Thy1-aSYN mice show a decreased response at 6 months of age compared with wild-type mice (Fleming et al., 2004).

Although these tests are mild stressors on the mouse, they do require some intervention and training by the investigator. We were also interested in tests that did not require direct contact between the investigator and the animal. Shredding behavior for nest-building is a gender-neutral, species-specific behavior that requires the use of orofacial and forelimb movements to grab the nesting material (cotton) when it is placed in the feeding bin of the home cage. It is possible to measure this behavior by weighing the amount of cotton left in the feeder of the cage in individually housed mice after a preweighed amount of cotton has been provided. Because animals must rear up in order to pull the cotton from the feeder, it is important to control for the absence of deficits in rearing. This can be done by using the cylinder test described earlier. In addition, it is important to control for lack of motivation by ensuring that mice build nests when provided with easily accessible cotton on the floor of their cage. The "bin cotton use test" proved to be surprisingly sensitive for detecting motor deficits in Thy1-aSYN mice. At 4 months of age, Thy1-aSYN mice showed reduced shredding behavior compared with their wild-type littermates; by 8 months, shredding behavior was reduced even further in the Thy1-aSYN mice (Fleming et al., 2004).

A frequently asked question is whether the behavioral deficits observed in Thy1-aSYN mice are reversed by L-dopa. We have shown that, in mice with a major loss of nigrostriatal dopaminergic neurons, motor deficits detected by the tests we have just described are reversed by L-dopa (Hwang et al., 2005). However, one must keep in mind that the Thy1-aSYN mice show deficits in these tests without loss of dopaminergic neurons, suggesting that the deficits result from dysfunction in neuronal circuits rather than cell death (Levine et al., 2004). L-dopa and dopaminergic agonists worsened some of the motor symptoms exhibited by Thy1-aSYN mice and did not affect others (Fleming et al., 2006a), which may have been the result of the detrimental effects excess dopamine can have on certain motor and cognitive function (Chudasama and Robbins, 2006).

Conclusions

In summary, we have shown that sensitive behavioral tests can detect motor anomalies in mouse models of basal ganglia diseases in which more traditional approaches fail to detect anomalies. Accordingly, one should use caution when describing a disease stage in

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these mice as "presymptomatic" or concluding that a particular mouse "does not show motor anomalies." Although their underlying mechanisms are usually not known, these functional anomalies provide useful end points for testing drugs that might be able to interfere with the disease process and prevent neuronal dysfunction. It should be noted that, although this review focuses on motor symptoms, nonmotor deficits reminiscent of PD symptoms (for example, olfactory deficits) have also been observed in Thy1-aSYN mice and could provide useful end points for drug studies (Fleming et al., 2006b).

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The Surprising Complexity of Pain Testing in the Laboratory Mouse

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Introduction

The phenotyping of mutant mice for pain-related traits (e.g., nociception, drug- and stress-induced antinociception, injury-induced hypersensitivity) is an active pursuit, both for pain researchers and for others studying phenomena in which pain sensitivity may affect results (e.g., learning and memory, tolerance, and dependence). We have recently compiled an interactive database of mutant mice tested for behavioral pain phenotypes: the *Pain Genes Database* (http://paingeneticslab.ca/4105/06_02_pain_genetics_database.asp) (LaCroix-Fralish et al., 2007). As of this writing, 212 null mutants (both transgenic knockouts and spontaneous mutants) display at least one significant difference compared with wild types on one or more pain-related trait, findings that are described in 456 published manuscripts appearing in the literature at a rate of more than 60 papers per year. Largely because of the continuing popularity of the transgenic knockout mouse, *Mus musculus* is rapidly overtaking *Rattus* as the "default" subject of basic pain research (Mogil et al., 2001; Wilson and Mogil, 2001).

Establishing the pain sensitivity of a laboratory mouse is far more difficult than it may first appear, and more an art than a science. We have extensive experience testing not only mutant mice (Rubinstein et al., 1996; Mogil et al., 2000b; Kest et al., 2001; Mogil et al., 2003; Mogil et al., 2005b,c) on pain traits, but also a large set of inbred strains providing the genetic background on which these mutations are placed (Mogil and Belknap, 1997; Mogil et al., 1998; Kest et al., 1999; Mogil and Adhikari, 1999; Mogil et al., 1999a,b; Kest et al., 2002a,b; Lariviere et al., 2002; Chesler et al., 2003; Mogil et al., 2003; Wilson et al., 2003a,b; Mogil et al., 2005a,d; Mogil et al., 2006). We have learned from these experiments that genotype robustly affects pain, but that interindividual variability is affected by a large number of additional organismic and environmental factors. In this syllabus, I present the state of this art, with an introduction to the myriad complexities that attend pain phenotyping in the mouse.

Algesiometry

Acute and tonic pain (seconds to days) is induced by noxious stimuli of three modalities: thermal (hot or cold), mechanical, and chemical (including protons released during inflammatory states, ATP released from damaged cells, and any number of exogenous and endogenous compounds that activate and/or sensitize nociceptors). The etiology of chronic pain (weeks to years) is less clear but can generally be classified as either inflammatory (e.g., arthritis), neuropathic (e.g., postherpetic neuralgia), or idiopathic/functional (e.g., fibromyalgia). Although most research attention, for reasons of practicality, is paid to somatic pain to the trunk and limbs, of equal or greater clinical importance are visceral pain and orofacial pain.

Reflecting the diversity of pain etiologies and characteristics is a panoply of available animal models (Walker et al., 1999; Le Bars et al., 2001; Wilson and Mogil, 2001; Negus et al., 2006). Table 1 provides information on popular models. The general trend over

Table 1. Common algesiometric assays (excluding orofacial models)*

Duration	Modality	Assay	Intensity Range[a]
Acute (seconds)	Heat	Hot plate Radiant heat paw withdrawal Tail-flick/withdrawal	46–56°C not reported 46–56°C
	Cold	Acetone drop Cold plate Cold tail-flick	20 µl 0–5°C –10–0°C
	Mechanical	Paw pressure (Randall-Selitto) Tail clip von Frey	variable 100–500 g 0.1–1.5 g
Tonic[b] (minutes to hours)	Chemical	Acetic acid Bee venom Capsaicin Formalin Magnesium sulfate	0.1–1.0% 0.05–0.5 mg 0.1–50 µg 1–5% 120 mg/kg
Chronic[c] (hours to weeks)	Inflammatory	Carrageenan Complete Freund's Adjuvant Prostaglandins Zymosan	1–5% 50% 10 ng 0.25–1.0 mg
	Neuropathic	Chronic constriction injury Partial sciatic nerve ligation Spared nerve injury Spinal nerve ligation	N.A. N.A. N.A. N.A.

[a]Based on a search of null mutant studies (Lacroix-Fralish et al., 2007).
[b]Typically, the dependent measure in these experiments is the total duration of licking or stretching responses of the affected part, though subsequent hypersensitivity to evoking stimuli can often be demonstrated as well.
[c]Typical dependent measures in these experiments, measured weekly or biweekly, include changes in sensitivity to acute evoking stimuli.
N.A.=not applicable.
*This list is not intended to be exhaustive.

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the past few decades has been favoring the use of chronic-pain models, especially those involving surgical injuries to peripheral nerves, over acute models. However, acute testing paradigms are still highly relevant, because surgical and inflammatory injuries produce robust hypersensitivity to acute pain–evoking stimuli (i.e., radiant heat, cooling stimuli, von Frey fibers), and thus, measuring "chronic pain" in rodents involves, *de facto*, the measurement of injury-induced changes in acute nociceptive sensitivity.

We have argued that the apparent lack of measurable spontaneous pain represents an important limitation of existing models (Mogil and Crager, 2004), given that spontaneous pain is likely the most important symptom of human clinical pain pathology. Others have complained that the reliance of the chronic pain models on purely reflexive, dependent measures ignores the important cognitive and emotional richness of the human pain experience, which might be better modeled in animals using operant techniques; indeed, such techniques are being increasingly adopted (e.g., Sufka et al., 1996; Jabakhanji et al., 2006; Neubert et al., 2006; Pedersen and Blackburn-Munro, 2006; King et al., 2007; Thut et al., 2007).

It is important to note that, although pain research in general is progressing rather quickly toward the use of more sophisticated models, transgenic knockout studies of pain are more often than not performed by scientists who are not pain researchers. As such, these investigations overwhelmingly employ more "simple" (but less clinically relevant) assays like the hot-plate and tail-flick tests (Mogil et al., 2006; LaCroix-Fralish et al., 2007).

Sex Differences

Women are greatly overrepresented as clinical pain sufferers (Unruh, 1996). Meta-analyses of controlled laboratory studies have revealed moderate-to-large (although modality-dependent) sex differences as well (Riley III et al., 1998), with women showing higher pain sensitivity, lower pain tolerance, and greater pain discrimination than men. In general, mice display equivalent sex differences, in that females usually display higher sensitivity across a number of stimulus modalities when differences are reported (Mogil et al., 2000a). The relevance of mice (or rats, for that matter) as a model species to the study of human sex differences in analgesic responses is less clear, since male mice usually display higher analgesic potency than females, though many studies suggest that women are more responsive to opioid analgesics than men (Craft, 2003). Of potentially far greater interest are the repeated demonstrations by us (Mogil et al., 1993; Mogil and Belknap, 1997; Mogil et al., 1997b; Mogil et al., 2003; Sternberg et al., 2004a,b) and other researchers (Liu and Gintzler, 2000; Tershner et al., 2000; Blednov et al., 2003; Mitrovic et al., 2003) of *qualitative* (i.e., genetically and neurochemically distinct) sex differences in the neural processing—possibly including differences in neuroanatomical circuitry itself—of pain modulatory mechanisms.

Although sex-specific pain processing represents a great opportunity for novel drug development, its existence also presents a great challenge to the conclusions of the existing literature. For example, an entire body of literature was amassed documenting the potentiation of morphine analgesia by N-methyl-D-aspartate (NMDA) receptor antagonists (Kozela and Popik, 2002), and the strength of this literature was sufficient to inspire a clinical trial of a morphine-dextromethorphan combination against postoperative pain. However, this clinical trial failed to show efficacy (Caruso, 2000). Virtually the entire preclinical literature used male rats; using male and female mice, we found that noncompetitive NMDA antagonists like dextromethorphan did indeed potentiate morphine analgesia in male mice but were completely ineffective in females (Nemmani et al., 2004).

Although sex differences in murine sensitivity to pain and analgesia are clearly demonstrable and need to be considered seriously, their impact is dwarfed by overall genotypic effects, and sex and genotype interact thoroughly (Mogil, 2003). Importantly, the existence of sex differences is *not* a good reason to avoid using female mice in the study of pain. In fact, the vast majority of basic science studies in the field use male mice only (Mogil and Chanda, 2005) despite the overwhelming epidemiological data suggesting that the modal human pain patient is female. The likely reason for the continued use of male rodents in basic studies of pain, besides sheer inertia, is the belief that estrous cyclicity in female subjects renders their data more variable than those of males. With very large data sets at our disposal, we tested that hypothesis directly, and found that the coefficients of variation in each sex were statistically equal (if anything, the trend was for *male* data to show higher variance) (Mogil and Chanda, 2005). If estrous cyclicity really does affect pain and analgesia (and overall, there appears to be very little evidence that it does) (Mogil et al., 2000a), we suggest that male mice exhibit a source of sex-specific variation as well: within-cage dominance hierarchies. Data from male mice may be affected by the dominance status of the tested subject, and by the time elapsed since there was last a fight in the cage, given that defeat in such encounters produces analgesia (Miczek et al., 1982).

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Genotypic Effects

Pain and analgesia are no different from other biological traits in demonstrating robust variability across a strain. We have concentrated on a set of 12 inbred mouse strains, and performed a systematic "phenome project" by testing pain sensitivity across more than 22 nociceptive assays (Mogil et al., 1999a,b; Lariviere et al., 2002) and analgesic responses to more than 10 different drugs (Chesler et al., 2003; Wilson et al., 2003a,b). Narrow-sense heritabilities ranged from $h^2 = 0.24–0.76$ (median: $h^2 = 0.46$) for nociception and $h^2 = 0.12–0.45$ (median: $h^2 = 0.34$) for drug analgesia.

Beyond the simple observation of robust effects of genotype in every assay considered thus far, and the identification of extreme-responding strains as an entrée to gene mapping efforts via quantitative trait locus (QTL) mapping (see below), our study of pain and analgesic responses across this set of inbred strains has allowed for the examination of genetic correlations across assays that have in turn illuminated some general principles of "pain genetics." For example, 20 of the 22 nociceptive assays "cluster" into five groupings when cross-correlations are analyzed using multi-variate techniques. These five groupings appear to be differentiated largely by the noxious stimulus modality used: (1) thermal assays, (2) chemical assays, (3) assays of mechanical hypersensitivity after injury, (4) assays of thermal hypersensitivity after injury, and (5) assays of thermal hypersensitivity after injury featuring spontaneous pain prior to the development of the hypersensitivity (Lariviere et al., 2002).

Genetic correlations among traits imply common genetic determinants of variability within those traits, and so this finding directly predicts the discovery of pain *symptom*–related genes rather than pain *etiology*–related genes. With respect to analgesia, we obtained compelling evidence that all centrally acting analgesic compounds tested thus far appear to be highly genetically correlated with each other, and furthermore highly correlated with the baseline sensitivity of that strain to the noxious stimulus (Wilson et al., 2003a). This suggests that "master" analgesia genes may be discovered, and that those genes are far more likely to be related to pain circuitry *per se* than related to the binding or metabolism of the drug itself. For peripherally acting ("over-the-counter") analgesics, a different pattern was observed, with two nonsteroidal anti-inflammatory drugs (NSAIDs; aspirin and indomethacin) showing considerable genetic correlation, but acetaminophen yielding a completely different pattern of strain sensitivity (Wilson et al., 2003b). Finally, we (Mogil et al., 1996a; Chesler et al., 2003) and others (Elmer et al., 1998) have observed that the pattern of strain sensitivities to drug inhibition of one noxious stimulus is entirely uncorrelated with the pattern of strain sensitivities of that *same drug* to a different noxious stimulus. Again, surprisingly, analgesia genetics seems to have more to do with the pain being inhibited and less to do with the drug itself.

As a practical matter, the mouse strain chosen for study will have a large impact on the data collected. Since most null mutants are engineered using 129-derived embryonic stem cells and are ultimately placed on a C57BL/6 background, a comparison of the sensitivities of these two strains is of particular importance. Unfortunately for pain researchers, the 129 and C57BL/6 strains diverge greatly on most nociceptive and analgesic phenotypes (Lariviere et al., 2001), rendering most transgenic studies of pain particularly subject to "hitchhiking donor gene" confounds (Gerlai, 1996). It can be argued as well that the behaviorally sensitive strain C57BL/6J, despite being the default biomedical research subject, is not well representative of inbred (or outbred, or wild) mouse strains (Lariviere et al., 2001), and thus the interpretation of knockout data in this strain is greatly affected by epistatic considerations. Although important differences among 129 substrains have been noted (Simpson et al., 1997), we have not observed any major substrain differences in pain or analgesia phenotypes (Mogil and Wilson, 1997).

Using F_2 intercrosses between extreme-responding strains (and recombinant inbred strains derived from such crosses), supplemented more recently by the use of congenic strains and haplotype mapping strategies, we have made considerable progress in the identification of genes responsible for nociceptive and analgesic variability in the mouse (Mogil et al., 1997a,b; Wilson et al., 2002; Mogil et al., 2003; Mogil et al., 2005d; Mogil et al., 2006, unpublished data). Other groups have also performed QTL mapping studies on pain-relevant traits (Seltzer et al., 2001; Furuse et al., 2003; Liang et al., 2006a,b). As a single example, we have provided compelling evidence that the gene primarily responsible for variability in thermal (heat) nociception across the entire set of 12 inbred strains is *Calca*, encoding the calcitonin gene-related polypeptide, α-subunit (CGRP) (Mogil et al., 2005d). QTL mapping in two separate crosses localized the gene to mid-chromosome 7, and an available congenic strain (A.B6-Tyr$^+$/J) confirmed the QTL. Electrophysiological recordings from primary afferent neurons from behaviorally sensitive (C57BL/6J) and resistant (AKR/J) strains suggested that the relevant genetic difference was contained within the nociceptor itself, and we went on to demonstrate strain

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differences in *Calca* expression in the dorsal root ganglion, differential CGRP content there, and differential release of CGRP upon noxious thermal stimulation. Pharmacological and antisense knockdown experiments confirmed that the strain difference could be "rescued" in each strain by mimicking the *Calca* expression level of the other. Finally, we showed the generalizability of this explanation by completely (albeit temporarily) abolishing strain differences in noxious thermal sensitivity by administering CGRP injections into the hindpaw of all 12 strains (Mogil et al., 2005d).

We have also demonstrated that genetic findings gleaned from QTL mapping studies in the mouse can be successfully "translated" in humans. Such studies have revealed the important role the *Mc1r* gene plays in opioid analgesia and nociception in the mouse, and in each case the prediction was confirmed in a counterpart human (MC1R) association study (Mogil et al., 2003; Mogil et al., 2005c).

Parametric Factors

Although genetic factors are robust in their modulation of pain and analgesic sensitivity in the mouse, much interindividual variability is left to explain. From one lab to another, it is likely that parametric differences in the precise application of these assays are primarily responsible, and that the most obvious varying factor is noxious stimulus intensity. The intensity of the noxious stimulus used has obvious effects on baseline nociceptive sensitivity; what is less well appreciated is that the stimulus has very important effects on the measurement of both analgesia and hypersensitivity (after inflammatory or neuropathic injury). Simply put, the more noxious a stimulus is, the harder it is to change responses to it, and by their very definition, analgesia and hypersensitivity represent changes in nociceptive responses. For example, if a hot plate is very hot, and baseline latencies to display nocifensive behaviors (e.g., hindpaw licking, hindpaw shaking, jumping) are very low, only high doses of opioids will produce measurable analgesia, and NSAIDs will be entirely ineffective. Furthermore, any attempts to demonstrate hypersensitivity will likely be foiled by a "floor effect." Lowering the stimulus intensity will solve some of these problems, but only to potentially replace them with others, such as "ceiling effects" (i.e., arbitrary cutoff latencies or pressures imposed by ethical constraints) and nonspecific behavioral and stress effects (see below). Another unappreciated problem is that of "Lord's paradox" (the "law of initial values"), which states that the analysis of correlations between baseline values and change values calculated using the baselines is inherently problematic (Harris, 1963). As mentioned above, there's no way around this problem in research on analgesia and hypersensitivity.

Another parametric factor related to many pain studies surrounds the use of intracerebroventricular (i.c.v.) and intrathecal (i.t.) injections. In the rat, administration of drugs via these routes is often preceded by the surgical installation of indwelling catheters or cannulae. In the mouse, by contrast, both intracerebroventricular and intrathecal injections are given acutely, under light gas anesthesia. Nonetheless, the procedure is very likely stressful, and acute stressors are well known to produce pain inhibition, a phenomenon known as stress-induced analgesia (SIA) (Terman et al., 1984; Yamada and Nabeshima, 1995). Failure to appreciate the possible existence of SIA can lead to misinterpretation of the results of pain studies. In a well-cited example, both groups that had originally isolated the endogenous ligand of the "orphan" ORL1 receptor initially investigated its possible biological role by intracerebroventricular injection into mice; both groups reported that the peptide, orphanin FQ/nociceptin (OFQ/N), produced hyperalgesia (Meunier et al., 1995; Reinscheid et al., 1995). We failed to replicate this finding, instead noting that OFQ/N was a functional anti-opioid (Mogil et al., 1996b). The original groups erred by not including a no-injection control; had they done so, they would have noted that the intracerebroventricular injection itself (i.e., the vehicle control) produced opioid-mediated SIA, which was simply being reversed by the OFQ/N (Mogil et al., 1996b; Suaudeau et al., 1998).

Environmental Factors

Generally speaking, within a laboratory, parametric factors are held constant. Even so, much variability remains. We investigated the possible sources of this environmental variability by compiling and analyzing a large set of 49°C tail-withdrawal baseline latency data from 8 years worth of experiments (Chesler et al., 2002a,b). This archival data set contained measurements from more than 8000 mice of 40 different genotypes, and featured a heritability of $h^2 = 0.24$. Besides strain (genotype) and sex, the following factors related to both husbandry and testing environment were both varied and recorded on the original data sheets: cage density, experimenter, humidity, season, time of day, and within-cage order of testing. Many other factors affect pain (e.g., age, light/sound levels), of course, but these were either strictly controlled in our data set or no records were available, and thus their influence could not be studied. Using classification and regression tree (CART) analysis followed by linear modeling of a data subset,

and confirmed by a fully balanced and crossed experiment performed on a single day, we were able to rank the relative importance of these factors in contributing to variability in the archival data set. We found that genotype was only the *second* most important factor, behind experimenter. This should serve to remind us of the importance of the experimenter-subject interaction, something that can never be exactly reproduced from one lab to another (Crabbe et al., 1999). The tail-withdrawal test is particularly affected by experimenter-specific factors, since the mouse is being actively restrained by the experimenter while being tested. (Restraining the mouse in Plexiglas may solve this particular problem, but it introduces another, since the prolonged restraint required yields significant SIA; Mogil et al., 2001.) We've gone on to demonstrate, however, that other nociceptive assays are similarly affected by experimenter, even those not featuring direct handling of mice during or immediately prior to data collection itself (Mogil et al., 2006).

Effect of Behavioral State

A potentially important factor modulating pain sensitivity in animals that has been entirely ignored in the literature concerns what the animal was *doing* immediate before and during the application of the noxious stimulus. This omission disregards the fact that attentional level can strongly modulate pain perception in humans (Bushnell et al., 2004), and a sleeping animal would traditionally be thought of as having reduced sensitivity to all external stimulation (Dement, 1965). In a study just completed (BL Callahan, ASC Gil, A Levesque, JS Mogil, submitted), we compiled an "ethogram" of mouse behavior within novel Plexiglas cubicles atop a glass or wire grid floor for several hours during the middle of their quiescent phase, which is essentially the modal testing situation in modern murine pain research. Mice are not testable in most assays while moving, and it generally requires several hours for them to reduce their exploration of the cubicle to acceptably low levels. Once this habituation has occurred, we find that CD-1 mice spend approximately 25% of their time grooming, 25% of their time resting or in light sleep, 25% of their time in deep sleep, but <15% of their time fully alert (Fig. 1A). The relevance of this breakdown is that if they are tested during grooming behavior, they are significantly less sensitive to noxious thermal stimuli (Fig. 1B) and profoundly less sensitive to mechanical stimuli (Fig. 1C). For mechanical stimuli, highest sensitivities are seen in resting and lightly sleeping mice (Fig. 1C). In general, C57BL/6 and 129 mice display similar patterns, except that C57BL/6 mice

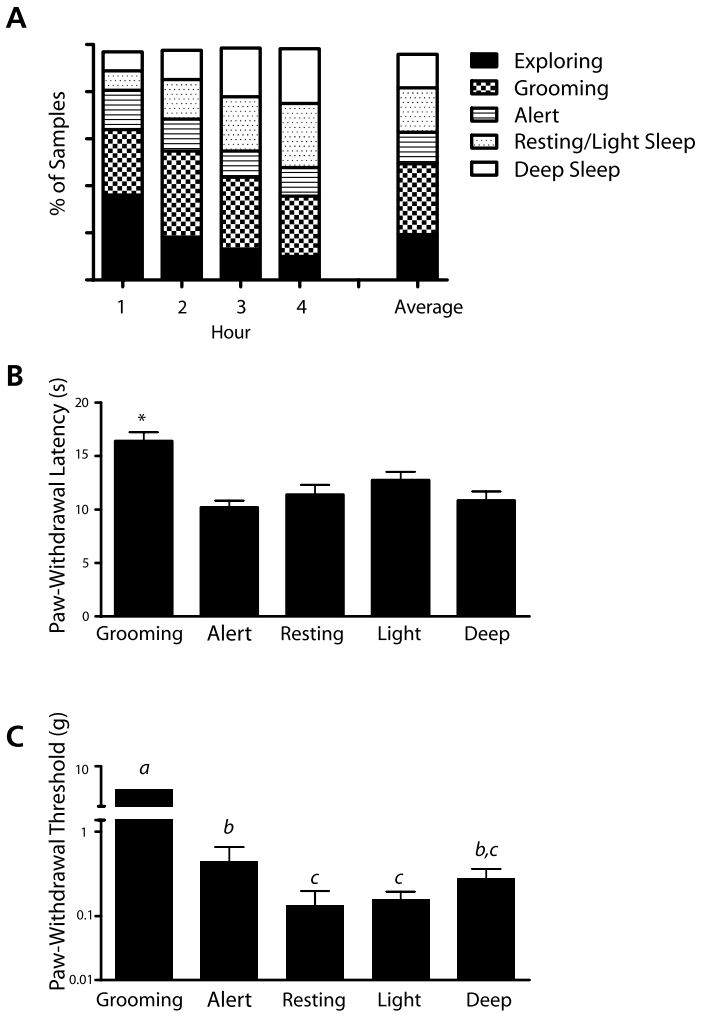

Figure. 1. ***A***, An ethogram of the behavior of naive, adult outbred CD-1 mice of both sexes in Plexiglas observation chambers (5 cm wide × 8.5 cm long × 6 cm high) atop a glass floor. Normally, we commence behavioral testing after 2 hours of habituation. Mice (n = 48) were videotaped and scored later by sampling (5 s of every minute) for the following behavioral states: Exploring (active locomotion), Grooming, Alert (standing on all four paws with eyes fully open, with active behaviors but no locomotion), Resting/Light Sleep (eyes half-open or closed), Deep Sleep (eyes closed, in a curled or hunched position). ***B***, Influence of behavioral state (A) on latency to withdraw hindpaw from noxious radiant heat from below (IITC Model 336; ≈45 W). Bars represent mean ± SEM withdrawal latency. *$p<0.05$ compared with all other groups. ***C***, Influence of behavioral state (A) on threshold to withdraw hindpaw from mechanical stimuli (von Frey fibers). The standard up-down psychophysical method was used (Mogil et al., 2006). Bars represent mean ± SEM withdrawal thresholds estimated using linear regression. Mice did not withdraw from any von Frey fibers while grooming; the threshold in this group is thus >2 g. Letters in italics indicate significantly different groupings.

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alone appear to be robustly analgesic during deep sleep (data not shown).

These observations have considerable implications. Not only do they likely represent a mouse model of attentional analgesia, but they suggest that null mutations that appear to affect pain sensitivity may actually do so by altering gross activity patterns, such that data are more or less likely to be collected in a particular behavioral state.

Social Context

The fact that within-cage order of testing had any measurable impact on hot water withdrawal latencies (Chesler et al., 2002a,b; see above) was a great surprise to us. We found that this effect could be abolished by not returning each mouse to its cage immediately after testing (Chesler et al., 2002b). This, of course, implied social communication among mice in the cage as the mediator of the effect: decreasing withdrawal latencies with each subsequently tested mouse. A serious difficulty in interpreting this and many other testing-environment variables is the demonstrated existence of both SIA (see Parametric Factors, above) and stress-induced hyperalgesia (SIH) (Imbe et al., 2006) as well as our very limited understanding of stress parameters leading to one or the other. Thus, the decreased nociceptive sensitivity displayed by later-tested cage members might represent either increasing SIA or decreasing (habituating) SIH. That is, mice returning to the cage might be saying to their neighbors, "Oh my God, that was horrible!", but they might also be saying, "No, really, I'm fine." An explanation of this phenomenon is not yet at hand, but our interest was piqued by the possibility of social communication among mice affecting their pain responses.

We ended up developing a number of paradigms in which to study this issue. In one such paradigm, the pain behavior of mice tested in dyads was compared with those tested in isolation. Two dyadic conditions were employed: (1) one mouse in pain/one mouse not in pain, and (2) both mice in pain. In the latter condition, of course, each subject was not only in pain (using the acetic acid writhing and formalin tests; Table 1) but observing another mouse in pain. Was this observation of pain enough to alter the observer's pain sensitivity? In fact, when the two mice in the dyad were familiar to each other (i.e., cage mates, for at least 14–21 days), a significant pain hypersensitivity was observed in both mice (Langford et al., 2006). Not only did we observe increased pain behavior, but we also observed a significant synchronization in the timing of the pain behavior of both mice in the dyad. These facts led us to conclude that mice were exhibiting "emotional contagion" of each other's pain, a rudimentary form of empathy (Preston and de Waal, 2002). In this particular case, stress was eliminated as a mediating factor, since stress levels (measured behaviorally and via corticosterone radioimmunoassay) were higher in stranger dyads than in cage-mate dyads, but only cage-mate dyads exhibited the emotional contagion. To our great surprise, the sensory modality implicated in this social communication was vision, since only a visual blockade abolished the hypersensitivity and synchrony. In another paradigm, we demonstrated that mere observation of writhing behavior in a cage mate led to hypersensitivity to withdrawal from radiant heat applied to the hindpaw (Langford et al., 2006). This latter finding is important not only because it eliminates mere imitation as the mediator, but because it suggests that social factors have the ability to sensitize pain circuitry in a general sense.

An intriguing finding from this same study was that, compared with mice tested in isolation, male (but not female) mice tested in a dyad with an unaffected stranger exhibited significantly *decreased* pain behavior (Langford et al., 2006). This result might represent a form of SIA, or might actually be evidence of a conscious decision to inhibit signs of vulnerability in the presence of a strange male mouse.

One way or another, the practical implication for pain testing is quite obvious: To avoid social confounds, mice should always be tested for pain behavior in visual isolation from all other mice. Of course, to completely abolish social confounds would require isolation housing, but this itself is a considerable stressor repeatedly found to alter pain sensitivity and analgesic response (e.g., Katz and Steinberg, 1970; Panksepp, 1980). The powerful effects of housing on pain behavior were elegantly demonstrated by Raber and Devor (2002), who observed that the extreme "autotomy" (self-mutilation after limb denervation) phenotypes of rats selectively bred over many generations for high autonomy (HA) and low autonomy (LA) levels could be dramatically altered simply by housing HA and LA rats together. This simple social manipulation, apparently mediated olfactorily, decreased autotomy behavior in HA rats and increased it in LA rats such that no phenotypic difference remained between the lines.

Our findings and those of Raber and Devor (2002) suggested to us the possibility that long-term exposure to cage mates in pain might itself produce hypersensitivity to pain. To test this theory, we subjected two mice per cage of four to either sham surgery or spared nerve injury (SNI), producing both thermal

and mechanical hypersensitivity (and, presumably, spontaneous pain, although this is difficult to confirm or quantify). Before surgery and on day 14 postsurgery, we tested all mice (in isolation) for thermal and mechanical sensitivity. A separate group of mice was also tested on day 15 using the acetic acid writhing test. As shown in Figure 2, our prediction was very clearly refuted; unoperated "neighbor" mice were unaltered by their constant observation of SNI-related pain behaviors. Although it is possible that our experimental paradigm was simply insufficiently supple to demonstrate the effect, we believe that social factors can likely modulate pain only in *real time*. This is no doubt good news for those trying to control the confounding influence of social factors.

Conclusions

Compared with other fields of inquiry within neuroscience (e.g., depression, schizophrenia), pain is generally regarded as relatively straightforward, and indeed our field has the advantage of possessing at least some models with clear face validity (i.e., I too will withdraw my finger from hot water within a certain number of seconds). Like many issues in biology, however, pain is far more complicated than it seems at first glance, and the animal models are far more subtle than we might suspect. Molecular and cellular techniques are far better funded, and thus more respected, than behavioral techniques in pain research (as in every other field), but ultimately drug development will not proceed without positive and trustworthy behavioral pharmacology data. Sir William Osler said in 1892: "If it were not for the great variability among individuals, medicine might as well be a science and not an art" (Roses, 2000). He was talking about humans, of course, but if we are to understand variability among humans, we must first understand variability in animal models of humans. For pain, this effort has just begun.

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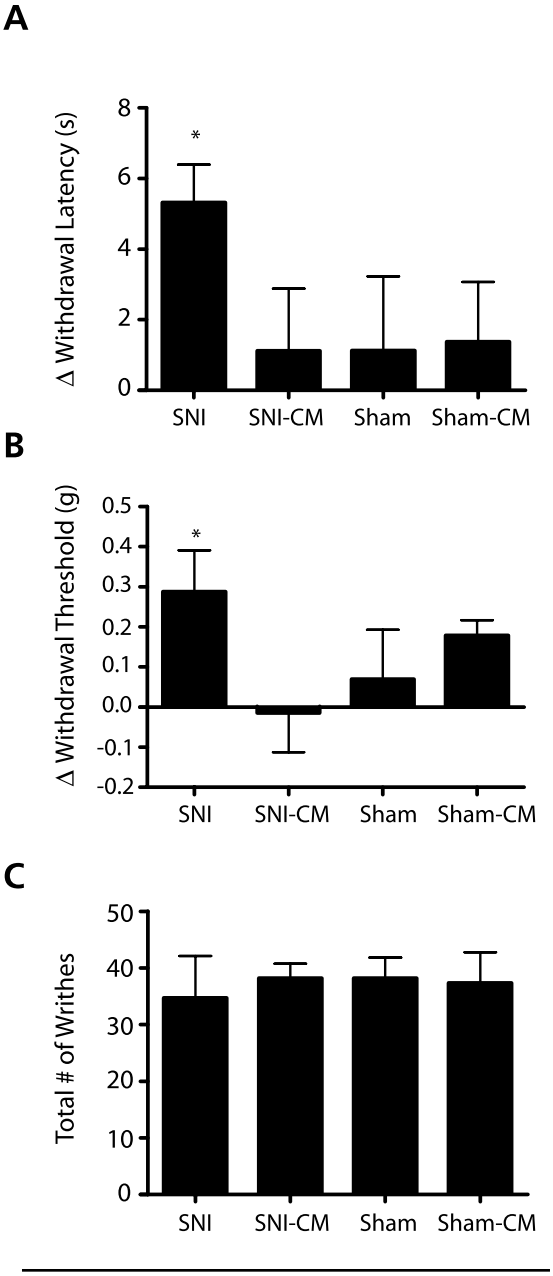

Figure. 2. Two-week co-housing with mice experiencing chronic pain does not produce hypersensitivity to pain in unoperated "neighbor" mice. Mice received either a spared nerve injury (SNI) or sham surgery, or were cage mates of SNI-operated mice (SNI-CM) or sham-operated mice (Sham-CM). Bars in ***A*** represent changes (mean ± SEM) in latency to withdraw from noxious radiant heat (Fig. 1*A* legend) on postoperative day 14 relative to baseline latencies; positive values indicate hypersensitivity. Bars in ***B*** represent changes (mean ± SEM) in threshold to withdraw from mechanical stimuli (Fig. 1*B* legend) on postoperative day 14 relative to baseline thresholds; positive values indicate hypersensitivity. Bars in ***C*** represent mean ± SEM total number of "writhes" in 30 minutes following intraperitoneal injection of 0.9% acetic acid on postoperative day 15. **p*<0.05 compared with zero (i.e., significant hypersensitivity observed).

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Features and Fundamentals of Pavlovian Fear Conditioning

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Introduction

The goal of this presentation is to give you a practical understanding of what Pavlovian fear conditioning is and how you can use it to answer your own questions about brain functions and mechanisms. In this regard, it differs from my typical lecture, which describes an in-depth analysis of a specific theory or empirical phenomenon. Fear conditioning most directly applies to questions about learning, memory, and anxiety, but it can also serve as an assay of general brain function. I will not give you a canned procedure or recipe; rather, my hope is that you will gain knowledge of how to adapt and design fear-conditioning experiments to address your own research questions.

What Is Fear Conditioning?

Fear is a natural phenomenon

Fear conditioning plays a normal and essential role in activating adaptive behavior whose function is to defend against environmental threats (Fanselow, 1984). Because there is tremendous selection pressure favoring effective defensive behavior, fear conditioning evolved as an extremely rapid and robust form of learning. A common misunderstanding is that the only reason for fear conditioning is that we quickly recognize a past threat when it occurs in the future. Certainly, such anticipatory behavior is one important function of fear conditioning. However, fear conditioning also is important for generating certain defensive behaviors the very first time a threat is experienced (Fanselow, 1980a). That is not to say that fear conditioning does not affect future behavior; indeed, fear conditioning produces memories that can last undiminished over a lifetime (Gale et al., 2004).

Fear conditioning also plays a role in anxiety disorders. The rapid and potent learning that occurs during a threatening experience means that anything capable of activating a fear memory may result in an overwhelming reaction. Fear conditioning can occur through either directly experiencing a traumatic event or watching another display a fear reaction (Mineka and Cook, 1993).

Fear in the laboratory

The speed and robustness that make fear learning such an effective natural adaptation also make it readily transferable to the laboratory, where it has been used successfully in a large number of species. Humans, rats, and mice are the most common subjects of such laboratory experiments.

Fear conditioning experiments use the standard nomenclature of Pavlovian conditioning. The stimulus that reinforces learning, Pavlov's unconditional stimulus (US), is usually a brief, mild electric foot shock. Neutral stimuli that are concurrent with the US may become conditional stimuli (CSs) capable of provoking defensive responses as a conditional response (CR). Note that I have used the term *conditional* and not the more common, but incorrect, term *conditioned*. Pavlov meant conditional, being synonymous with dependent, because he believed that a neutral stimulus had to be in a conditional relationship with the US for it to acquire the ability to produce a learned response (CR). Many years later, Rescorla (1967) proved this point. Not every stimulus contiguous with a US comes to produce a CR. Only stimuli that are dependent on the US become a CS capable of producing a learned response. For example, in Rescorla's experiment, he held the number of tone–shock (CS–US) pairings constant for several groups of rats and degraded the conditional relationship between tone and shock by adding different numbers of unsignaled shocks for the different groups. Under these conditions, fear responses to the tone were directly related to the strength of the dependent (conditional) relationship between tone and shock.

Another common misunderstanding about Pavlovian conditioning is that the CR to the CS is the same behavior as the unconditional response (UR) to the US. A CR is any response that emerges to the CS because it is in a dependent relationship with the US. The CR and the UR are sometimes the same (Cason, 1922), sometimes opposite (Siegel, 1975), and sometimes simply different than US-elicited behavior (Timberlake and Grant, 1975; Holland, 1977). Fear conditioning provides a case in point. The UR to a shock US is a vigorous burst of activity, but this activity burst does not occur in response to stimuli paired with shock (Fanselow, 1982). Indeed, stimuli paired with the same shock that causes an activity-burst UR produce an immobile "freezing" response as a CR. Importantly, freezing is never produced as a UR to shock (Fanselow, 1980a, 1986).

The CS in most conditioning experiments is a temporally discrete event such as a sound or light. However, conditioning occurs in a specific environment (e.g., a conditioning chamber in a particular room), and the unique features of that environment are in a dependent relationship with the shock insofar as, relative to the normal environment, they provide a context for the shock. This context also acts as a CS that can elicit a fear CR. Whether or not there is a discrete CS, there will be a context CS as well. Unlike the discrete CS, the context is usually present before, during, and

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after the US. Since the context is usually present immediately before the US, it becomes a CS capable of producing a CR. Because it usually continues after the US, the behavior that immediately follows the US may not be a UR to the US but rather a CR to the context CS. For example, the freezing that immediately follows a shock is a CR to the context and not a UR to shock (Fanselow, 1980a, 1986). Earlier, I said that fear conditioning contributes to the behavior that occurs immediately after the first presentation of an aversive or threatening stimulus. Having a context cue that is statically present before and after the shock mediates this learned response. Testing for conditional behaviors immediately after the shock provides a convenient measure of short-term memory. Testing either the context or a discrete CS at a time point remote from the training session is used to gauge long-term memory.

Why Use Pavlovian Fear Conditioning?

Features

In general, Pavlovian conditioning procedures are well suited for rigorous laboratory analysis because the events are highly specified and directly controlled by the experimenter. Fear conditioning offers a number of features that make it particularly adaptable in the laboratory. Fear behaviors are rapidly acquired, and there are several robust, easily quantifiable measures. One-trial learning is reliable for conditioning fear, which makes possible specification of the exact instant of memory formation.

Another attractive feature of fear conditioning is that the neural circuitry and relevant sites of plasticity within those circuits have been thoroughly described. Figure 1 is a rough schematic of this circuit, and highlights pathways for different classes of CS and regions where CS–US integration occurs. The fact that the hippocampus is important for context but not for auditory conditioning has attracted substantial attention (Kim and Fanselow, 1992). The fact that dissociable hippocampus-dependent (context fear) and hippocampus-independent (tone fear) memories are formed at the same instant and can be measured with the same responses provides a great deal of analytical power. Figure 2 shows the design and outcomes of such an experiment. The knowledge that specific sites of integration of CS and US can be identified (Romanski et al., 1993) has enabled researchers to make remarkable progress on detailing the molecular mechanisms of plasticity that support this type of learning (Apergis-Schoute et al., 2005).

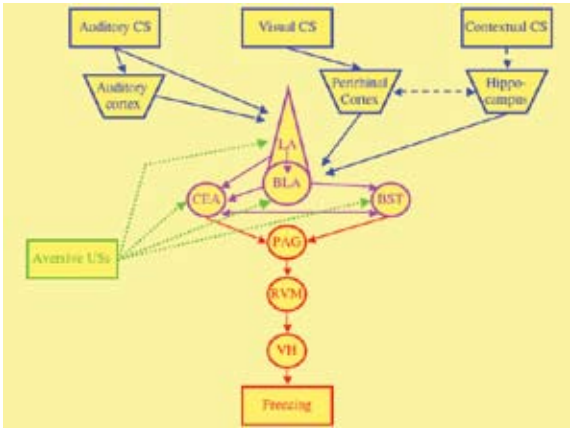

Figure 1. A simplified diagram of the neural circuitry responsible for conditional fear. Abbreviations denote: periaqueductal gray (PAG); rostral ventral medulla (RVM), ventral horn (VH), central nucleus of the amygdala (CEA), basolateral amygdala (BLA), lateral amygdala (LA), and bed nuclei of the stria terminalis (BST). Predictive stimulus (CS) inputs, starting at the thalamic level, are in blue, and aversive reinforcing US inputs are in green. Regions of CS–US integration are in purple and response generation pathways in red.

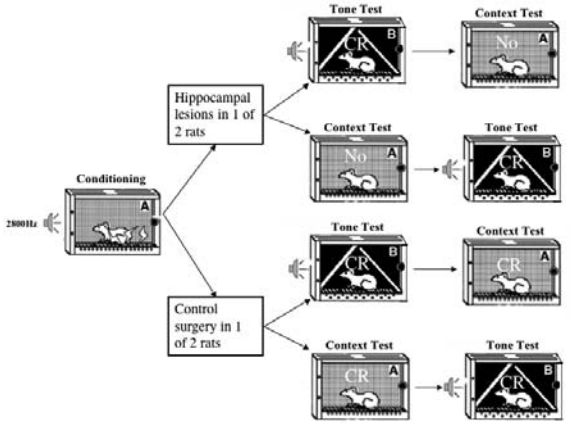

Figure 2. A schematic of a basic design for testing tone and context fear. Typically, 1 day is given between each phase, except for a period of 10–14 days for recovery from surgery. Note how a different context is used to test tone from that of training and context testing. If tone and context fear are to be compared, a counterbalanced testing order, as shown here, is recommended; if no direct comparison of tone and context is to be made, testing context first is the preferred approach. Presence of a fear CR (typically freezing) is indicated by "CR" in the box. Loss or reduction of fear is indicated by "No" in the box. After Kim and Fanselow, 1992.

Measures

Several measures have been used to assess the acquisition of fear. These include fear-potentiated startle, increased blood pressure, defecation, analgesia, and freezing. For several reasons, I will concentrate on freezing. First, it is a robust response to fear in both rats and mice and is readily observed in natural fear-provoking situations in both these species. Relative to the other measures, it is highly sensitive and thus easily detects the learning

acquired in a single trial. Freezing can be reliably and directly detected by a human observer, although automated systems also can approach the sensitivity of the human observer. Another important advantage of freezing is that it does not require constraint or administration of probe stimuli that can influence behavior. For example, potentiated startle requires presentation of a loud noise that can condition fear on its own, and analgesia testing requires administrating a painful stimulus. This is not to say that other measures do not have their own advantages. The latency to freeze after a CS presentation is relatively long, and reliably measuring it takes a significant amount of time. Thus, if very short CSs (5 seconds or less) or precisely timed CRs are of interest, fear-potentiated startle may be more appropriate.

Since we are concentrating on the freezing measure, it is worth defining. It is the absence of all visible movement except for breathing. In the freezing rat or mouse, even whisker movement ceases. This rigid definition makes it easy for a human observer to objectively score freezing. Sometimes freezing is punctuated by a period of small movements, such as keeping completely still except for sniffing (including whisker movement) or a slight slow, side-to-side sway. These motions are not freezing, but they often present the most difficult behaviors for automated scoring systems. Further, the definition of freezing says nothing about the posture of the rodent. While freezing usually occurs in a crouching position (some early work used crouching rather than freezing as a measure; Blanchard and Blanchard, 1969), these two phenomena are independent. For example, freezing can occur while rearing, although the animal usually slips into a crouch position and resumes freezing. Since a crouching animal may make small head movements, usually the rate of crouching exceeds the rate of freezing.

Parameters Matter

When designing a conditioning experiment, many decisions need to be made: when should the CS occur, how long should it be, how many trials should take place, how intense they should be, how long the shock should last, etc. The novice may despair when I say that every one of these parameters has the potential to greatly influence the outcome of the conditioning experiment, especially because I will not present a single set of optimal procedures. The reason I do not is that every experiment has its own goal. Researchers testing a cognitive enhancer may want to use weak parameters that barely support learning so that plenty of room is left to detect enhancement. But that set of parameters would be inappropriate for studying a knockout model that is expected to reduce learning.

The task at hand is not as daunting as it may seem. Learning theorists have intensively studied why these parameters matter, and their work has resulted in many models of conditioning. The most influential of these has been the Rescorla–Wagner model (Rescorla and Wagner, 1972). For our purposes, the model is incredibly useful because it provides a rudimentary equation based on a set of simple ideas that accounts for almost all the effects of manipulating the basic parameters (Fig. 3, *A*). One needs only a paper and pencil (not a computer) to run a simple simulation and, with a few examples, one may easily intuit the model's predictions. This is not to say there is no theoretical debate on the merits of the Rescorla–Wagner model relative to other theoretical models.[a] However, as a day-to-day tool to aid thinking about conditioning experiments, its usefulness is unsurpassed.

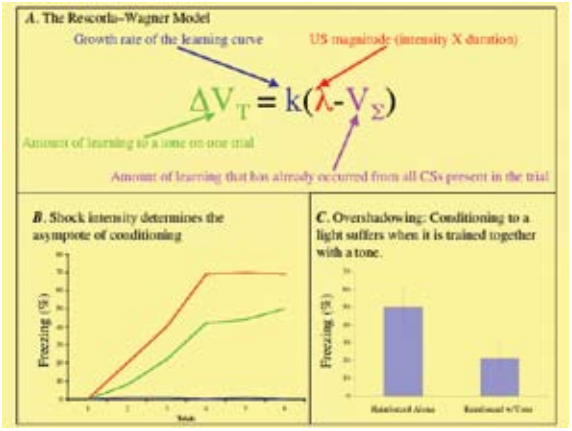

Figure 3. ***A.*** The Rescorla–Wagner (1972) equation. ***B.*** An example of how λ (here manipulated with shock intensity) influences asymptote. The data were generated by measuring preshock freezing in a context in which 1 shock trial was conducted per day using 0.8 mA (red), 0.6 mA (green), or no shock (blue) as a US in rats (Young and Fanselow, 1992). ***C.*** An example of a form of stimulus competition known as overshadowing. Rats were tested for fear of a light after they received pairings of just the light and shock, or light and tone together with shock.

The Rescorla–Wagner Model

The Rescorla–Wagner model begins with the idea that a US can support a certain limited amount of conditioning (λ) that is determined by the magnitude of the US. In fear conditioning, the magnitude of the shock is roughly equivalent to the product of the intensity and the duration of shock. The strength of conditioning (associative strength) to a CS is indicated by a variable (V) that starts at 0 when the CS is novel but changes over the course of conditioning. The major function of λ is to set the asymptote of the learning curve. Figure 3*B* shows data from a conditioning experiment that generated

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learning curves with two different intensities of shock as a context CS (Young and Fanselow, 1992).

In any trial in which the CS is present with a US, the CS will grab a proportion (k) of λ. Thus, the parameter k serves as a learning rate parameter, rendering learning fast or slow. Since a US can support only λ worth of conditioning, the amount of conditioning supported has to be reduced by the amount of conditioning that has already occurred. The most important aspect of the Rescorla–Wagner model (which sets it apart from earlier models) is that reduction in the amount of conditioning possible in a trial is determined not just by the CS in question but by all the CSs present. To obtain this value, V_Σ, you simply add all the current V values of all the stimuli present in a trial. This summation rule means that all the available CSs will compete for conditioning. If you have one CS consistently paired with a US, the CS will approach λ for that US. But if two consistently reinforced CSs are present (e.g., a tone and a light), they will split that λ between them, a phenomenon called overshadowing. Figure 3C shows the effects of such competition between 2 CSs. A light CS that was reinforced alone produced more conditioning than one that was reinforced together with a tone. Putting these results together, we can express the change in conditioning to a Tone (ΔV_T) on a conditioning trial as $k(\lambda - V_\Sigma)$. A learning curve is generated by repeating the calculation of ΔV_T for each trial. If there is more than one CS, the calculation needs to be made separately for the other CS. That is, for a light in compound with a tone, you would also calculate $\Delta VL = k(\lambda - V_\Sigma)$; for this case, $V_\Sigma = V_T + V_L$.

Rescorla and Wagner suggest that k has two components: one related to the CS (α) and one related to the US (β). The parameter β is used to explain several phenomena: why some types of conditioning are faster than others (e.g., fear and taste aversion versus eye blink); more complex information processing situations with multiple US intensities (Fanselow, 1980b; Young and Fanselow, 1992); and CSs that undergo a mixture of reinforcement and nonreinforcement (Wagner et al., 1968).

Extinction (the repeated presentation of a CS with no US) is another area where β is useful. Rescorla and Wagner view extinction as a series of conditioning trials with a US of 0 intensity. Having a low β for a US of 0 intensity slows the rate of extinction relative to acquisition, which is commonly observed empirically. Rescorla and Wagner proposed that α corresponds to stimulus salience. Originally, stimulus salience was related solely to the physical intensity of the CS, which predicts that a louder tone conditions the subject more rapidly than a softer one. However, another factor influences stimulus salience: We tend to ignore stimuli that we experience repetitively with no outcome. Stimuli that predict no change in reinforcement conditions have an α lower than that predicted merely by their intensity (Rescorla, 1971; Mackintosh, 1975; Pearce and Hall, 1980). The k value is the product of α and β, although β is often ignored in simulations. Since α corresponds to CS salience, it will be different for different CSs. The salience of any CS will depend on the configuration of each lab; therefore, no one has ever generated a reference table for α. It usually suffices to pick an arbitrary value that reflects differences in salience among cues.

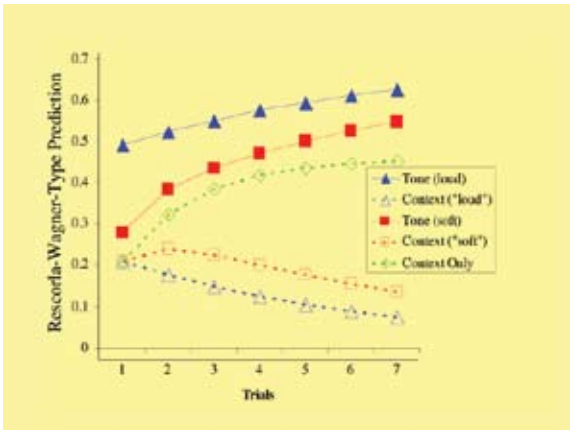

Figure 4. A simulation plotting V (associative strength) as function of trials generated by the Rescorla–Wagner model (see test for details of the parameters used).

Even if you are investigating the conditioning to a single discrete CS, at least two stimuli will be present — the explicit CS and the context. Since a context is present between trials (intertrial interval, or ITI), its associative strength will change during this period. The same equation is used, but since there is no US, λ is set to 0. The ITI will often differ from the duration of the CS. The typical practice is to break up the ITI into CS length blocks and to repeat the calculation of ΔV for each block.[b] Because context acts as a CS, it is critical to test the tone in a context quite dissimilar from the training context so that tone conditioning will not be confused with context conditioning.

Figure 4 shows a simulation that incorporates most of the features we have discussed. It looks at the results of a trial of a tone tested in both a novel context and the original training context. Three simulations are run that differ with respect only to the tone. There is a loud tone, where a high α results in a high k (0.7), and a soft tone, where k is low (0.4). Note that the 2 tones display different amounts of conditioning when

tested after a few trials, but that these differences diminish with further training. Three context curves are also shown: for the loud tone, the soft tone, and one in which no tone is used. These tests would return to the original training context, but with no tone or shock. All 3 curves were generated with the same k. For reinforced trials (tone–shock pairings), k was 0.3, but it fell to 0.05 during the ITI because nonreinforcement has a low βvalue. Although the context is treated with identical parameters for each curve, the context curves differ a great deal. This is because the competitive rules of the Rescorla–Wagner model result in an interaction between tone and context fear. There is less context conditioning with the loud tone because it is so effective at competing with the context. Also note that both these context learning curves actually decrease with continued conditioning: There is more conditioning after 2 than after 7 trials. However, the unsignaled context, because it is free from competition with the tone CS, continues to increase over the course of trials and shows a learning curve quite similar to the soft tone. Table 1 lists several relevant parametric manipulations.

The practical point of this analysis is that it provides a highly useful tool for choosing a set of parameters that can best test the hypothesis of interest. It can also help diagnose a procedure that is producing a less than ideal level of responding. For example, if you find that your auditory conditioning is too strong and your context conditioning too weak, it is easy to choose a parameter to adjust. For example, to address the hypothesis that hippocampal lesions selectively affected context conditioning (because auditory conditioning is stronger than context conditioning), Anagnostaras and colleagues (1999) used these principles to enhance context conditioning relative to tone conditioning. Specifically, they decreased the number of trials and increased CS duration.

Context Is the Violator

When using a context (especially when there is no discrete CS), some very interesting exceptions appear to some of these rules. For an unsignaled shock, the context–US interval (CS duration) would be the time between placement in the context and the US (placement-to-shock interval, or PSI). If multiple trials are used, CS duration is basically the same as the interval between shocks (ITI). Contrary to what happens for discrete CSs, and contradictory to the Rescorla–Wagner model, increasing the PSI (i.e., CS duration) or ITI enhances conditioning—to an unsignaled shock (Fanselow 1986, 1990; Fanselow and Tighe, 1988). Furthermore, pre-exposing a context CS facilitates context conditioning—the exact opposite of the latent inhibition effect found with discrete CSs (Fanselow, 1990). This leads us to the view that, in order for contexts to become effective CSs, the subject has to integrate the many features of the context into a mnemonic representation during exploration (Fanselow, 1990). This interpretation was energized by the finding that the hippocampus—a region especially well situated anatomically for integrating multimodal information—is important for contextual fear conditioning (Kim and Fanselow, 1992; Rudy and Sutherland, 1995; Fanselow, 2000).

Conditioning Controls

To conclude that conditioning occurs, one must compare the level of the CR to a condition where no CR is expected. Because we define conditioning as changed behavior brought about because of experience, with a dependent relationship between events, the appropriate control would be to provide similar experience but without a dependent relationship (Rescorla, 1967). For fear conditioning to a discrete CS, an unpaired control in which there is equal experience with the CS and US, but these are kept separate in time, provides an excellent option.[c] For context conditioning, a test in a novel condition is used. If one is testing a tone in a novel context, the baseline period preceding the tone provides such a control.

Table 1. The effects of parametric manipulations on conditioning and their Rescorla–Wagner interpretation

Increasing parameter	Discrete CS	Context	Reason from a Rescorla–Wagner-type perspective
CS intensity	↑	↓	Increases α of CS
US magnitude	↑	↑	Increases λ
Time between USs (ITI)	↑	↓	More context-only periods produce more context extinction; the tone more easily outcompetes the context for λ
CS duration (CS–US interval)	↓	↑	Fewer context-only periods leads to less context extinction; the context does better in the competition for λ
Trial number	↑	↓	As discrete CS approaches asymptote, context loses competition with the CS
CS pre-exposure (latent inhibition)	↑	↑	Decreases α of CS

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Another useful control procedure is a discrimination design in which an individual subject is exposed to two CSs (or two contexts): one that is reinforced and one that is not (e.g., Fanselow, 1981). This procedure conveniently offers a conditional and control stimulus in the same subject. Obviously, the rapidity of such discriminations depends on how different the two stimuli are. It has long been hypothesized that the dentate gyrus of the hippocampus is a region that allows memories of similar patterns to be stored individually (Marr, 1971). To test this pattern separation hypothesis, we used a discrimination design of two different contexts having significant overlapping features (McHugh et al., 2007). Mice with a deletion of NMDA receptors from the dentate gyrus and their littermate wild-type controls were compared for rate of discrimination. The knockouts were much slower at acquiring the discrimination, taking about 10 days to catch up to their siblings.

Arrangement of the CS and US

Typically the CS starts and, after a short delay, the US begins. Pavlov called this delay conditioning, and it is the arrangement of CS and US that supports the best CR performance. In delay conditioning, the CS may terminate at either the start or the end of the US and makes little difference in the strength of the CR. One can reverse this arrangement (backward conditioning) or have CS and US completely overlap (simultaneous conditioning). While these procedures are helpful for addressing certain theoretical issues, they are rarely used, for they produce less robust performance.

An arrangement that has received a great deal of theoretical attention is trace conditioning. In it the CS terminates before the start of the US, leaving a gap (trace interval) between the two stimuli. Pavlov named the procedure trace conditioning because he believed that a "trace" of the CS must be held in memory in order for conditioning to occur. Pavlov appears to have been prescient — later it was discovered that, with sufficiently long trace intervals, trace conditioning depends on the hippocampus (the quintessential memory structure) even when delay conditioning does not (Weisz et al., 1980). For conditioning fear in mice, the necessary trace interval seems to be about 20 seconds (Chowdhury et al., 2005).

Trace conditioning can be a powerful tool for assessing hippocampal function. We were interested in a mouse with a knockout of the Δ subunit of the $GABA_A$ receptor. This subunit has very limited expression compared with the more ubiquitous γ2 subunit, which it replaces in the $GABA_A$ pentomer. What attracted our attention was the Δ subunit's very heavy expression in dentate gyrus. While standard contextual fear-conditioning procedures did not detect a phenotype in the knockout, trace (though not delay) conditioning was markedly enhanced in these mice (Mihalek et al, 1999; Wiltgen et al., 2005).

The Learning/Performance Distinction

No discussion of conditioning would be complete without giving at least some attention to the learning/performance distinction. We can assay learning and memory only by measuring a change in behavior, but factors other than learning and memory are likely to affect behavior. A genetic or drug manipulation may have nonspecific or unintended effects on sensory, motivational, or motor function. We need to be able to rule out such effects by taking measures that supplement our measures of primary interest. Table 2

Table 2. Some performance confounds and how to assess them

Result you are seeking	Potential confound	Control assay
Hippocampus-specific effect	General influence on other brain region(s)	Test fear response to tone—there should be no effect
Manipulation that affects the duration of memory (i.e., retention or consolidation)	General learning impairment	Post-shock freezing during training is a short-term memory that is mediated by a context-shock association but does not require any long-term memory
Drug impairs learning or memory formation	**State-dependent learning** drug state (or any other manipulation) is different during training and testing. Change in conditions from training to testing can lead to a loss in the stimulus support of behavior	Factorially manipulate drug between training and testing. If effect on learning reverses when drug is given during testing, you have a problem (indicated by a reliable training X testing interaction in ANOVA)
An effect on learning, memory, or anxiety	**Hyperactivity** reduces ability to freeze	Measure the number of crossovers from one side of the box to the other during the preshock period. This is a sensitive index of hyperactivity.
An effect on learning or memory	Manipulations alter **anxiety**, which in turn changes freezing levels	General anxiety should be reflected in preshock freezing, thigmotaxis (staying near walls), and fewer crossovers
An effect on learning	Altered **motivation**	Generate a learning curve: motivational variables typically affect the asymptote but not the rate of learning
Change in learning, memory, etc.	Alteration in **pain** sensitivity/ reactivity alters impact of shock	Measure movement during the shock; the activity burst is very sensitive to shock

provides a list of common performance problems that might arise in fear conditioning, examples of where they arise, and convenient ways of assaying them.

Footnotes

a. While specific components of the fear and eye blink circuits appear responsible for Rescorla–Wagner-like calculations (Fanselow, 1998), there are also a few conditioning effects that the model does not account for (Arcediano, 2004). However, if the Rescorla–Wagner model accounts for 95% of the variance that occurs in conditioning experiments, in recent years, learning theorists have concentrated their research almost exclusively on the 5% of the variance it does not account for.

b. There are models that make Rescorla–Wagner-type predictions based on time rather than arbitrary trials (e.g., Wagner, 1981; Sutton and Barto, 1990). These models are theoretically important, but for the purpose of getting a quick sense of what changing a basic conditioning parameter does, the effort required for these other models may not be justified.

c. Rescorla (1967) originally advocated a random control, but this was before he developed the Rescorla–Wagner model. Based on this model and empirical experience, unpaired controls are appropriate when few trials are used, as in most conditioning experiments. When large numbers of trials are used, however (say over 20), the random control is more appropriate. This is because with large numbers of trials, unpaired subjects learn that the CS predicts the absence of the US.

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Drug Self-Administration and Microdialysis in Rodents

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Drug Self-Administration in Rodents

Introduction

Operant behavior maintained by intravenous drug delivery is the most direct, and perhaps the most relevant, animal model of human drug self-administration. The observation that more than 20 psychoactive drugs that are abused by humans also function as reinforcers in rodents supports the hypothesis that drug self-administration in animals may be a reliable predictor of abuse liability in humans (Collins et al., 1984). Self-administration also provides a powerful experimental model for developing therapeutic interventions for human drug abuse and dependence and for identifying the genetic basis of abuse liability. Evidence suggests that the neural circuitry activated by drugs of abuse overlaps with that underlying reward-related processes relevant to motivation.

The aim of this chapter is to introduce some practical aspects of drug self-administration in rodents. Although previous studies may be referred to for the purpose of providing demonstrative examples, a review of findings regarding the neural substrates of drug self-administration is beyond the scope of this paper (Koob and Goeders, 1989; Katz and Goldberg, 1991). The technical challenges involved in setting up the appropriate apparatus, assembling intravenous catheters, and achieving reliable surgical implantation are tedious but essential. The sections at the end of this chapter examine unit dosage and scheduling variables, sample results, and interpretation as well as revealing the practical considerations that determine what the data are and what they mean. Materials described in detail are intended specifically for intravenous self-administration procedures in rats and mice.

Materials and apparatus

Most materials described herein are available from a variety of commercial distributors.

The operant chamber

The most versatile operant chamber is one that allows for temporary and repeated attachment of the subject to the self-administration apparatus. Detachment permits maximum versatility of environmental variables, such as group housing compared with social isolation, and the introduction of novel environmental stimuli concurrent with drug availability. The major components of a simple self-administration chamber are displayed in Figure 1.

(a) For rats and mice, the typical manipulandum is a lever. For other species, a more natural operant may be preferred, such as the nose-poke, which is commonly used for mice. In both cases, it is best to equip the chamber with at least two of these devices, allowing for the employment of complex schedules or controls.

(b) Of particular importance is the swivel system, which must be extremely easy to turn. Resistance to movement not only endangers the delicate catheter assembly but invariably disrupts the animal's performance. The cylindrical swivel depicted in Figure 1 is anchored in an assembly that is counterbalanced with an adjustable weight so that the lead remains gently taut to prevent tangling.

(c) The lead, which delivers the drug solution and attaches to the catheter, must be flexible but coated or wrapped with a chew-resistant material such as wire. An internal cannula at the tip of the lead inserts into the externalized cannula guide of the implanted catheter (lead, internal cannula, and external cannula guide supplied by Plastics One, Roanoke, VA).

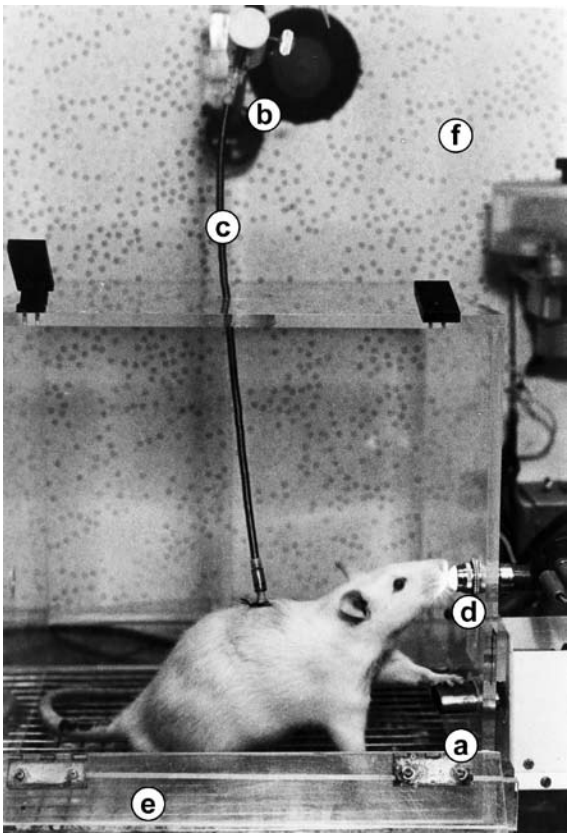

Figure 1. Components of a typical self-administration chamber for the rat: (a) manipulandum, (b) swivel system, (c) spring-bound lead, (d) discrminative stimulus (e.g., light), (e) grid floor, and (f) sound-attenuating chamber.

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(d) Other helpful devices include lights or tones to be used as discriminative or conditioning stimuli, and food hoppers to assist in operant training or for use in multiple schedules.

(e) Most operant chambers employ a grid floor, which allows waste to collect in a removable tray. Particularly for self-administration, it is best if all floor components (including the grid) are removable for systematic cleaning.

(f) A worthwhile amenity is soundproofing of the chamber to prevent the effects of unintentional environmental stimuli (e.g., ultrasonic vocalizations of other animals).

The homemade chronic intravenous catheter

Preassembled but costly catheters have recently become available, as have animals commercially implanted with chronic indwelling catheters. However, such catheters may require modification for temporary and repeated attachment to the lead in an operant chamber such as the one shown in Figure 1. The following description outlines a procedure for assembling catheters suitable for implantation in rats and mice, based on previously established methods (Caine et al., 1999, 2002; Thomsen and Caine, 2005, 2007) (Fig. 2).

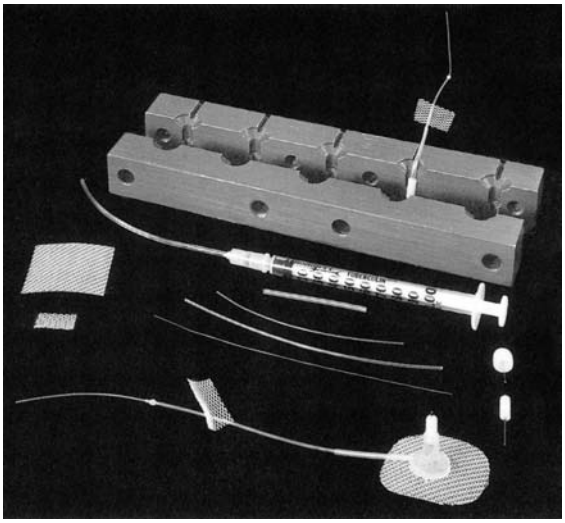

Figure 2. Materials for assembling and maintaining a chronic intravenous catheter for the rat. From the top: mold for constructing the catheter base; flushing syringe; polyethylene (PE) 160, PE10, and PE50 tubing; 33 gauge wire for insertion into tubing during heat-fusion of PE10 and PE50 tubing; dummy stylet and cannula guide; and completed catheter assembly (shown at 75% actual size).

Materials and Methods

To assemble your own catheters, you will need:

- Monofilament Cloth–Nylon Mesh, 500 µm, 12" × 24"; cat# CMN-500-D (Small Parts, Miami Lakes, FL)
- Scissors, scalpel, cotton swabs
- A standard hot plate
- Medical Grade Tubing, Silastic Brand; cat# 602-105, size 0.012"/0.31 mm ID × 0.025"/0.64 mm OD, Non-Sterile (Dow Corning Medical Products, Midland, MI)
- Medical-grade silicone adhesive
- Cannula guides with threaded plastic base: Cannula Guide 22 GA 5 mm; cat# C313G-5UP (Plastics One)
- Needle-nose pliers
- Xylene solvent or a less toxic substitute (e.g., Hemo-De organic solvent; Scientific Safety Solvents, Keller, TX)
- Silicone oil lubricant
- Permanent nontoxic fixative such as dental cement or resin (e.g., cranioplastic powder and liquid (Plastics One)
- Molds for making the catheter base (e.g., aluminum, as shown in Fig. 2)
- Spatula or small spoon to mix the fixative
- A flushing syringe with sterile water

Alternative materials for the intravenous catheters for mice include:

- Medical-grade Silicone Catheter Tubing, Non-Sterile, 1.2 French (0.007"/0.018 mm ID × .16"/0.41 mm OD) × 25 ft; cat# BC-1S (Access Technologies, Skokie, IL)
- Cannula guides with threaded plastic base, 26 gauge 5 mm; cat# C315G-5UP (Plastics One)
- All other materials as for rat catheters

Catheter assembly (Fig. 2) (Caine et al., 1993)

(1) (a) For rats: Cut a 3 × 3 cm square of mesh for each catheter, and cut off the corners to yield a rounded shape. Then melt the edges of the mesh piece by holding it down onto the hot plate and dragging it as you turn it, to sear the mesh. This will reduce irritation to the animal both by preventing the mesh from fraying and by rounding the sharp ends of the nylon threads.

(b) For mice: Cut a 2 × 2 cm square of mesh for each mouse catheter and proceed as in step 1a.

(2) (a) For rats: Prepare the catheter tubing by cutting a 13 cm length of silicone tubing for each

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catheter using a scalpel to produce a smooth, straight cut. Some authors suggest beveling the catheter tubing in order to provide a larger opening, thereby possibly reducing the risk of blocking. However, the bevel may increase irritation of the vein endothelium during insertion, which may accelerate atrophy of the vein and/or thrombus formation.

(b) For mice: Cut a 6 cm length of silicone tubing for each catheter, as described in step 2a.

(3) (a) Drip an anchoring "bead" of medical silicone adhesive onto the catheter tubing approximately 1.5 to 2.0 mm in diameter and at least 3.7 cm from the end of the tubing. The bead should encircle the catheter rather than sit on one side (Fig. 2). Hang the end of the tubing to let the bead air-dry.

(b) Drip an anchoring "bead" of medical silicone adhesive onto the catheter tubing approximately 1.5 mm in diameter and at least 1.2 cm from the end of the tubing. The bead should encircle the catheter rather than sit on one side. Hang the end of the tubing to let the bead air-dry.

(4) Using needle-nose pliers, bend the long end of the cannula so that the tip points at a 90° angle from the threaded base, but avoid a sharp bend that would pinch the cannula and narrow the fluid channel. Start 1–2 mm from the threaded base and bend the cannula approximately 15°, then move the pliers farther up and bend again. Repeat until the cannula curves smoothly to the desired shape.

(5) Soak the tip of the silicone tubing in Hemo-De (Scientific Safety Solvents) or similar solvent for a few minutes, and dip the cannula in silicone lubricant to facilitate insertion of the cannula guide.

(6) Work the silicone tubing onto the bent end of the cannula guide, leaving a little space for the cement to attach to the cannula under the threaded base. Do not apply excess force to the tubing because you may pierce or score it, making it prone to leak at the cannula–tubing junction. Rather, soak the tubing in solvent again if it does not easily slide onto the cannula guide. Also, the tubing should extend straight from the cannula guide, and kinks should be removed by gently pushing the tubing straight.

Assembly of the Catheter:

(7) Spray the mold with silicone lubricant.

(8) Position the cannula guide in the mold so that the bent end of the cannula is in line with the top of the mold (that is, the bottom of the catheter base, where the mesh will be). The cannula guide and tubing should not touch the sides of the groove in the mold. The threaded base should extend two-thirds of the way into the fixative, leaving about 3 mm of threading free.

(9) Mix and pour fixative, letting it run down one side of the mold to avoid entrapping air bubbles. The fixative should be thin enough to fill out the bottom of the mold, but not so thin that it will contract excessively when the solvent evaporates. Avoid getting fixative in the groove around the catheter tubing. You may have to fill up the mold in two phases, using thinner fixative for the bottom and thicker fixative for the top.

(10) Place a piece of mesh centered on the base, and drip fixative through it to secure it and to provide a smooth surface. Remember that the fixative will contract as it hardens, so it should cover the mesh well to ensure that this will not come detached. The loose edges of the mesh should remain free of fixative to allow tissue to grow into it. Let the fixative harden almost completely, then open the mold gently without tugging on the delicate catheter tubing.

(11) Test the catheter for leaks: Attach the flushing syringe, filled with air and sterile water, to the cannula guide; clamp the tip of the catheter; and gently depress the plunger of the flushing syringe. You should feel resistance, and no fluid should leak along the catheter. When releasing the plunger, it should recede back to its original position owing to the compressed air. Now unclamp and test for patency: Flush a small volume of water through the catheter; it should flow easily.

(12) Before the fixative hardens completely, remove any sharp ridges or points that would irritate the animal, particularly if fixative seeped into the groove around the tubing.

(13) Most leaks appear around the cannula guide–tubing junction, and it is advisable to stabilize this part of the apparatus with a droplet of silicone adhesive.

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(14) Measure the length of the catheter from the anchoring bead to the tip. It should be 3.7 cm for male rats, 3.2 cm for female rats, and 1.2 cm for mice. Trim with a scalpel blade if necessary.

Catheter accessories

To prevent contamination and backflow, the catheter cannula must be capped at all times, except during self-administration. Also needed are syringes fitted with a line for flushing the catheters, as well as protective covers to prevent the animals from chewing the caps. This section also describes how to make the needle used in the catheter implantation surgery.

Materials List:

- Rat flush/cap tubing: Tygon 0.02"/0.51 mm ID × 0.06"/1.52 mm OD (Saint-Gobain Performance Plastics); monofilament nylon (e.g., 50 lb capacity fishing line)
- Mouse flush/cap tubing: flexible plastic tubing, ID 0.010"/0.25 mm, OD 0.030"/0.76 mm, Tygon cat#AAQ04091
- Mouse syringe: #5200 TB syringe glass tip (Popper & Sons, New Hyde Park, NY) (supplier: PGC Scientifics, Frederick, MD)
- Rat luer hub: NDL S/S 22GA, 1/2" w/ luer hub, NE-221PL (Small Parts Inc.)
- Mouse luer hub: NDL S/S 26GA, 1/2" w/ luer hub, NE-221PL
- Protective covers: S6-8 Round Standoff Aluminum 6-32 × 1/2" 0.250" OD; order #2838 (Small Parts Inc.)

The Flushing Syringe

For rats:

(1) Insert a 22 gauge luer hub into a 15–25 cm length of Tygon tubing (0.51 mm ID). Take care not to damage the tubing.
(2) Connect this modified needle to a 1 ml syringe.
(3) The flushing syringe is fitted to the catheter by working the Tygon tubing onto the catheter cannula guide, providing a tight fit. To maintain this tight fit, the tip of the flushing tubing should be trimmed periodically using a razor blade or scalpel, with a hard, flat surface as support.

For mice:

(1) Insert a 26 gauge luer hub into a 12–15 cm length of flexible plastic tubing (0.25 mm ID). Take care not to damage the tubing.
(2) Connect this modified needle to a 0.1 ml syringe. The syringe itself should be graded in 0.01 ml increments and should work smoothly to allow you to detect resistance to flow in the catheter. One way to achieve this is to lubricate a 0.25 ml glass syringe with Vaseline.

The Cap and Protective Cover

(1) To make the cap for rats, cut a 10 mm length of 0.51 mm ID × 1.52 mm OD Tygon tubing and close one end by inserting a 3–4 mm length of nylon monofilament that will provide an airtight fit. A less elegant and easier method of sealing the cap is to melt one end of the tubing with heat (see step 2).
(2) To make the cap for mice, cut a 4 mm length of flexible plastic tubing (ID 0.010"/0.25 mm, OD 0.030"/0.76 mm) and close one end by melting it briefly in a gas flame and clamping it firmly.
(3) A threaded metal protective cover fitted onto the base of the catheter will prevent animals from chewing on the cap. Light aluminum hoods that fit both rat and mouse catheters are commercially available.

Subjects

Reliable intravenous self-administration has been reported in rats and mice (Table 1). Detailed descriptions of materials and procedures presented here are intended for rats and mice. Catheter implantation can be performed fairly easily in rats weighing 180–400 g, although 260–320 g is optimal because large jugular veins are easier to manipulate, though excess fat and muscle tissues hamper access to the vein. Animals are normally maintained in a temperature-controlled environment (approximately 21°C) with a 12-hour light/dark cycle. Rats will self-administer drugs during any period of their diurnal cycle, although they are most active during the dark phase, and may acquire self-administration activity more expediently during this period. Newly arrived animals are routinely allowed 1 week to habituate to the environment before the start of behavioral or surgical procedures.

Surgical procedures

Catheter implantation

The implantation of a jugular catheter in the rat may require fewer than 15 minutes for the expert surgeon. For this reason, a short-acting vapor anesthesia such as a halothane/oxygen mixture is highly recommended. This is a microsurgical procedure, and a binocular surgical microscope is recommended but not essential. The surgery is an invasive procedure with a reasonable risk of infection, which can be reduced by employing sterile techniques when possible. A particularly useful precaution is to wrap in absorbent paper all materials that will touch the animal and to steam-autoclave this package prior to surgery. The catheters cannot be steam-autoclaved but may be gas-autoclaved (e.g., with ethylene oxide) or, at the very least, rinsed and flushed with 70% ethanol prior to surgery.

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Table 1. Drug self-administration in knockout mice

Intravenously self-administered drug	Gene knockout	Effect of knockout on self-administration	Reference
Cocaine	Cocaine-regulated and amphetamine-regulated transcript (CART)	—	Steiner et al., 2006
	Muscarinic M_5 acetylcholine receptor	↓	Thomsen et al., 2005
	Adenosine A_{2A} receptor	↓	Soria et al., 2006
	CART *Carttm1Amgen*	↓	Couceyro et al., 2005
	Glucocorticoid receptor	↓	Deroche-Gamonet et al., 2003
	Kir3 potassium channel	↓	Morgan et al., 2003
	Neurokinin NK_1 receptor	—	Ripley et al., 2002
	Serotonin 5-HT_{1B} receptor	↑	Rocha et al., 1997
	Serotonin 5-HT_{2C} receptor	↑	Rocha et al., 2002
	Cannabinoid CB_1 receptor	—	Cossu et al., 2001
	Tissue plasminogen activator	—	Ripley et al., 1999
	Dopamine transporter	—	Rocha et al., 1998
	Dopamine D_2 receptor	↑	Caine et al., 2002
Amphetamine	CART *Carttm1Amgen*	↓	Couceyro et al., 2005
	Cannabinoid CB_1 receptor	—	Cossu et al., 2001
Morphine	Protein kinase Cε	↑	Newton et al., 2007
	Dopamine D_2 receptor	↓	Elmer et al., 2002
	Neurokinin NK_1 receptor	↓	Ripley et al., 2002
	Cannabinoid CB_1 receptor	↓	Cossu et al., 2001
Methylenedioxymethamphetamine	Serotonin transporter	↓	Trigo et al., 2007
WIN 55,212-2 (cannabinoid CB_1 agonist)	Prodynorphin	↑	Mendizabal et al., 2006
Nicotine	Cannabinoid CB_1 receptor	—	Cossu et al., 2001
	β2 nicotinic acetylcholine receptor	↓	Epping-Jordan et al., 1999
Ethanol	β-endorphin	↑	Grahame et al., 1998

↑ = increased responding; ↓ = decreased responding; — = no change.

To perform a catheterization, you will need:

- Inhalant or injectable anesthesia
- A hair trimmer
- Betadine scrub (Purdue Pharma), Betadine, 70% ethanol and gauze pads
- A scalpel blade
- Large surgical scissors, rounded at the tip
- A hemostat, curved at the tip
- Two pairs of fine forceps (microsurgical tweezers), curved at the tip, with blunted points
- Cotton swabs
- A suture needle, holder, and suture
- Sterile physiological saline, heparinized (30 USP units/ml)
- One 18 gauge and one 22 gauge needle, both filed down to remove half the needle shaft along its length but leaving the sharp point intact (the needle tip should be made to resemble a canoe)
- A catheter
- Cyanoacrylate (referred to commonly as Super Glue)
- A flushing syringe
- A catheter cap
- Topical antibiotic

For jugular catheterization, you will need to follow these steps: (1) Anesthetize the animal, (2) shave the animal, (3) prepare it, (4) incise, (5) position the catheter subcutaneously, (6) expose the vein, (7) catheterize, (8) flush, and (9) suture.

Recatheterization

It is difficult to predict how long an implanted catheter will remain patent. The best remedy for catheter failure is to recatheterize the animal on the contralateral jugular vein. This procedure is identical to the Jugular Catheterization procedure described in Caine et al. (1993) but is preceded by removal of the first catheter. In general, it is best to reopen the dorsal

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incision and to cut and remove the base of the first catheter, leaving the majority of the tubing in the animal in order to prevent any further tissue damage from removal procedures. Then proceed with jugular catheterization, this time using the contralateral jugular vein. Because there is little recourse after failure of the second catheter, recatheterization is recommended only when necessary (e.g., for valuable animals or animals that are already trained and reliably self-administer). Recatheterization of the same vein can be accomplished on occasion but is not reliable enough to warrant elaboration here.

Catheter evaluation, maintenance, and failure

The methohexital test

The methohexital test is a brief, simple, and repeatable procedure for determining catheter patency (Caine et al., 1993). Methohexital sodium is an ultra-short-acting barbiturate anesthetic that, when flushed through the catheter, produces overt signs of acute anesthesia within seconds. It is recommended that a methohexital test be performed (1) on animals that demonstrate unusually poor acquisition or unusual patterns of self-administration and (2) at the end of an experiment to confirm the integrity of the self-administration before interpreting the results. To perform a methohexital test, you will need the following:

- 1% methohexital sodium (e.g., Brevital Sodium; King Pharmaceuticals, Bristol, TN)
- Two flushing syringes
- Sterile physiological saline, heparinized (30 USP units/ml)

Catheter maintenance

After catheterization, it is best to flush the catheters at least every 12 hours for 2 days, and then once daily. It is crucial to flush the catheters immediately before and after self-administration. Sterile physiological saline is adequate, but the routine use of saline containing heparin (30 USP units/ml) may prolong catheter life.

Catheter failure

The most common cause of catheter failure is blockage by a fibrin thrombus within the tubing that leads to one of two symptoms: (1) a total block of flow or (2) apparently normal or enhanced flow but erratic self-administration behavior. If the catheter is totally blocked, it may be cleared by flushing with heparin (1000 USP units/ml), especially if the block is detected early. Applying too much pressure to a blocked catheter may cause it to leak, but it is worth the attempt because there is no alternative. Occasionally, flushing with heparin may not work the first time but may work in a subsequent attempt, perhaps because enough heparin reached the block in the first attempt to begin remedying the block. Attempts to clear the catheter by inserting a cleaning wire into the cannula guide are not advised. Most blocks occur in the PE10 portion of the tubing within the vein, and so puncturing the block will also risk damaging the vein, the catheter, or worse.

If there is reason to suspect that a catheter is faulty but flow is apparently unimpaired, it is likely that an undetected block (e.g., during a self-administration session) resulted in a leak somewhere along the length of the catheter. This may be the most common symptom of catheter failure for the type of catheter described in the Catheter Assembly procedure in Caine et al. (1993). Use the methohexital test described earlier in this section to evaluate catheter patency. Two less common causes of catheter failure that does not exhibit impaired flow are as follows: the PE10 tubing has slipped out of the vein or the vein has collapsed. The only recourse for a leaky catheter or collapsed vein is recatheterization of the contralateral jugular vein.

Self-administration training

Apart from studying the acquisition of self-administration as a behavioral measure (Deneau et al., 1969; Deminiere et al., 1989), a variety of approaches are used to establish stable and reliable self-administration behavior. The three most common training approaches are as follows: (1) to train food-restricted animals on a food-reinforced operant and then replace food with drug as the reinforcer; (2) to train food-restricted animals on a food-reinforced operant first, then allow the animals access to food *ad libitum*, and next reintroduce the same operant with drug as the reinforcer; and (3) to train the animals on a drug-reinforced operant with no prior operant training.

Although (1) and (2) seem similar, in procedure 1, the animals continue to be food-restricted even after they "acquire" self-administration. The common argument that this condition does *not* influence self-administration is that the animals are not "food-deprived" but rather "food-restricted" because they are given the recommended daily requirement of food (usually 20 g/rat). However, procedure 2 suggests much more convincingly that the operant behavior is truly maintained by drug reinforcement alone. The hypothesis goes that the previous training with food is an efficient method only to condition animals to use the operant, but the previous motivational state is now absent because the animals are allowed access to food *ad libitum*.

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Clearly, the preferred approach is 3, and surprisingly, animals quite readily acquire self-administration for highly reinforcing drugs such as cocaine without any prior operant experience. A few tricks can be used to accelerate or aid in the process of training, especially for approach 3, in the event that this becomes necessary. Most importantly, the likelihood that operant-naive animals will acquire self-administration is determined mainly by the number of times they accidentally engage the operant and receive a reinforcement. This frequency is often determined by the following simple variables, which can be maximized: level of exploratory activity, accessibility of the operant, unit dose (the dose delivered per injection), injection speed, and schedule of reinforcement.

An extended discussion of schedules can be found below, but for training purposes, the most efficient schedule to begin with is one of continuous reinforcement (fixed-ratio 1) with a brief time-out following each injection. Once stable responses are maintained on a fixed-ratio 1, the fixed-ratio value can be increased fairly quickly (Risner and Goldberg, 1983; Swerdlow and Koob, 1987; Robbins et al., 1989) in subsequent self-administration sessions or even within a single session. For animals that do not readily acquire self-administration, two common approaches are taken. The first regards animals that are not already food-restricted (training approaches [2] and [3] above). Depriving these animals of food for 24–48 hours often increases their level of exploratory activity. This approach is aimed at animals that do not initially explore the box enough to activate the operant even occasionally. The second alternative is appropriate for all animals that do not readily acquire self-administration and is loosely termed "priming." Priming involves automatically activating (by remote control if possible) the operant for several injections, ensuring that any discriminative or conditioning stimuli associated with activation of the operant (e.g., a light) are also activated. Then the animal is allowed to acquire self-administration for at least 30 minutes, and if no injections are earned, the priming procedure is repeated. The priming procedure is repeated every 30 minutes during alternate training sessions until animals respond on their own. If many animals occasionally activate the operant but do not acquire self-administration, it may be necessary to change the unit dose.

Analysis and interpretation of self-administration behavior

For most schedules of self-administration, unit doses that fall within a certain range maintain stable responding. However, unit doses below a certain threshold fail to maintain responding behavior, presumably because they fail to be adequately reinforcing; in contrast, unit doses above a certain threshold produce erratic patterns of self-administration or cessation of responding.

Some typical patterns of cocaine self-administration in a rat maintained on a simple fixed-ratio schedule are shown in Figure 3.

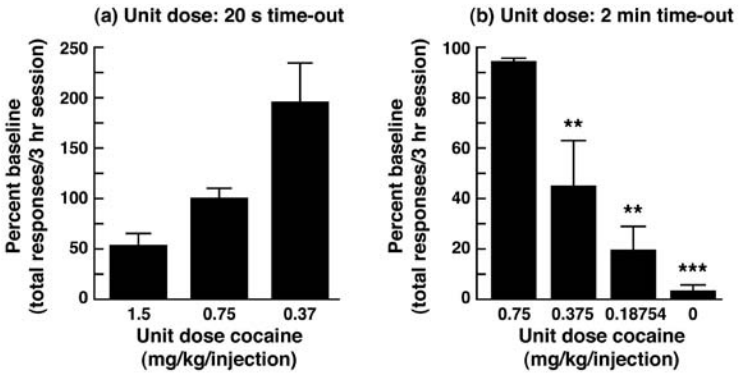

Figure 3. Effects of decreasing the unit dose on the total number of cocaine injections self-administered by rats maintained (a) on a fixed-ratio schedule with a 20 second time-out period ($n = 6$). (Taken with permission from Koob et al., 1987) and (b) on a fixed-ratio schedule with a 2 minute time-out ($n = 8$). Asterisks indicate significance by individual comparison (**$p < 0.01$, ***$p < 0.001$, Dunnett's t-test) with the training dose condition (0.75 mg/kg/injection) following significant main effect by ANOVA.

Within the range of doses that maintain stable responding, as the unit dose is decreased, animals increase their self-administration rate, apparently compensating for decreases in the unit dose. Conversely, as the unit dose is increased, animals reduce their self-administration rate. Thus, manipulations that increase the self-administration rate on this fixed-ratio schedule resemble decreases in the unit dose and may be interpreted as decreases in the reinforcing potency of cocaine.

A variety of evidence supports the hypothesis that the reinforcing properties of cocaine are the result of its properties as an indirect dopamine agonist. As would be predicted by the unit-dose response model, low to moderate doses of dopamine receptor antagonists increase cocaine self-administration maintained on this schedule in a manner similar to decreasing the unit dose of cocaine. This result suggests that partial

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blockade of dopamine receptors by competitive antagonists reduces the reinforcing potency of cocaine. Conversely, some dopamine agonists decrease cocaine self-administration in a manner similar to increasing the unit dose of cocaine, suggesting that the effects of dopamine agonists, together with cocaine self-administration, can be additive, perhaps because they mutually activate the same neural substrates.

As mentioned earlier, below a certain unit dose, drugs will not maintain stable self-administration. Replacing the drug with saline (zero unit dose) often produces a decrease in self-administration similar to the "extinction" effect observed in other reinforcement paradigms following removal of the reinforcer. According to the unit dose–response model, manipulations that completely abolish drug reinforcement should produce an extinction effect resembling that observed with the zero unit dose.

In both rats and monkeys, under a given schedule of reinforcement, dopamine receptor antagonists have decreased response rates for low unit doses of cocaine but increased response rates for higher unit doses of cocaine. The unit dose, in conjunction with the minimum inter-injection interval, determines the animals' overall immediate access to cocaine. Higher unit doses facilitate compensatory increases, allowing animals to overcome the effects of dopamine receptor antagonists by increasing their self-administration rate. In contrast, self-administration of lower doses is more easily disrupted, leading to decreased self-administration behavior. These types of changes are predictable based on the inverted U-shaped relationship between unit dose and rate of responding (Koob et al., 1987). In summary, a mutual antagonism between cocaine and dopamine receptor antagonists appears to exist such that animals increase their self-administration when given sufficient immediate access to cocaine, but decrease their self-administration when the effects of dopamine antagonists are insurmountable owing to dose and time constraints on self-administration.

The use of different schedules of reinforcement for studying self-administration increases the number of controls that can be incorporated. These controls improve the understanding of the behavior being measured, as well as the effects of manipulations on this behavior. Many drugs that maintain self-administration behavior, such as opiates and psychostimulants, also increase exploratory activity and locomotion. One approach for distinguishing between nonspecific increases in activity within the self-administration chamber and increased responding to a drug is to include a second, inactive manipulandum in the chamber that enables researchers to measure selective increases in responding on the active manipulandum. Another approach is to increase the fixed-ratio value required for obtaining reinforcement.

One approach for examining the selective effects of manipulations on drug reinforcement is to incorporate self-administration into a multiple component schedule. Behavior maintained alternately by food or cocaine in the same test session and with identical reinforcement requirements has been reported for various species (Balster and Schuster, 1973; Woolverton and Virus, 1989; Kleven and Woolverton, 1990; Winsauer and Thompson, 1991). Such schedules may be used to evaluate the selectivity of manipulations that apparently reduce the reinforcing efficacy of cocaine.

Progressive-ratio schedules are designed to directly evaluate the reinforcing efficacy of the self-administered drug by increasing the response requirements for each successive reinforcement and determining the "break point" at which the animal will no longer respond. A variety of evidence supports the hypothesis that this schedule is effective for determining relative reinforcement strength. The unit dose–response model demonstrates the principle that increasing the unit dose of self-administered drugs on a progressive ratio schedule increases the break point (Griffiths et al., 1978; Bedford et al., 1978; Roberts et al., 1989). In addition, dopamine receptor antagonists have been shown to decrease the break point for cocaine self-administration (Roberts et al., 1989; Hubner and Moreton, 1991).

Experimental design

Most self-administration studies use a within-subjects design. This design reduces cost and technical work, but more importantly, provides the most appropriate control group for testing the effects of manipulations, owing to the marked individual differences observed in self-administration behavior.

The length of the self-administration sessions should be determined somewhat by the choice of schedule and the objectives of the study. For schedules that directly assess self-administration (e.g., fixed-ratio schedules), longer sessions (3–4 hours) may be a more accurate animal model of human drug self-administration than shorter sessions (e.g., 1 hour). Longer sessions also permit researchers to evaluate the time-course of effects of manipulations on self-administration behavior. Extended sessions are also necessary for unlimited access studies (e.g., withdrawal or "binge" studies).

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A common procedure is to begin each self-administration session with the automated delivery of two injections, after which the operant becomes active (e.g., a lever extends into the chamber). Some drug is required to replace the saline in the catheter (approximately 0.05 ml for the catheter described above) so the first responses of the animal will be immediately reinforced. Beyond this dose, a "starter" injection of drug serves as a powerful discriminative stimulus for the beginning of drug availability and increases the probability that the animal will initiate self-administration (de Wit and Stewart, 1981). However, depending on the experimental objectives, starter injections may not always be appropriate.

Group sizes

For rodent studies, a group size of 4 can be considered small, and group sizes of 6 to 8 deemed optimal. However, it is best that the group size be determined by the number of animals required to obtain reliable results, confirmed by rigorous statistical analyses. These studies normally employ drug-naive subjects and report data for various self-administration drugs or manipulations in different sets of subjects.

Establishing baseline data

The establishment of baseline self-administration behavior varies among studies, but the most common procedure requires a minimum number of consecutive self-administration sessions with less than 10% variation in the dependent variable (e.g., total injections earned or average response rate). Test data may be calculated as the percent of an individual's mean baseline value for within-subjects designs.

Manipulations of baseline behavior

Once baseline behavior is established, manipulations such as drug pretreatment comprising numerous doses should be administered using a Latin square design to control for the effects of order. Pretreatments are normally separated by at least two baseline self-administration sessions that similarly vary by less than 10%, which also prevents the systemic accumulation of the pretreatment drug. Studies that examine only a single-dose drug pretreatment as a manipulation are not advised for self-administration studies. Single manipulations such as lesions are compared with prelesion baseline data, but a sham group should be treated in an identical manner to control for the effects of such variables as the recovery period from the surgery itself, independent of the lesion effects.

Analyses

Detailed information regarding the appropriate choice of statistical analysis can be found elsewhere (Keppel, 1982). As a typical example, a study of multiple doses of a pretreatment drug may include a vehicle condition as a dose and an overall ANOVA in which repeated measures on dose are performed. Following a significant main effect, further individual comparisons of doses with the vehicle condition may be explored using the appropriate *post hoc* test.

Mouse intravenous self-administration procedures

Anesthetize mice with halothane or isoflurane mixed with oxygen vapor, and prepare them with chronic indwelling intravenous catheters as previously described (Caine et al., 1993; Caine et al., 1999; Thomsen and Caine, 2005, 2007). Fit a 6 cm length of silastic tubing (0.25 mm i.d., 0.76 mm o.d.) to a 26 gauge stainless steel cannula bent at a right angle, and then embed it in a cement disk with an underlying nylon mesh. Insert the catheter tubing 1.2 cm into the right external jugular vein and anchor with a suture (Barr et al., 1979). In some cases, if implantation in the right jugular vein is unsuccessful owing to vein constriction during the procedure, implant the catheter in the left (or contralateral) jugular vein. Run the remaining tubing subcutaneously to the cannula in order to exit at the midscapular region. Suture all incisions and coat with triple antibiotic ointment. Administer ticarcillin disodium or cefazolin (20 µl of 67 mg/ml saline) through the catheter immediately after surgery to forestall infection. For the next 4 to 7 days, allow the mice to recover from surgery, and administer antibiotics as before, but with 30 units per ml heparin in the solution. Thereafter, flush catheters with saline containing heparin only (30 units per ml).

Periodically evaluate the patency of intravenous catheters (usually once per week after behavioral testing, and/or at the completion of each experimental phase, and whenever drug self-administration behavior appears to deviate dramatically from that observed previously). Always evaluate catheters at least 2 hours or more before or after a drug self-administration test session. Infuse approximately 20 µl of 1% methohexital sodium (Brevital) or a cocktail containing 15% Ketaset (ketamine, 100 mg/ml), 15% Versed (midazolam, 5 mg/ml), and 70% saline through the catheter. If prominent signs of anesthesia are not apparent within 3 seconds of infusion, and the left jugular vein has not been previously implanted (see above), then surgically remove the catheter from the right jugular vein and implant a new catheter in the left jugular vein using the procedures described above.

Experimental sessions are conducted in mouse conditioning chambers (ENV-307W; Med

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Associates, St Albans, VT) located within ventilated sound attenuation chambers. Each chamber is equipped with two levers, above which are cue lights. Responses on the active lever result in the simultaneous activation of the infusion pump and the stimulus light above the active lever. Responses on the inactive lever and during the time-out period are recorded but have no programmed consequences. Activating the infusion pump results in the delivery of various doses of cocaine hydrochloride in a 15 µl volume. Intravenous drug infusions are delivered by a software-operated infusion pump (Med Associates) placed outside the sound attenuating box, through a liquid swivel and a syringe. Tygon microbore tubing connects the components of the infusion apparatus to each other and to the exit port of the catheter.

The following is an example of an experimental design that allows for fairly high-throughput mouse self-administration testing. For the first 6 days of intravenous self-administration testing, 1-hour sessions using a fixed-ratio 1 schedule of reinforcement with a 33 second time-out period are employed; a different dose of cocaine is available on each day. The doses we have used are 0.15, 0.3, 0.6, 1.2, 2.4, and 4.8 mg/kg delivered in this ascending order to minimize the potential for mice developing aversion early in the experience of cocaine. The intake of cocaine at each dose, as well as the total cocaine intake across all doses, is calculated relative to body weight. For consistency of analysis across experiments, subsequent testing takes place using the 0.6 mg/kg/infusion dose. Both fixed-ratio 1 and progressive-ratio responding is assessed. The 2 progressive-ratio tests abide by the following equation: [5e(injection number × 0.20)] – 5 (rounded to the nearest integer). The sequence of lever press requirements would begin as follows: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, etc. The break point is defined as that ratio at which the mouse fails to receive a reinforcer for 60 minutes. The maximum test session is 6 hours. After these tests, mice are returned to fixed-ratio 1 tests to re-establish a continuous reinforcement schedule and to record a more stable response rate at this dose. Finally, mice receive ten 1 hour sessions in which the cocaine pump is turned off in order to examine the process of response extinction.

Issues Unique to Mice

Several issues are unique to mice that warrant mention. For example, concerns may arise that small mice could have difficulty depressing the levers (which typically require about 5 g of downward force) and that this may lead to problematic results. Hundreds of mice have been tested over the years for both cocaine and ethanol self-administration using this system, and researchers have found that mice as small as 12 g can lever-press successfully. In fact, in cases where mice under 20 g have been used, we have observed rates of more than 100 lever presses within a 30 minute period. In our experience, mice tend to place both front paws, and sometimes their forearms, on the levers, thereby using their entire upper body to depress the levers. We recommend against decreasing the force requirement because doing so could likely lead to spurious presses as the mice ambulate around the operant chambers.

Another key factor when studying drug self-administration in mice is their genetic backgrounds. Not only will there be genetic involvement in drug self-administration, but other behaviors that may be unrelated or peripherally related might influence experimental results. For example, genetic differences in activity level, anxiety state, sensory function, learning, and memory can each affect self-administration behavior. These issues should be considered when designing mouse studies (Thomsen and Caine, 2006).

Concluding remarks

Variation on the techniques described herein is encouraged. Examining the primary sources from the relatively small list of studies for mice shown in Table 1 will provide technical and theoretical information on self-administration that goes far beyond the scope of the present discussion.

Microdialysis in Rodents

Microdialysis as a technique

Microdialysis of brain tissue is a method for measuring unbound *in vivo* tissue concentrations of endogenous and exogenous compounds in the brain (Parsons and Justice, 1994; Plock and Kloft, 2005). A microdialysis system consists of a microdialysis probe, a microdialysis pump, and a microvial in which the sample is collected. During the procedure, the probe is implanted into tissue; then, perfusion fluid enters the probe through the inlet tubing at a constant flow rate, passes through the membrane, and is transported through the outlet tubing and collected in a microvial (Fig. 4).

Microdialysis is based on the principle that substances will diffuse from a higher to a lower concentration; thus, if the composition in the probe were identical to the extracellular fluid of the tissue, no net exchange would take place. Therefore, the optimal perfusate is an aqueous solution that mimics the composition of the surrounding medium. Ringer's solution has commonly been used as a perfusate but more closely

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resembles plasma than extracellular brain fluid. Current perfusates for rodents are likely to be artificial CSF consisting of 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.25 mM ascorbic acid, and 5.4 mM D-glucose (pH 7.2–7.4).

Probe design

Probes used for microdialysis in rodent studies have evolved from the original linear hollow-fiber design with monodirectional flow (Ungerstedt and Pycock, 1974) to concentric probes with a thin dialysis tube having an inner diameter in the range of approximately 0.15–0.3 mm and a semipermeable membrane at the tip with bidirectional flow (Fig. 4). Perfusion fluid enters the probe through the inlet tubing at a constant flow rate (usually 0.5–5 µl/min) and is collected via the outlet tube.

Relative recovery

Relative recovery describes the ratio between the concentration of a substance in the dialysate to that of the fluid around the probe. Relative recovery depends on several factors: velocity of the diffusion across the membrane, composition of the perfusate, the nature of the sample tissue, and flow rate.

Calibration procedures

Because relative recovery never reaches 100% (except in the procedure known as no-net flux), a microdialysis probe must be calibrated to make accurate predictions

Figure 4. Sampling of interstitial neurochemicals by *in vivo* microdialysis.

about concentrations in the fluid around the probe. One method involves varying the perfusion rate and is called the "method of flow rate variation" (Jacobson et al., 1985). Other procedures for calibration include no-net flux, retrodialysis, and endogenous reference substance (Plock and Kloft, 2005).

It should be noted, however, that these types of procedures are not required for experiments in which the desired goal is assessment of *relative* changes in a dialysate analyte (e.g., evaluations of drug-induced changes in neurotransmitter content). In these instances, the effects of a manipulation (e.g., drug challenge) are evaluated as a change from pre-manipulation baseline, and knowledge of the *in vivo* probe recovery is less important. Although *in vitro* estimates of probe recovery can be useful for verifying similar probe performance among experimental groups, *in vitro* estimates of probe recovery should not be used to transform or "normalize" data gathered *in vivo* (Parsons and Justice, 1994).

General advantages and disadvantages of microdialysis

Advantages

- Allows repeated sampling from conscious animals (e.g., provides a temporal profile), often during an ongoing behavioral task or pharmacological challenge.
- Samples are "pre-cleaned" by virtue of the semipermeable membrane. Very large proteins and similar molecules do not diffuse across this membrane; as such, the samples are typically well-suited for analysis without additional cleanup.
- Fluid samples are suitable for a variety of analytical techniques that will provide unambiguous identification and quantification of the neurochemical of interest.
- Microdialysis probes can be used to locally administer a drug while monitoring the neurochemical consequences of its administration.

Disadvantages

- Provides relatively poor temporal resolution owing to the need for several microliters of dialysate for most analytical techniques. This results in sampling intervals typically ranging from 5 to 20 minutes (though 30 second intervals have been achieved for certain analytes). Thus, even under the most rapid circumstances, a microdialysis sample reflects a summation of many neuronal events.
- Offers poor sampling efficiency for some analytes, such as larger-molecular-weight substances (e.g., peptides) and nonpolar analytes such as fatty acids and endogenous cannabinoids. For these analytes, the careful selection of the probe membrane material can be critical. Different membrane materials

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can provide substantially different sampling efficiencies depending on the analyte type. Moreover, in some circumstances, the inclusion of certain additives to the perfusion media can improve sampling efficiency. Examples include the addition of bovine serum albumin to improve the recovery/retention of large peptides and the addition of cyclodextrin moieties to assist in the solubilization/recovery of nonpolar analytes. The impact these perfusate additives will have on the subsequent analytical method employed is a critically important consideration.

Possible limitations of microdialysis

Numerous limitations to microdialysis procedures must be considered when interpreting the results (Table 2).

Table 2. Advantages and limitations of microdialysis sampling

Advantages	Limitations
Collection of a substance at a site of action	Size limitation for molecules to be determined
Direct delivery to the target tissue	Determination of mean concentrations over a time interval
No biological fluid loss (continuous sampling even in small animals)	Calibration necessary because recovery does not reach 100%
Protein-free dialysate (complex and time-consuming sample preparation becomes unnecessary)	Requirement of an analytical method with a low limit of quantification capable of dealing with small volumes
Measurement of unbound drug concentrations	Risk of tissue trauma, gliosis influence on blood–brain barrier function
Cessation of enzymatic degradation	
Online measurement of unbound tissue concentrations	
Simultaneous sampling at multiple sites	
Applicability to almost every organ	
Applicability in conscious animals	

Reprinted with permission from Plock and Kloft, 2005.

Tissue trauma can result from probe insertion. Also, gliosis around the microdialysis probe can block the membrane. Using a guide cannula and hours of presampling insertion times can mitigate these concerns. The blood–brain barrier can be compromised. Finally, bacterial infection can result, but aseptic handling of the probe can lessen this possibility.

Microdialysis procedures—mouse

The advent of transgenic mouse lines and the growing array of genetic manipulations possible in mice have prompted the adaptation of rat microdialysis techniques for use in mice. Surprisingly, beyond the obvious limitations of reduced brain size, few impediments appear when performing microdialysis in mice as compared with rats. Minor modifications to bear in mind include the need for liquid swivels with reduced resistance to motion. Owing to the animals' size and weight, mice are unable to easily rotate standard swivels, and the use of rat-suitable swivels with mice can result in tangling the dialysis lines and immobilization (leading to increased stress). Fortunately, liquid swivels designed specifically for mice are available from most commercial vendors such as Instech Laboratories (Plymouth Meeting, PA).

Another consideration is that the mouse skull is less robust than a rat's, and as such, one cannot rely as heavily on skull screws to secure the guide cannula prosthetic to the mouse skull. A more reliable approach is to use an adhesive or cement that "grips" skull bone more heartily than does methylmethacrylate, and to apply this adhesive to as large an area of skull as possible. A good adhesive for this purpose is Geristore resin ionomer (Den-Mat, Santa Maria, CA), which is quite fluid, easily spreads across skull bone, and is rapidly cured to its solid phase upon exposure to ultraviolet light. The adhesion of this ionomer to bone is stronger than is the mouse skull, and cranial prosthetics made with an initial layer of Geristore will typically pull bone off with them when removed for postmortem cannula retrieval. Although the initial layer or two of Geristore is of substantial value when securing guide cannulae/probes to mouse skull, it is unnecessary to construct the entire prosthetic assembly using Geristore. Traditional (and less costly) methylmethacrylate cement can be used on top of the Geristore to build up the skull prosthetic bulk as needed.

The much smaller size of mouse versus rat brain also puts obvious restrictions on the number of specific brain regions suitable for microdialysis sampling. Thus, it is unlikely that one can practicably use microdialysis to monitor neurochemical events specifically in the mouse inferior olive. Although all microdialysis experiments should be designed in a manner that produces the least possible disruption of the interstitial environment, regardless of the species being examined, these considerations are of particular importance in mice. For example, as previously discussed, the volume of tissue surrounding the active probe membrane that is altered by microdialysis sampling is directly proportional to the perfusate flow rate. Faster flow rates result in greater disruption of the neurochemical environment for greater distances from the dialysis

probe than do slower flow rates. As such, it stands to reason that the slowest possible perfusate flow rates should be employed in mouse dialysis experiments to reduce the risk of sampling neurochemicals from nearby but distinct brain regions. Selecting the optimal perfusate flow rate will require one to balance the goals of producing the least tissue disturbance while acquiring the sample volumes needed for neurochemical analysis.

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Depression-Related Tests

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Introduction

Depression is a prevalent psychiatric illness, afflicting approximately 16% of all Americans (Kessler et al., 2003) and placing a great economic burden on individuals, families, and the health care system overall (Simon, 2003). The precise etiology of depression is unknown, though it likely involves a combination of genetic and environmental causes. Pharmacological treatments are available that can treat depression. However, many of these treatments are effective for only a portion of depressed patients, take weeks to become active, and cause dose-limiting side effects. There is an urgent medical need to develop new drug treatments or strategies that are more effective, work more rapidly, and confer fewer side effects than currently available medications. The development of more effective therapies targeting disease etiology will depend on the emergence of a greater understanding of the pathophysiology of depression.

Modeling Depressive Behavior in Mice

Studies with mice can be helpful for identifying physiological mechanisms underlying depressive behavior or for identifying new treatments for depression. Behavioral tests in rodents for tasks that comprise a spectrum of depression-sensitive behaviors have emerged from two intersecting lines of investigation. First, animal tests can be used to study the physiological basis for components of depression because they measure behaviors that are similar to the formal symptoms of depression. Several recent reviews have included tables of depression symptoms in humans and how they might be modeled in rodents (Cryan and Mombereau, 2004; Cryan and Holmes, 2005). For example, the symptom of diminished interest or pleasure (anhedonia) can be modeled in rodents by measuring the response to rewards. Behavioral tests in rodents can measure equivalent changes in appetite and body weight, sleep disturbance, reductions in cognitive abilities, and increased fatigue or loss of energy in humans. Even behaviors that are not formal symptoms but are changed in depressed patients can be the basis for tests of depressive behavior in rodents. For example, excessive passive coping or failure to maintain escape-directed behavior in response to inescapable stress is the basis for a number of tests of depressive behavior, such as learned helplessness, the forced swim test (FST), or the tail suspension test (TST) (Cryan et al., 2005a, 2005b). Note, however, that rodents cannot be used to measure symptoms of depression such as internalized feelings that remain classically human, e.g., depressed mood, feelings of worthlessness, or excessive guilt, and it is incorrect to infer internal mood states in rodents.

Second, behavioral tests in rodents have emerged from drug discovery because of their ability to predict which compounds have potential activity as antidepressant drugs. One of the criteria for validating any traditional animal model of depression is to demonstrate that the depressive behavior is altered by drugs that are effective in treating depression in humans. Evaluating predictive validity for any depressive behavior test or model is not simple because of the varied types of treatments and drugs that are available to treat clinical depression. In order to be validated, depressive behaviors must demonstrate sensitivity to antidepressant treatments from a number of different drug classes. These may include tricyclic antidepressants, monoamine oxidase (MAO) inhibitors, selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors, and atypical antidepressants (drugs that produce their antidepressant effects through an undetermined mechanism and are not monoamine transporter inhibitors). Somatic treatments, such as electroconvulsive shock, exercise, or transcranial magnetic stimulation, have also been used to validate drugs' effectiveness on depressive behavior. The selectivity of behavioral response to antidepressant drugs is important and can be demonstrated by showing that drugs with side effects of antidepressants, or classes of drugs that do not improve the symptoms of depression (e.g., antipsychotics or anxiolytics), do not alter the targeted test results for depressive behavior.

More recently, concern has been raised as to whether depressive behaviors respond to a single or a few drug treatments, or whether the behavior demonstrates changes only following the chronic administration of antidepressants (Table 1). Rapid onset of action is advantageous when behaviors are being used as a screen for assessing antidepressant potential. However, these responses do not simulate the time course of clinical treatment for depression. Slowly developing responses that emerge with repeated administration of many antidepressants include changes in receptor regulation, signaling mechanisms, neurotrophin secretion, gene expression, and neurogenesis. These responses may represent changes in functional neuroplasticity of key processes affected by stress. Behavioral responses, too, have been described that emerge following chronic, rather than acute, antidepressant drug treatments. These behavioral responses may be the result of changes in plasticity that emerge following chronic antidepressant drug treatment.

Physiological challenges must often be modeled in order to produce a predisposition for depressive behavior based on some of the best-known factors that correlate with clinical depression. For example, maternal deprivation or stress during early

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Table 1. Tests used most commonly to measure depression-Related behavior

	Advantages	Key questions or problems
Acute tests		
• FST • TST • Learned helplessness	• Works acutely or rapidly • Ideal for drug discovery or genetic phenotype • Sensitive to antidepressant treatments • Can be used in conjunction with risk factors for depression to create models • Genetic sensitivity	• Can they be used to discover new compounds? • What are the effects in diseased subjects? • Are critical effects produced or missed by chronic treatment?
Chronic tests		
• Chronic mild stress • Social defeat • Novelty-induced hyponeophagia • Olfactory bulbectomy	• Simulates how antidepressants are given clinically for treatment • Can measure time course of onset of drug action • Can be based on an etiological mechanism with theoretical implications (chronic stress or illness)	• Difficult implementation • Work is slow • Limited database of reference compounds • Limited pharmacology • Based on an individual risk factor

development was used to develop one of the first models of depression (McKinney and Bunney, 1969). Because stress is one of the precipitants most closely associated with depression, the application of stressors to adult rodents has become a common method for modeling the development of depressive behavior and is the basis of the chronic mild stress model of depression. Depression is frequently comorbid with other medical illnesses. Using models of physical illness, such as cardiovascular disease, diabetes, stroke, and gonadal dysfunction, many studies have demonstrated the increase of depressive behaviors in rodents. Lines of rats and mice have been identified or deliberately bred that demonstrate exaggerated predisposition to depressive behaviors, and these lines have been used as animal models for depressive behavior in drug discovery studies (Crowley and Lucki, 2005). Mice can now be generated with genetically modified key proteins for transmitters, hormones, receptors, enzymes, or signaling molecules. Some of these genetic mutants have demonstrated alterations in depressive behaviors (Cryan and Mombereau, 2004). Human genetic studies have not yet identified consensus targets for the etiology of depression. Once these targets are identified, genetic engineering will provide the methodology for producing analogous mutations in mice for investigating correlated behavioral changes.

In the following sections, we will review the different behavioral procedures most commonly used with mice for measuring depressive behavior. Applications for these studies include the measurement of differences among inbred strains, the evaluation of genetically modified mice for changes in stress-related behaviors, and the demonstration using mice of antidepressant efficacy for established and novel compounds in different target behaviors. A number of recent reviews are available on these general topics (Cryan et al., 2002; Cryan and Mombereau, 2004; Cryan and Holmes, 2005; Cryan and Slattery, 2007; Jacobson and Cryan, 2007).

Behaviors Responsive to Acute Antidepressant Treatments

Forced swim test

The FST is the most frequently used behavioral test for measuring depressive behavior in rodents. The FST was developed by Roger Porsolt (Porsolt et al., 1977) as a convenient way to measure the effects of antidepressant drugs in mice. Modifications of the FST have been described for other rodent species (Cryan et al., 2002). The procedure places mice in individual cylinders containing water (Fig. 1). Although they attempt escape at the start of the session, the mice rapidly become immobile, demonstrated by floating passively or making only movements necessary to remain afloat. As developed originally, a 6-minute session was employed, and immobility time was scored only during the final 4 minutes, to eliminate the period of escape activity. Antidepressants administered just prior to the session reduced the amount of immobility during the test period.

Figure 1. A mouse demonstrating immobility in the FST. Mice show immobility when they passively float across the cylinder and stop making climbing movements aimed at the side of the cylinder, spontaneous swimming movements, or engaging in exploratory behaviors. Photorealistic image by Gina R. Colaizzo.

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Antidepressant treatments from a variety of pharmacological classes reduce immobility time; these include tricyclic compounds, MAO inhibitors, atypical antidepressants, and SSRIs (Borsini and Meli, 1988). Somatic treatments for depression, such as electroconvulsive shock and exercise, also reduce immobility in the FST.

Test performance depends on a variety of procedural parameters, including water temperature, water depth, cylinder diameter, injection schedule, and number of exposures. The mouse FST is conducted typically in a single session (Porsolt et al., 1977), whereas the rat version is conducted usually in two sessions—an induction session that facilitates the development of immobility and a second test session that evaluates the effects of drug treatments (Cryan et al., 2005b). However, the mouse FST has also been conducted using multiple sessions, to assess, e.g., the effects of a drug (Conti et al., 2002) or the development of immobility in genetically mutant mice (McLaughlin et al., 2003). Exposing rodents to multiple sessions facilitates the development of stress-related immobility and may be important for assessing the effects of the stress-induction procedure on certain biological variables. Since scoring immobility or other behaviors in the FST are subjective, the test sessions should be recorded and raters should be blind to drug pretreatment conditions. Automated devices are available for providing objective scoring but should be used only if validated using an antidepressant drug. However, procedural parameters continue to vary among laboratories, making precise replication of results more difficult.

Genetic background of the mouse strain can have dramatic effects on baseline immobility and response to different classes of antidepressant drugs in the FST (Lucki et al., 2001; Petit-Demouliere et al., 2005). Interestingly, baseline immobility values across inbred strains did not correlate with response to antidepressants. For example, the mouse strain with the greatest immobility time, C57BL/6, was not the most responsive to antidepressant treatments (Lucki et al., 2001). Clear strain differences also appeared in the response to different types of antidepressants in the FST (Lucki et al., 2001). Further, genetic modifications have produced lines of mice with greater immobility (pro-depressive) and less immobility (antidepressant-like) in the FST (Cryan and Mombereau, 2004).

Amphetamine and other psychomotor stimulants reduce immobility in the FST but are not used clinically as antidepressants. Therefore, locomotor activity is usually employed with the FST as a secondary screen for identifying compounds with potential behaviorally activating effects (Porsolt et al., 1977). If a novel drug or genetic mutant mouse demonstrates locomotor hyperactivity, then effects obtained in the FST cannot be attributed unambiguously to stress-related depression. Most antidepressant drugs actually reduce locomotion in activity tests in rodents.

Tail suspension test

The TST is a second behavioral test developed to measure depressive behavior (Steru et al., 1985). Mice are suspended by the tail from a horizontal bar or from a platform; their activity can be videotaped for later scoring or measured using an automatic monitoring device (Crowley et al., 2004). Mice usually display escape-like limb and body movements immediate after suspension, followed by the onset of immobility (Fig. 2). Antidepressant treatments reduce the duration of immobility during the common 6-minute test (for review, Cryan et al., 2005a). In other words, antidepressant treatments delay the onset of immobile behavior, and this effect can be measured when mice are followed up for a longer period.

Figure 2. A mouse demonstrating immobility in the TST. Mice usually display escape-like limb and body movements immediately after suspension, followed by the onset of immobility. Photorealistic image by Gina R. Colaizzo.

The TST shares a common theoretical basis with the FST in that it studies the development of immobility after exposure to inescapable stress. Nevertheless, differences between these tests can be obtained when comparing the response to drugs or the behavior of genetic mutants. For example, fluoxetine given to 129/Sv mice effectively reduced immobility in the TST (Crowley et al., 2004) but increased immobility in the FST (Lucki et al., 2001) because of the development of hindlimb ataxia in the water. Some lines of C57BL/6 mice may climb up their tails onto a horizontal bar during the TST (Mayorga and Lucki,

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2001), but C57BL/6 mice did not climb a vertical attachment bar if they were attached at the base of their tail (Crowley et al., 2005). As for the FST, results with the TST require confirmation that the drug or genetic mutation would not be expected to produce locomotor hyperactivity.

Using the TST, mice with genetic mutations can be assessed for changes in depression-related behavior. Genetic mutants have been described with increases or decreases of baseline immobility in the TST (Cryan and Mombereau, 2004). The reason that genetic mutants demonstrate an antidepressant-like phenotype could be a persistent alteration of neural systems that are altered by antidepressant drugs (Jones and Lucki, 2005).

Performance on the TST has also been used to identify gene candidates that might be associated with depressive behavior or the behavioral effects of antidepressants. CD-1 mice were selectively bred with high or low immobility values in the TST and were then bred for multiple generations as a genetic model of depressive behavior. These lines of mice have been thoroughly characterized as to behavioral, neurochemical, and electrophysiological differences, and researchers have described similarities to clinical depression (El Yacoubi et al., 2003). Mice from inbred strains with different baseline performance on the TST and FST were also intercrossed for a quantitative trait locus study in order to map areas containing genes associated with differences in their performance on these tests (Yoshikawa et al., 2002). In a more recent study, mice from inbred strains with innate differences in the TST response to citalopram were intercrossed in order to map gene candidates associated with differences in the behavioral effects of citalopram (Crowley et al., 2006). Using mouse models, these studies developed evidence for candidate genes involved in depression that will be tested in gene association studies for their applicability to clinical depression.

Learned helplessness

Learned helplessness is a model of depression in which animals exposed to unpredictable and uncontrollable stress subsequently develop coping deficits for aversive but escapable situations (Maier and Seligman, 1976). Similarities have been drawn between deficits in affective, motoric, and cognitive behaviors produced by exposure to learned helplessness, and depressed patients (Seligman et al., 1980). Behavioral tests have been conducted most frequently in rodents using electroshocks and shuttle avoidance behavior. Only a few studies have been published using learned helplessness procedures in mice, and several protocols have been described (Shanks and Anisman, 1988; Caldarone et al., 2000; Chourbaji et al., 2005).

Behaviors Responsive to Chronic Antidepressant Treatments

Chronic mild stress

The chronic mild stress (CMS) model of depression involves the presentation, in an unpredictable sequence, of a series of environmental stressors to mice for a prolonged period of time—usually 1–2 months. Chronic exposure to stress produces many long-term behavioral, neurochemical, and neuroendocrine changes resembling the dysfunction observed in depressed patients (Willner, 1997, 2005). Key to this field of study have been behavioral assessments that measure aspects of anhedonia, such as sucrose consumption, or reward mechanisms, such as cranial self-stimulation behavior. These behavioral measures, produced by CMS, have been shown to reverse after chronic treatment with a number of different antidepressant drugs, including tricyclic antidepressants and SSRIs (Willner, 1997). The CMS model has many characteristics that make it ideal for evaluating depressive behavior. However, it also has had difficulty producing similar outcomes among laboratories.

Relatively fewer CMS studies have been conducted in mice than in rats (Cryan and Mombereau, 2004). However, because the range of mice available demonstrates substantial baseline differences in depressive and anxiety behavior, much work is required to determine the correspondence of behavior in CMS mice to these innate variations (Mineur et al., 2006). Mice have shown reductions in sucrose preference following CMS that were reversed by chronic treatment with imipramine (Monleon et al., 1995; Harkin et al., 2002). One modification of the CMS procedure for mice examined the physical state of the fur or coat, because mice subjected to stress do not groom normally (Griebel et al., 2002). For these reasons, any systematic program of research should include a demonstration that reference antidepressants actively reverse behavioral responses produced by exposure to CMS, before concluding that a stress procedure is associated with depressive behavior.

Novelty-induced hyponeophagia or novelty-suppressed feeding

Exposure to a novel environment produces an emotional reaction in the mouse that results in inhibition towards the approach and consumption of food. This behavioral response is termed

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hyponeophagia. Hyponeophagia may be a good paradigm for behavioral tests that correlate exclusively with chronic antidepressant drug treatments (Dulawa and Hen, 2005).

Hyponeophagia-based paradigms either present food to food-deprived animals (referred to as novelty suppression of feeding) or present a highly palatable and familiar food to satiated animals (referred to as novelty-induced hyponeophagia). The latency to feed and the amount eaten in a novel environment is then measured and compared with similar behavior measured in the home cage or a familiar environment. Interest in this paradigm has intensified recently because of demonstrations that hyponeophagia in mice was attenuated following the chronic, but not acute, administration of antidepressant drugs (Santarelli et al., 2003; Merali et al., 2003; Dulawa et al., 2004). Furthermore, the effects of chronic antidepressant treatments on hyponeophagia may be associated with increases in hippocampal neurogenesis, because this behavioral effect cannot be produced in mice that are unable to increase new cell proliferation in the hippocampus (Santarelli et al., 2003).

Attenuation of hyponeophagia, however, is not restricted to chronic antidepressant drug treatments (Dulawa and Hen, 2005). Previous studies have shown that benzodiazepine anxiolytics also attenuate hyponeophagia and do so following acute administration (Soubrie et al., 1975). Many antidepressants are used to treat anxiety disorders (Nutt et al., 2006). This suggests that hyponeophagia may be a good model for studying the anxiolytic effects associated with chronic antidepressant drug treatments. However, since benzodiazepines are sufficient to produce effects on hyponeophagia, and benzodiazepines are generally not effective as antidepressants, it is unclear whether effects in this model may be associated with the effects of antidepressant drug treatments on depressive behaviors.

Social defeat

Defeat arising from social competition and interaction has been described as a potential contributor to the development of depressive behavior (Sloman et al., 2003). Mice experiencing defeat from repeated aggression develop enduring behavioral deficits, including an aversion to social contact measured in an open field (Avgustinovich et al., 2005; Berton et al., 2006). The aversion to interaction is reduced by chronic, but not acute, administration of imipramine and fluoxetine (Berton et al., 2006). This mouse model has not yet received much pharmacological characterization or replication. However, its use of social interaction and dominance to produce persistent emotional disturbance in mice incorporate a number of clinical conceptions of depression and other psychiatric conditions in humans.

Olfactory bulbectomy

Olfactory bulbectomy (OB) is a model for producing behavioral, endocrine, and morphological changes that resemble features of patients with major depression (Cryan and Mombereau, 2004). Two key behavioral changes following OB are increased locomotor activity and deficits in avoidance response. These behavioral changes produced by OB are reversed in rats following chronic treatment with antidepressant drugs. Although most research with this model has been done with rats, these behavioral effects can also be produced by OB in mice. Both of these behavioral changes were reversed following chronic treatment with the antidepressant drugs amitriptyline, imipramine, and trazodone (Otmakhova et al., 1992; Jarosik et al., 2007). However, pharmacologically diverse antidepressant drugs have not been extensively validated in mice using the OB models. In addition, C57BL/6 mice, but not DBA/2 mice, displayed the deficits in avoidance behavior following OB, emphasizing the importance of background strain in this model (Gurevich et al., 1993).

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Social Behavior Tests for Mice

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Mice Are a Social Species

If you are interested in the biology of social behaviors, are generating mouse models of psychiatric disorders, or simply enjoy watching and understanding animal behavior, you are in luck. Many excellent assays of mouse social behaviors are well-established in the behavioral neuroscience and behavioral neuroendocrinology literature. The references listed below include enlightening reviews and step-by-step protocols. This overview will briefly describe some of the most commonly used methods for quantitating social interaction, affiliation, sexual behaviors, parental behaviors, juvenile play, social dominance, aggression, social recognition, social memory, and social communication in mice. These tasks apply to phenotyping mouse models of human social dysfunctions, such as those found in autism, schizophrenia, aggression, and social phobias.

Social Interactions in Mice

Two unfamiliar mice placed in a neutral arena will usually display high levels of sniffing, following, crawling over and under each other, nose-to-nose sniffing, and anogenital sniffing (Fig. 1A). Allogrooming, in which one mouse grooms the other, is frequently observed in a neutral arena and when two or more mice reside in the same home cage. Scoring of social interactions is commonly performed using videotapes of social interaction sessions in the home cage, empty novel cage, structured novel environment, or in an open-field chamber. A freestanding digital video camera or a computer-assisted video tracking system is used to record and store an electronic version of the session for subsequent analysis. The researcher scores the video sessions for frequency and duration of carefully defined behavioral events. Scoring can be conducted using either pencil and paper, a keyboard event recorder, or an automated video tracking software system. Automated and semiautomated systems that measure social approach and social recognition usually calculate the number of approaches, total time spent together, and specific components of interaction by each individual (Kwon et al., 2006; McFarlane et al., 2007) (Fig. 1B). Each behavioral parameter is analyzed independently using the appropriate statistical tests. For some purposes, a composite score of total social interactions is employed (Bolivar et al., 2007). On most parameters of social interaction, juvenile mice tested at 20–25 days of age display play behaviors that resemble adult social interactions (Terranova and Laviola, 2005; Panksepp and Lahvis, 2006; McFarlane et al., 2007).

Aggressive Behaviors

Dominance hierarchies are common among groups of male mice. For example, introducing a new male mouse into the home cage of an unfamiliar adult male mouse is likely to lead to aggressive behaviors. The standardized resident-intruder test is used to score tail rattling, following behaviors, latency to first attack, number of attacks, duration of fighting, and body scars (Miczek et al., 2001). A second approach used to quantitate aggressive tendencies is isolation-induced fighting. Isolating male mice in individual housing cages for several weeks will result in high levels of attack and fighting when the isolated males are subsequently placed together in a test arena (Valzelli et al., 1974). Fighting is more common among male mice than female mice (Compaan et al., 1993; Miczek et al., 2001). If it is important to avoid actual fighting and scarring, the tube test is a good choice for measuring dominant-subordinate status. Two male mice are placed in a cylindrical plastic tube, and the individual that retreats to one end is scored as the subordinate (Hahn and Schanz, 1996; Spencer et al., 2005).

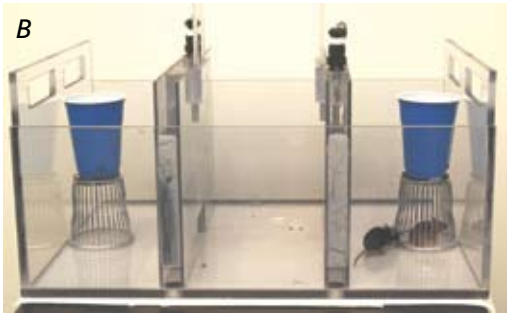

Figure 1. ***A,*** Nose-to-anogenital sniffing is commonly seen when unfamiliar mice are placed together. Olfactory pheromones in the anogenital area are detected by the vomeronasal organ as cues used for social recognition in rodents (Keverne, 2002). ***B,*** Automated 3-chambered social approach task, with photocells embedded in the openings between chambers, tallies the amount of time the subject mouse spends in the middle start chamber, the side chamber containing a wire cup novel object, and the side chamber containing a new mouse (Moy et al., 2007; McFarlane et al., 2007).

Photographs by Janet Stephens, NIH Photography, and the author.

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Sexual Behaviors

Introducing two normal adult mice of the opposite sex into the same cage is likely to lead to sexual behaviors. Males are scored for following, sniffing, mounting, thrusts, and intromissions (Scordalakes et al., 2002). Females are scored for lordosis and subsequent presence of a vaginal plug indicating insemination (Keller et al., 2006). Well-validated and standardized methods are available for conducting surgical ovariectomy, followed by hormone implants and injections to regulate receptivity and estrus in the females (Sisk and Meek, 1997), and for scoring sexual behaviors (Sisk and Meek, 1997; Scordalakes et al., 2002; Keller et al., 2006).

Parental Behaviors

Both male and female mice contribute to parenting. Both parents build nests and huddle with their pups (Bult and Lynch, 1996; Lijam et al., 1997; Moretti et al., 2005). Nests are scored for height, shape, quality, and utilization. Maternal care is scored for licking, sitting with, crouching, nursing, and retrieving the pups. When a pup strays from the nest or is removed by the experimenter, the pup emits ultrasonic vocalizations (Branchi et al., 2001; Hofer et al., 2001). Both parents locate the calling pup and retrieve it, returning it to the nest.

Social Recognition, Preference, and Memory

Individual recognition is interpreted to have occurred when the subject mouse displays more investigation of an unfamiliar mouse and less investigation of a familiar mouse upon repeated exposures to these conspecifics. The observer scores time spent in social interactions during brief exposures sessions, e.g., for 5 minutes. Preference for a specific individual, gender, strain, or genotype is demonstrated when the subject mouse spends more time interacting with one individual than with another in a choice test (Winslow, 2003) (Fig. 2A). Preference for social novelty is demonstrated when the subject mouse spends more time with a new mouse than with a familiar mouse (Crawley et al., 2007; Moy et al., 2007) (Fig. 2B). Social memory is evaluated by inserting a time delay, e.g., 30 minutes, between repeated exposures to the same and different mice (Ferguson et al., 2000).

Social Communication

Olfactory

Most communication between mice appears to employ olfaction (Keverne, 2002). Urine deposits elicit high levels of investigative sniffing. Interest in urine scents is measured in terms of the frequency and duration of sniffs directed at urine, which is delivered through various means, including an olfactometer delivering a stream of volatile odors into a port in an operant chamber, or cotton swabs soaked in urine (Wersinger et al., 2006). The olfactory habituation/dishabituation test employs cotton-tipped applicator swabs soaked in nonsocial and social odors, such as water, almond, banana, lemon, mouse urine, and floor wipes from soiled mouse cages (Luo et al., 2002; Wrenn et al., 2003; Wersinger et al., 2006; Crawley et al., 2007) (Fig. 3A). Olfactory communication of new food flavors on the breath of a cage mate is measured using the social transmission of food preference test (Wrenn et al., 2003; Wrenn 2004; McFarlane et al., 2007) (Fig. 3B)

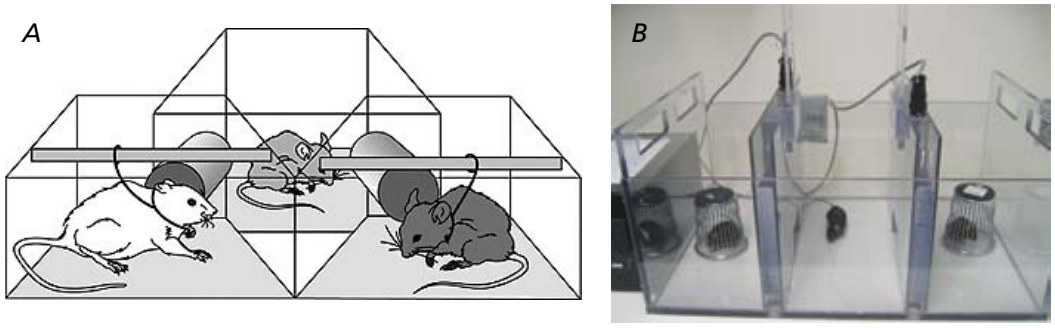

Figure 2. ***A,*** Social choice test (from Winslow, 2003). The subject mouse in the center chamber walks through the cylindrical tunnel to interact with one of the two tethered target mice. ***B,*** Preference for social novelty test. The subject mouse in the center chamber walks through the doorways to spend time interacting with either a familiar mouse in one side chamber or an unfamiliar mouse in the other side chamber. Photocells embedded in the panels around the doorways count time spent in each chamber and number of entries.

Photographs by Ms. Selen Tolu, NIMH, and the author.

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Auditory

Auditory communication among mice is an emerging research field. Complex vocalizations are emitted by juveniles engaged in social interactions and by adult males in response to female pheromones (Panksepp et al., 2007; Guo and Holy, 2007). As described above, ultrasonic vocalizations emitted by separated pups serve as distress calls the parents use to detect and locate the pup and retrieve it, bringing it back to the nest (Winslow et al., 2000; Hofer et al., 2001; Branchi et al., 2001) (Fig. 3C). Maternal potentiation of pup ultrasonic vocalizations is a modification that may incorporate more cognitive components. Rat pups twice separated from the dam display more ultrasonic vocalizations during the five minutes after the second separation than during the five minutes after the first separation (Hofer et al., 2001). There is some evidence for maternal potentiation of ultrasonic vocalizations in mice (Moles et al., 2004; M.L. Scattoni and J.N. Crawley, NIMH, unpublished observations). Since the separations are identical in every other way, and occur five minutes apart, it seems likely that the pup is regulating its response based on a cognitive interpretation of its previous separation experience. Intentionality and functional significance of mouse ultrasonic vocalizations remain to be determined. Studies are needed that feature playback tape recordings of salient vocalizations in mice, similar to those studies used to investigate vocal communication among birds (Konishi, 2004).

Motivation for Social Interactions

Another area of mouse social behaviors that requires new ideas is the measurement of motivational level for engaging in social interactions. Conditioned place preference for the chamber in which a social partner was previously located has been validated as a test for social reinforcement among rats as well as among juvenile C57BL/6J, A/J, and DBA/2J inbred strains of mice (Everitt, 1990; Panksepp and Lahvis,

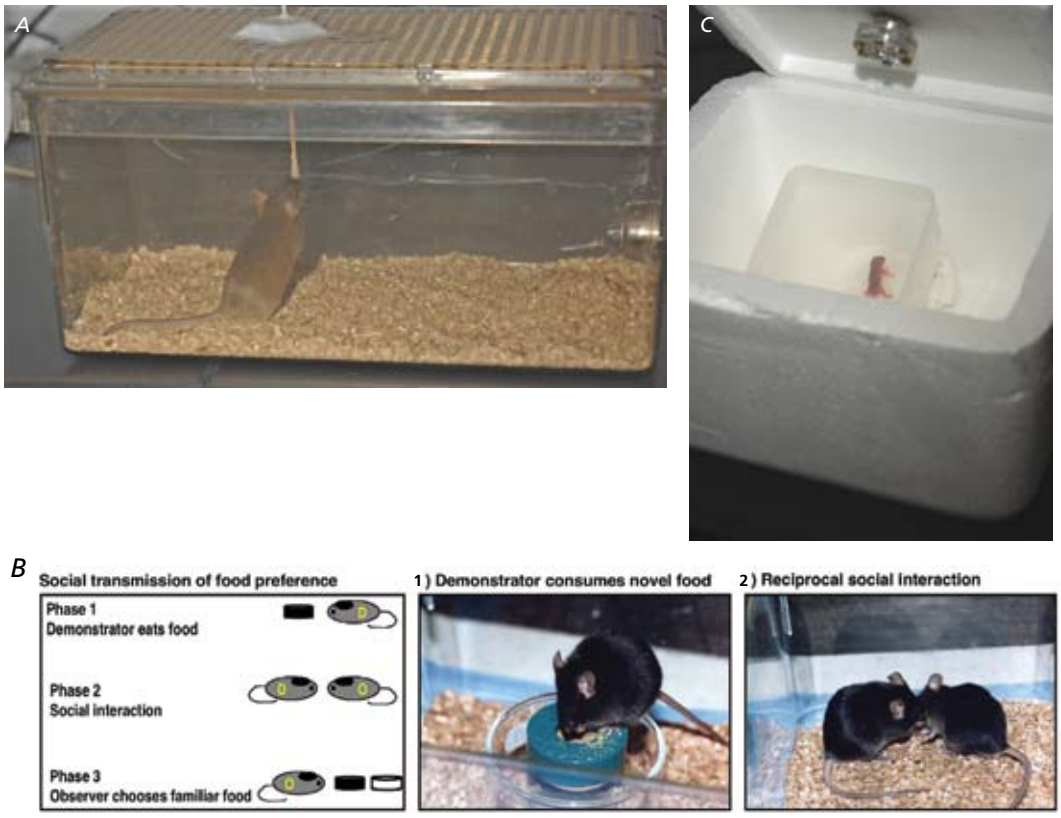

Figure 3. Social olfactory tests. ***A,*** Olfactory habituation/dishabituation. An observer measures the time spent by the subject mouse in sniffing new and familiar social smells, such as mouse urine or cage swipes, presently sequentially. Photograph by Janet Stephens, NIH Photography, and the author. ***B,*** Social transmission of food preference. (1) One cage mate (demonstrator) consumes a novel flavored food and (2) communicates the odor on its breath to its cage mates (observers). The observer eats more of the flavored food detected on the mouth and whiskers of the demonstrator than it does a completely new flavored food. Photographs by Valerie Bolivar, Wadsworth Center, Troy, NY; diagram by Valerie Bolivar, Wadsworth Center, Troy, NY, modified by Hewlet McFarlane, Kenyon College, Gambier, OH. Adapted from McFarlane et al., 2007. ***C,*** Ultrasonic microphone in the lid of a Styrofoam box records ultrasonic vocalizations emitted by a mouse pup separated from its nest. Photograph by Janet Stephens, NIH Photography, Dr. Maria Luisa Scattoni, NIMH, and the author.

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2006). Maternal motivation for retrieving rat pups is measured in terms of responses of the mother using an operant lever to deliver pups from a carousel (Lonstein and Fleming 2001) (Fig. 4A). A rat operant chamber has been modified to allow the investigator to open a trapdoor and deliver a social partner when the subject rat presses a lever on a fixed ratio schedule (Everitt, 1990) (Fig. 4B). Automated two-chamber systems with electronic access doors that open and close according to a reinforcement schedule are needed in order to allow researchers to quantitate the number of nose-pokes a mouse is willing to make to gain access to a social partner.

Mouse Models of Aberrant Social Behaviors

Aberrant social behaviors or low levels of social interaction are symptoms of several psychiatric disorders, including autism, anxiety, depression, schizophrenia, and social phobias. Genetic, pharmacological, and lesion models of neuropsychiatric disorders are increasingly available. In these models, transgenic and knockout mice with mutations in candidate genes for a disease are phenotyped for behavioral traits with face validity, i.e., conceptual analogy to the human symptoms (Crawley, 2007). Some of the first laboratory protocols for measuring social interactions in rodents came from studying models of anxiety. Two rats placed in an unfamiliar environment will display less sniffing and following behavior under high levels of illumination than when the ambient light is dim (File and Hyde, 1978). Anxiolytic drugs increase social interaction in the brightly lit arena (File, 1997). Social cognition deficits in schizophrenia could be modeled using some of the tests described above. It is interesting to speculate that the social anhedonia seen in some forms of depression, in which the patient gains no pleasure from engaging in social interactions and avoids social environments, could be modeled using some of the social motivation tasks described above.

Autism is diagnosed on the basis of aberrant social interactions as well as impaired communication and repetitive behaviors. Given the strong genetic component to autism spectrum disorders, mouse models of autism are increasingly focusing on knockouts of candidate genes that have been identified in human association studies, as well as inbred strains with unusual background genes that may be relevant to social behaviors (Crawley, 2004). Measures of social interactions between mice tested in a variety of ways offer complementary approaches for quantifying abnormal levels of sociability. Examples include mice tested in an empty arena (Spencer et al., 2005; Bolivar et al., 2007), in a 3-chambered apparatus (Winslow, 2003;

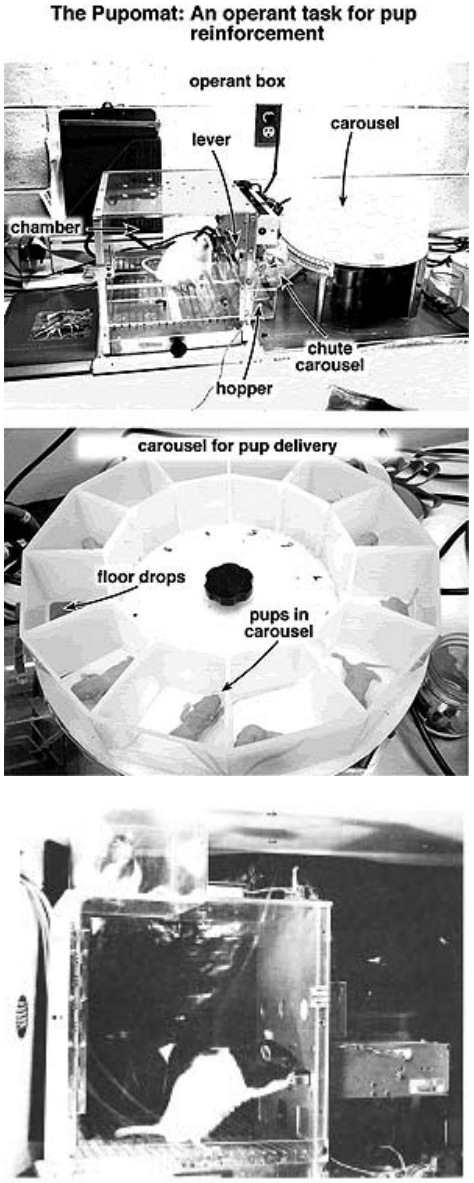

Figure 4. Social motivation equipment. ***A,*** Pupomat carousel delivers a pup when the mother presses a lever (from Lonstein and Fleming, 2001). ***B,*** Male rat presses a lever on a fixed ratio schedule to gain access to a familiar female rat located above the operant chamber.

Photograph kindly contributed by Professor Barry Everitt, University of Cambridge, UK; adapted from Everitt, 1990.

Brodkin, 2007; Moy et al., 2007; McFarlane et al., 2007) (Figs. 1B, 2A, 2B), in a visible burrow (Arakawa et al., 2007), in a socially conditioned place-preference chamber (Everitt, 1990; Panksepp and Lahvis, 2006), and using video tracking systems (Kwon et al., 2006).

Aberrant forms of reciprocal social interactions detected in mouse models of autism, Rett syndrome,

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fragile X syndrome, schizophrenia, and other disorders characterized by social deficits provide translational phenotypes for testing hypotheses about biological mechanisms and for evaluating the therapeutic efficacy of proposed treatments.

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Studying the Epigenetic Influence of Maternal Care in Rodents

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Overview

Environmental influences on adult gene expression and behavior have been explored extensively in rodents. This research suggests that experiences early in development can result in long-term changes to neural systems involved in stress responsivity and reproductive behavior. In particular, the maternal care provided to offspring during the first week of life can have profound effects on their development. These effects, which have been studied in laboratory rats, suggest that maternal care is associated with epigenetic regulation of gene expression that results in stable individual differences in behavior. These studies illustrate the use of a longitudinal approach to analyzing the effects of environmental experiences at a behavioral, neurobiological, and molecular level. The purpose of this chapter is to describe the methodology for studying epigenetic effects in rodents associated with postnatal maternal care, with particular emphasis on the challenges to taking this type of life-history approach in a laboratory setting.

Assessing the Quality of the Early Environment

In mammals, the interactions between mother and infant are essential for survival and play a critical role in shaping infant development. Evidence from a variety of species shows that the quality of the external environment influences mother-infant interactions during the prenatal and postnatal period, and thus influences offspring via maternal care. Therefore, experimental manipulations, such as postnatal maternal separation and handling, have been found to result in changes to numerous neural systems and behaviors (Levine, 1957; Lehmann et al., 1999); these changes are thought to be mediated in part by changes to the behavior of postpartum females. However, to determine the role that maternal care plays in regulating offspring development, it is not necessary to use separation paradigms. Even among inbred laboratory animals, one may observe stable individual differences in maternal behavior that can be associated with offspring characteristics (Champagne et al., 2003; Champagne et al., 2007).

This approach has been explored in Long–Evans rats and suggests that variation within the normal distribution of maternal care can have profound effects. Implementing this strategy involves 3 steps: (1) observing a cohort of postpartum females during the first week following parturition, (2) selecting females that engage in levels of a behavior that deviate by 1 SD from the mean of the cohort, and (3) comparing the offspring of females that engage in levels of maternal care that are either 1 SD above or 1 SD below the mean. Following these 3 general steps raises numerous methodological problems that can be addressed by answering the questions:

- *What protocol should be used when observing postpartum females?* and
- *What aspects of maternal care should be used to select females?*

Quantifying postpartum mother-infant interactions

Characterizing postpartum maternal behavior in any species is challenging. In rodents, this process is complicated by the nocturnal nature of these species. Though initial work with Long–Evans rats suggests that observations made during the light phase of the light-dark cycle can yield significant data, the value of assessing behavior during this period tends to vary among strains and species. Certainly, considerable differences in maternal behavior will be seen across the light cycle. What is critical in these studies is to be able to distinguish between females that exhibit high or low levels of care. To do this requires extensive observation.

The approach we have used is to conduct 4–5 hours of observation per day for 6 consecutive days; during this span, a time sampling of behavior is taken every 3 minutes within each 1-hour period. Comparison with a focal sampling procedure suggests that this observation method captures the degree of detail necessary to differentiate among females. Following this protocol will result in 480–600 observations per female. We based the choice of number of days of observation on the natural decline in maternal behavior over this period and on the finding that the first week of life represents a critical period in rodents.

To obtain a sample of females that can be characterized, approximately 40–50 females will need to be mated. Near the end of the gestational period, females need to be checked repeatedly in order to accurately record the date of birth. Owing to the lengthy birthing process, it is usually preferable to begin observations on the day after birth. To be observed, females will have to be housed in clear plastic cages so that mother-offspring interactions can be viewed. Though one might be tempted to automate the observation process, using a video camera or computerized system, the approach we have used involves directly observing the home-cage behavior and recording the data with paper and pencil. Thus, observers need to be trained to a high level of inter-rater reliability such that they are able to classify behavior consistently. Since mother-infant interac-

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tions do vary considerably in response to environmental change, it is best to avoid any disturbances to the home cage during the observation period and, in particular, to avoid changing the bedding during the first week postpartum.

What aspect of maternal care is critical for offspring development?

Many aspects of postpartum maternal care in rodents can be observed during the first week postpartum. There is a high frequency of nursing, in which the female crouches over the pups to provide thermoregulation and allow them to suckle. Nest-building, self-grooming, eating, and drinking are also observed; the frequency of these behaviors varies over consecutive days. Postpartum females also lick pups, which serves to groom and stimulate the altricial young. Initial studies examining the role of maternal behavior in regulating the offspring's stress responsivity have focused on the role of pup licking, which likely serves as a critical source of tactile stimulation (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999). Among Long–Evans female rats, there is a substantial amount of individual variation in licking/grooming (LG) behavior. Analysis of large cohorts of lactating females suggests that average LG is approximately 10–11% of time spent, and that this behavior is normally distributed (Fig. 1). As such, females can be selected as High LG or Low LG based on the relative frequency of LG observed. Thus, if the average LG for a cohort is 10.5%, with a standard deviation of 2.5%, then females engaging in LG at a frequency of 13% or greater would be classified as High LG, whereas females engaging in LG at a frequency of 8% or lower would be classified as Low LG. Generally, the mean and standard deviations do not fluctuate significantly from one cohort to another, allowing the use of the cutoffs to select females.

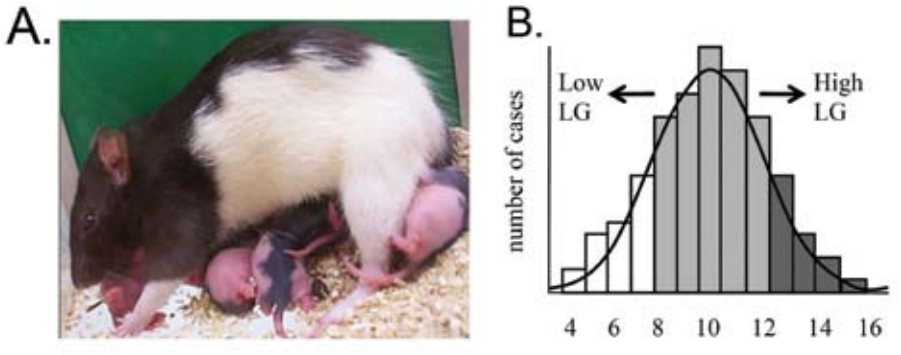

Figure 1. ***A***, Long–Evans lactating female and pups. ***B***, Normal distribution of licking/grooming (LG) behavior indicating selection of High LG and Low LG females.

Though LG may be a critical behavior when considering some aspects of offspring development, this fact does not lessen the contribution of other behaviors exhibited during the postpartum period. These are particularly important when considering effects in other species or strains within a species. For example, frequency of time spent nursing varies considerably among individual females and between species and may also influence brain development and behavior. For this reason, it is best practice to record as many behaviors as possible. These data can be entered into a spreadsheet, and a profile of maternal behavior can be created for each female.

Cross-Fostering

To determine the role of the maternal environment in shaping gene expression and behavior, it is critical that other potential sources of influence be eliminated. One strategy to achieve this goal is to cross-foster offspring between mothers. For example, to explore the role of postpartum LG on offspring development, pups born to High LG dams can be transferred to Low LG dams on the day of birth, while pups born to Low LG dams can be transferred to High LG dams. For this manipulation to succeed, you must be able to reliably predict the quality of maternal care that a female will provide. In the case of Long–Evans rats, LG behavior exhibits a high degree of stability, such that females who are High LG toward their first litter will be High LG toward subsequent litters (Champagne et al., 2003). The same is true of females that exhibit Low LG. Thus, the first step in a cross-fostering experiment would be to characterize the primiparous behavior of a cohort of females and to select High LG and Low LG dams.

Lactating female rats and mice will not typically cannibalize cross-fostered offspring. Though the female can likely distinguish between biological and adopted offspring, based on olfactory cues, she will readily retrieve and care for any pups placed in the home cage. Ideally, the transfer of pups would occur within 12 hours of birth to reduce the potential influence of the biological mother. This requires that biological and adoptive mothers give birth at approximately the same time. To achieve this timing, many females will have to be mated

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simultaneously. In addition, a decision must be made regarding the number of pups to transfer. In previous cross-fostering studies (Francis et al., 1999), only 2–4 pups have been transferred to adoptive females, while the remaining biological offspring remain with the mother so as to limit potential disruption to the patterns of maternal care. If this strategy is employed, it will be necessary to distinguish between adopted and biological offspring at the time of weaning. To this end, adopted pups can be marked on the hind region with surgical ink once weekly. Alternatively, whole litters can be swapped between females. This swap eliminates the need to mark individual pups and increases the number of potential fostered offspring. There has been no systematic comparison of these two methods, however; instead, the illustration of the epigenetic influence of maternal care has relied on the fostering of 2–4 pups rather than whole litters.

Postnatal cross-fostering can be a very effective method for determining whether a characteristic in offspring is genetic in origin or environmentally mediated. However, this manipulation does not eliminate the influence of the prenatal environment. In mice, evidence suggests that both the prenatal and the postnatal environments are critical in shaping offspring's stress responsivity (Francis et al., 2003). Thus, in addition to postnatal cross-fostering, it may be necessary to use embryo transfer to illustrate epigenetic effects.

Behavioral Assessment

The ultimate goal of this research is to understand the origins of individual differences in behavior. After characterizing the early maternal environment that offspring experience, the next step is to assess phenotype. Countless behavioral measures can be used in this context. Our primary focus has been on stress responsivity and behavioral indications of fearfulness. One of the measures we have found particularly effective in differentiating between the offspring of High LG and Low LG mothers is the open-field task. During this assessment, the test animal is placed in a square box, which serves as a novel environment, and allowed to move freely for a 10-minute period. Typically, mice and rats placed in this environment engage in thigmotaxis, wherein the animal stays in the periphery of the field, moving along the sides near the walls of the field but not venturing toward the inner area of the field (Crawley, 1985). During the 10-minute period there is usually a reduction in overall activity, and in some cases, the animal will venture into the inner area of the field. There is debate about how to interpret the behavioral patterns observed during this test; however, there is also agreement that animals that do not explore the inner area of the field are inhibited, and thus, reduced time spent in the inner area is often taken as a measure of anxiety-like behavior. These interpretations are based on the finding that anxiolytic drugs increase exploration of the inner area, whereas anxiogenic drugs increase thigmotaxis.

To use this test to examine the effects of maternal care on behavior requires a system for determining the litter of origin of each animal. We typically house offspring with littermates and assign ID numbers based on litter of origin. Alternatively, ear punching done at weaning is an effective way of tracking individual animals. We typically test rodents at 60–90 days of age to investigate the long-term consequences of maternal care. However, tests like the open field can be used at a variety of ages. In planning a study, it is best practice not to test animals repeatedly in the open field, as this will reduce the novel nature of this task. In Long–Evans rats, there is a significant level of individual variation in open-field behavior. Comparison of the offspring of High LG and Low LG mothers suggests that increased levels of maternal LG in infancy result in offspring that are more exploratory in the inner field (Fig. 2). We have not observed any differences in the activity level of these offspring.

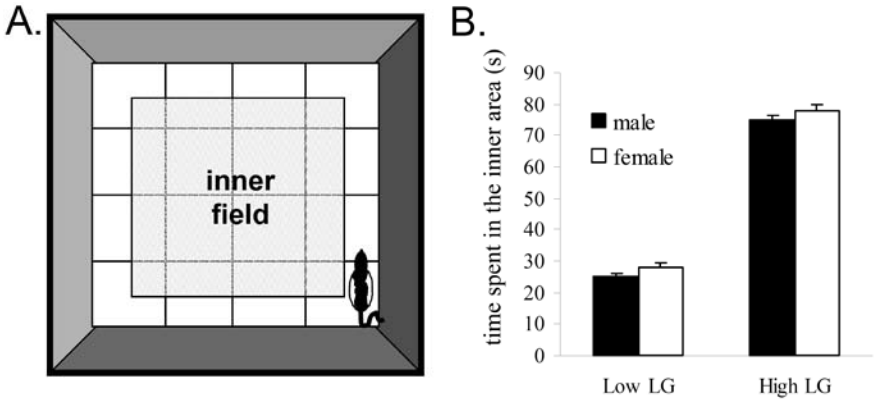

Figure 2. *A,* Schematic of open-field apparatus indicating inner area. *B,* Mean ± SEM time spent in the inner area of the open field (in seconds) by male and female offspring of High LG and Low LG mothers.

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For purposes of analysis, we divided the open field into grids, to facilitate the distinction between inner and outer area, and indicated the activity level by the number of transitions an animal makes from one grid to another. Group differences in activity make interpreting measures of exploratory behavior very difficult. For example, some strains of mice are particularly hyperactive. A reduction in anxiety-like behavior in a hyperactive strain may reduce exploration by reducing movement within the field.

A few considerations should be made when conducting any type of behavioral assessment, but particularly of behaviors that are sensitive to stress. Do not test animals on the day they have undergone cage cleaning. This aspect of animal husbandry is usually very disruptive and increases activity levels in rodents. Time of day is also important: If animals are on reverse lighting, such that the dark phase is during the daytime, then assess their behavior during the dark phase. Avoid testing them near the time of transition between light phases and, if testing over consecutive days, test at the same time each day. There are significant changes in corticosterone from the morning to the afternoon that could affect the behavioral response observed. Finally, if you are testing multiple animals from one cage, it is best not to reintroduce a recently tested animal back into its home cage until all its cage mates have been tested. Reintroduction usually initiates bouts of aggressive behavior as dominance hierarchies are reestablished.

Environmental Regulation of Gene Expression

Many strategies are available for studying the expression of genes. Rather than use global measures of change in gene expression, we have chosen to focus on particular genes. Among the offspring of High LG and Low LG dams are found differences in the levels of stress-induced corticosterone (Liu et al., 1997). Though both these groups respond to stress, levels of corticosterone are elevated for a prolonged period in the offspring of Low LG mothers. This finding has led to an investigation into the role of the glucocorticoid receptor (GR) in mediating individual differences in stress responsivity. Levels of GR in the hippocampus have been found to exert a negative feedback effect on the corticosterone response to stress (Sapolsky et al., 1985). Thus, higher levels of hippocampal GR are associated with a more rapid decline in corticosterone following a stressor.

To test the hypothesis that the attenuated response to stress among the offspring of High LG mothers is a consequence of elevated hippocampal GR, we used *in situ* hybridization. This technique allows us to quantify the density of GR expression and provides us with a detailed map of locations in the brain where these differences in expression occur. We typically compare expression in the brains of 5–6 animals per group (ideally, this tissue is not taken from subjects that have undergone behavioral testing). The protocol for conducting GR *in situ* hybridization has been described previously (Liu et al., 1997) and involves the use of nonperfused frozen tissue that is sliced coronally at a thickness of 16 µm and mounted on poly-L-lysine–coated slides. The hybridization protocol takes 2–3 days, after which the slides can be exposed to film for visualization.

Investigations into the role of maternal care in regulating expression of GR have indicated that, indeed, the offspring of High LG dams have higher levels of hippocampal GR compared with the offspring of Low LG dams. Results from cross-fostered offspring confirm that it is the quality of the postpartum environment that is critical for mediating this difference. These studies illustrate how best to implement a longitudinal study in a laboratory setting where, in the span of 4–5 months, the effect of an early experience on adult gene expression and behavior can be demonstrated. It is difficult to determine whether there are critical periods in which these effects set in. Cross-fostering is always done immediately after birth, so we do not yet know whether postnatal experience at day 2 or 3 would likewise shift patterns of behavior and gene expression. However, it is clear that the effects of postnatal care influence gene expression and behavior long after offspring have been weaned. Observing the stability of the effects of this early experience has led us to explore the factors that regulate gene expression—and that possibly maintain changes to it in the long term.

Epigenetic Regulation of Gene Expression

Studying epigenetic effects is a popular new approach in the neurosciences. However, the term *epigenetics* has been used very broadly and can have widely different meanings depending on the context in which it is used. At a molecular level, *epigenetics* refers to the modifications to DNA and chromatin that alter the functioning of a gene without changing the sequence of its DNA. Numerous mechanisms are involved in the process of gene expression. In order for RNA polymerases to initiate transcription, there must be a change in the structure of the nucleosome, such that DNA is exposed and no longer firmly wrapped around histone proteins. There are many modifications that alter the accessibility of DNA. In addition to histone modifications, such as acetylation and phosphorylation, DNA can be modified

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by attaching a methyl group to cytosine bases. This DNA methylation can exert stable effects on gene expression and is the primary mechanism through which cellular differentiation occurs (Razin, 1998). Attaching a methyl group to DNA effectively blocks access to the DNA, thereby inhibiting the binding of transcription factors in the promoter region of a gene, leading in most cases to transcriptional repression. Thus, DNA methylation is a logical candidate when considering the molecular mechanisms that mediate long-term effects on gene expression.

The process of exploring the role that maternal care plays in shifting patterns of DNA methylation within the hippocampal GR promoter region involved 3 main steps: (1) characterizing levels of DNA methylation within the GR promoter in the offspring of High LG and Low LG mothers, (2) confirming the maternal mediation of these effects by comparing methylation patterns among cross-fostered offspring, and (3) testing the functional consequences of this differential methylation for the binding of transcription factors to the GR promoter. The analysis of methylation within a specific DNA sequence is best achieved using a technique known as *bisulfite mapping* (Clark et al., 1994). After purifying DNA samples in which the promoter region is isolated, the samples are treated with sodium bisulfite. This treatment results in the conversion of unmethylated cytosines to uracil, whereas methylated cytosines are not converted. Following PCR amplification, the resulting sequence will contain thymine at the location of unmethylated cytosines, whereas the sequence will contain a cytosine where methylation is present. Thus, by locating cytosines in the resulting sequencing gel, one is able to create a map containing the precise location of methylated cytosines.

Methylation patterns vary considerably within and among tissues. As such, it is best to sequence multiple samples from each animal. Graphically, these methylation patterns are usually presented as a bead-on-string figure. The string represents the sequence of DNA being analyzed, and the beads along the string represent each site at which a methyl group can bind to the DNA. In the case of the GR promoter region (Fig. 3), there are 17 potential sites within the promoter at which methylation can occur. If methylation is detected within the sequencing gel, the bead is colored black; if the bead is white, there was no methylation detected. In the analysis of GR-promoter methylation in the offspring of High LG and Low LG mothers, we included results from 10 sequencing gels per animal. Thus, in Figure 3, there are 17 beads per string and 10 strings per animal. Visually, this presentation is very effective because the differences in methylation can be clearly observed. Offspring of Low LG mothers have elevated methylation within the GR promoter region corresponding to the decreased levels of GR expression previously described (Weaver et al., 2004). To analyze these data statistically, rather than visually, requires that an average methylation value be calculated for each of the 17 sites for each animal. To do this you simply create a spreadsheet in which unmethylated sites are assigned a value of 0, whereas methylated sites are assigned a value of 1. The average methylation will thus range from 0 to 1. This analysis will allow you to determine locations within the DNA sequence whose differential methylation is significant.

To determine the functional consequences of the differential methylation of a promoter region of DNA, one strategy is to use a chromatin immunoprecipitation assay (ChIP). In the GR promoter region, among the offspring of High LG and Low LG mothers, we can use ChIP to quantify the level of binding of a transcription factor to a region of differentially methylated DNA. Among the offspring of Low LG mothers, there are considerably higher levels of methylation at site 16 within the promoter, a region that contains an NGFI-A binding site. Thus, we predicted that less NGFI-A binding would take place in the heavily methylated GR promoter of the offspring of Low LG mothers compared with the offspring of High LG mothers. Using ChIP we were able to dem-

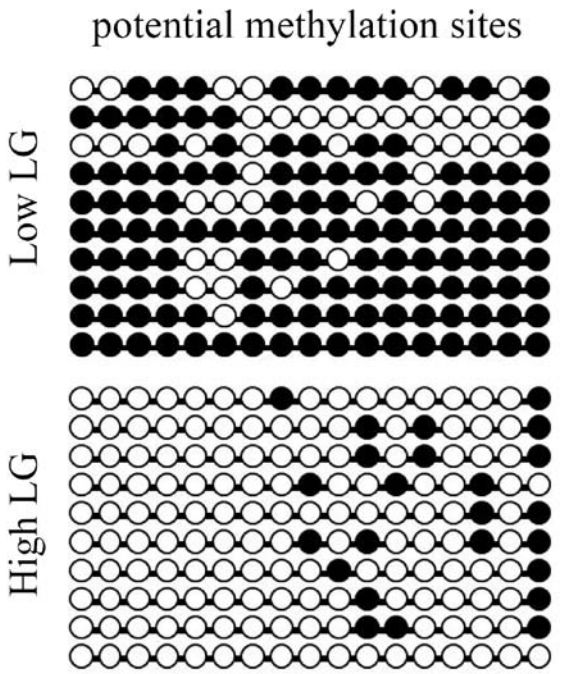

Figure 3. Bead-on-string schematics indicating the methylation patterns in the hippocampal GR promoter of offspring of High LG and Low LG mothers. Black circles indicate methylated cytosines.

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onstrate the reduced binding of NGFI-A to the GR promoter of Low LG offspring (Weaver et al., 2004).

In addition to ChIP, the functional consequence of a gene promoter's methylation status can be determined by pharmacologically manipulating methylation levels. Drugs such as trichostatin-A (TSA) and valproate have been demonstrated to reduce levels of methylation, whereas methionine administration has been shown to increase levels of methylation. Following administration of these compounds, the effects on gene expression and behavior can be evaluated. Offspring of Low LG mothers that are treated with TSA as adults demonstrate a profound alteration in phenotype, such that they resemble the offspring of High LG mothers (Weaver et al., 2004). Conversely, offspring of High LG mothers that are treated with methionine display patterns of gene expression and behavior that are not significantly different from the offspring of Low LG mothers (Weaver et al., 2005). These tools can provide a highly effective means for illustrating the role epigenetic mechanisms play in regulating individual differences in phenotype.

Transgenerational Effects

In addition to demonstrating the effects of maternal care at a molecular level, these studies illustrate a mechanism that can mediate the transmission of behavioral patterns across generations. One of the best predictors of the level of care a female will provide to offspring is the level of care provided to her during infancy (Champagne and Meaney, 2001). Thus, in the case of Long–Evans rats, female offspring born to mothers that provided high levels of LG are themselves High LG mothers, whereas the offspring of Low LG mothers exhibit low levels of LG toward their own offspring. Using cross-fostering, this transmission has been determined to be behavioral rather than genetic. As a consequence of the transmission of maternal care behaviors across generations, there is also a transmission of stress responsivity from one generation to the next (Champagne and Curley, 2005). This epigenetic transmission results in similarities between grandparental, parental, and offspring generations that would normally be attributed to inherited genetic polymorphisms. Thus, in any research design it is critical to explore the origins of individual differences in behavior and to consider both genetic and epigenetic sources of variance.

Implications for Studies with Transgenic Mice

The role of maternal care in mediating epigenetic changes in offspring has been explored most fully in rats. The translation of this work to a mouse model is currently in progress, and so far, research suggests that maternal effects are critical in the development of stress responsivity in mice (Francis et al., 2003). These effects are very important to consider when working with transgenic mice. If variations in maternal care result from manipulating a gene in a transgenic line, there may be aspects of phenotype that are not necessarily the product of the gene itself but are instead an effect on development that is due to prenatal or postnatal care. In order to make this distinction, the procedures outlined in the previous sections can be applied. Cross-fostering offspring between wild-type and transgenic females, either prenatally or postnatally, can give some indication of a maternal effect. This can be complemented with detailed observation of postpartum maternal care. Following these procedures will allow you to explore the role of gene expression in regulating physiology and behavior and to incorporate both a genetic and an epigenetic approach.

Summary

Studying the developmental consequences of maternal care on offspring development is a topic of broad scientific interest. The approach we have taken to study these effects combines detailed behavioral observation with molecular techniques; this method allows us to better understand the mechanisms through which maternal effects are mediated. Implementing this method is challenging and can best be achieved by creating a multidisciplinary research group with expertise in the different levels of analysis we have described. However, despite these challenges, these methodologies can provide a wealth of data that addresses fundamental questions about the origins of individual differences in behavior.

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Mouse Startle Tests Relevant to Schizophrenia

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Introduction

In the context of models relevant to schizophrenia and related psychotic disorders, assessments of behavior in mice have comprised a variety of tasks. Most of these tasks have been adapted from similar paradigms used previously in the more extensive rat literature. Owing to the historical dominance of the dopamine hypothesis of schizophrenia, the vast majority of the classical tests in this area have focused on measures of the effects of psychostimulant drugs. Similarly, most of the validation of these tests has relied on pharmacological isomorphism, in terms of measuring the ability of clinically effective antipsychotic drugs to reverse the behavioral effects of psychostimulants (Geyer and Markou, 2002). With the advent of atypical antipsychotics, the hypoglutamatergia hypothesis of schizophrenia (derived from the psychotomimetic effects of acute administrations of glutamate antagonists such as phencyclidine) has received more attention and prompted researchers to utilize additional behavioral tests. Several reviews are available that provide overviews of the rodent tasks classically related to schizophrenia, using either dopaminergic or glutamatergic manipulations (Crawley, 2000; Geyer and Moghaddam, 2002; Segal and Geyer, 1985). With the increased interest in identifying treatments for the cognitive deficits in schizophrenia, which are largely insensitive to typical or atypical antipsychotics, current efforts are focusing on the further development and validation of rodent tests of cognition (Floresco et al., 2005). To this end, recent overviews of rodent behavioral tasks relevant to assessing models of schizophrenia-linked cognitive deficits have become available (Powell and Geyer, in press; Young and Geyer, 2007).

The present discussion focuses on the use of measures of startle response in the context of murine models related to schizophrenia. Recapitulating the history of schizophrenia-related tests in rats, mouse startle testing began with the characterization of pharmacological effects on startle in mice that had been validated to some degree in terms of the effects of typical and atypical antipsychotic drugs (Dulawa and Geyer, 1996; Curzon and Decker, 1998). In work related to hearing deficits, rather than schizophrenia, this effort was presaged by assessing strain-related differences in the startle behavior of mice (Hoffman and Ison, 1980; Ison and Hoffman, 1983; Willott et al., 1994, 1995). The creation of mutant lines of mice that have hypothesized relationships to schizophrenia prompted the use of startle testing as a phenotypic marker, which in turn was based on the demonstrated abnormalities in startle behavior among patients with schizophrenia (Ralph et al., 1999; Geyer et al., 2002). As discussed elsewhere (Geyer and Moghaddam, 2002; Geyer, 2006a), abnormalities in startle response in rodents have conceptual relevance to the effects of classical antipsychotics on the positive symptoms of schizophrenia (presumably mimicked in animals by the effects of psychostimulant drugs) as well as the cognitive deficits characteristic of schizophrenia that are not treated adequately with existing antipsychotics. Hence, phenotypic characterizations of startle response in murine models related to schizophrenia provide some of the most direct tests of the model's potential validity for studying the condition in humans.

The Startle Reflex

Centrally mediated reflexes are highly modifiable by several events: ambient conditions, organismal factors, stimuli occurring concurrently with or shortly before reflex elicitation, and prior exposure to the eliciting stimulus. Thus, studying the plasticity of reflexes provides a window into a wide variety of physiological processes that modify their expression. The startle reflex (the fastest centrally mediated reflex) provides a highly quantifiable and reliable operational measure of several physiological processes that can be easily assessed across species. Hence, measures of the startle response and its plasticity provide powerful research tools for examining the neural control of behavior. Specifically, a variety of information-processing functions, including sensorimotor gating, affective modulation, and habituation, modify the expression of startle. Startle measures can be used to assess these processes easily and reliably across a variety of species, including humans, nonhuman primates, and rodents.

The startle reflex consists of involuntary contractions of whole-body musculature elicited by sufficiently sudden and intense stimuli. The primary acoustic startle circuit consists of three synapses at and below the level of the pons; these include the cochlear root neurons, the nucleus reticularis pontis caudalis, and motoneurons in the facial motor nucleus and spinal cord (Davis et al., 1982, 1999). Although startle is mediated at the brain stem level, several forms of startle plasticity, such as prepulse inhibition (PPI) and habituation, are regulated largely by forebrain structures, including the striatum, ventral pallidum, and hippocampus (Swerdlow and Geyer, 1998; Swerdlow et al., 2001).

Prepulse Inhibition of Startle in Schizophrenia and Rat Models

PPI is a form of startle-response plasticity considered to be a form of sensorimotor gating, since it refers to the ability of a sensory event to suppress a motor

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response. PPI is the normal reduction in startle magnitude that occurs when a weak pre-stimulus or "prepulse" precedes the startling stimulus by 30–500 msec (Ison et al., 1973; Graham, 1975; Hoffman and Ison, 1980; Ison and Hoffman, 1983). Since PPI can be either intramodal or cross-modal, a variety of stimulus modalities can be used as either the prepulse or the startling stimulus to affect PPI (Braff et al., 1992; Kehne et al., 1996; Brody et al., 2004a). The theoretical construct of sensory or sensorimotor gating refers to putative neural mechanisms that inhibit the processing of extraneous sensory input, cognitive information, and motor programs. The normal inhibition, filtering, or gating of extraneous information is thought to permit mental and behavioral integration (Braff and Geyer, 1990). In contrast, the symptoms of schizophrenia have been theorized to result in part from deficiencies in filtering or gating mechanisms. Accordingly, PPI has been evaluated in a number of psychiatric populations. Using a variety of testing procedures and stimulus parameters, several laboratories have reported significant deficits in PPI among schizophrenia, schizotypal, and presumably psychosis-prone subjects (Braff et al., 2001). As reviewed elsewhere (Braff et al., 2001; Geyer, 2006b), however, PPI deficits are not unique to patients diagnosed with schizophrenia; rather, deficits have also been reported in other psychiatric disorders involving abnormalities of gating in the sensory, motor, or cognitive domains.

The neuroanatomical substrates that contribute to the modulation of PPI in rats have been studied extensively (Swerdlow et al., 2001), providing an excellent model of the regulation of behavior by integrated neuronal circuits. Similarly, a wide range of developmental and pharmacological manipulations have been found to alter PPI in rats, leading to multiple rat models that are useful in the identification of antipsychotic medications (Geyer et al., 2001). PPI has also shown good predictive validity as a screen for antipsychotic drugs. For example, PPI deficits can be induced in animals by administering psychotomimetics such as amphetamine or phencyclidine. These drug-induced deficits in PPI can then be attenuated using antipsychotic drugs (Geyer et al., 2001).

One important aspect of animal models of schizophrenia is their ability to distinguish between typical and atypical antipsychotic drugs. PPI deficits induced by apomorphine are reversed by both typical and atypical antipsychotics. Thus, although the ability of antipsychotics to restore PPI in apomorphine-treated rats strongly correlates with the drugs' clinical potency (Swerdlow et al., 1994), when used with the dopamine agonist apomorphine, this paradigm clearly fails to show the important distinction between these two classes of antipsychotic drugs. In contrast, the PPI disruptions produced by NMDA antagonists (e.g., phencyclidine, dizocilpine, and ketamine) react somewhat differently to typical and atypical antipsychotics. Specifically, typical antipsychotics such as haloperidol do not attenuate the PPI-disruptive effects of NMDA antagonists in rats, whereas clozapine and some other atypical antipsychotics do reduce the disruption in PPI produced by NMDA antagonists in both rats (Geyer et al., 2001; Geyer and Ellenbroek, 2003) and mice (Brody et al., 2004b).

Habituation of Startle in Schizophrenia and in Rat Models

Habituation is another example of startle-response plasticity. It refers to the exponential decrement in response to repeated presentations of an initially novel and intense stimulus, and constitutes the simplest form of nonassociative learning (Thorpe, 1956; Duerr and Quinn, 1982; Hawkins et al., 1998). In rodents and humans, habituation can be quantified by measuring the magnitude of the startle response to repeated presentations of startling stimuli. As for PPI, several groups have reported startle habituation to be reduced in schizophrenia and schizotypal patients (Geyer and Braff, 1982; Braff et al., 1992; Bolino et al., 1992; Cadenhead et al., 1993; Taiminen et al., 2000; Meincke et al., 2004). Also paralleling observations for PPI, some psychiatric populations other than schizophrenic patients reportedly exhibit deficits in startle habituation. For example, patients with panic disorder exhibit reductions in both PPI and startle habituation (Ludewig et al., 2002). Interestingly, in unmedicated panic disorder patients, the cognitive dysfunctions were correlated with the reductions in startle habituation but not with a deficit in PPI (Ludewig et al., 2005). Deficits in startle habituation can be induced in rats by treating them with psychotomimetic drugs such as phencyclidine, mescaline, or LSD (Davis, 1987; Geyer et al., 1978, 1984).

Startle

Startle reactivity, PPI, and habituation have been well characterized in many murine strains. Mice exhibit orderly increases in PPI levels, as shown by increasing prepulse intensities and gradual reductions in startle magnitudes in response to repeated presentations of the same stimulus (i.e., habituation). Along with appropriate measurement systems, startle reactivity values are large relative to baseline levels of recorded movements; thus, drug effects on overall motor activity should not confound the measurement of startle, habituation, or PPI in mice. As in other

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species, the time interval between the prepulse and pulse; the prepulse duration; and the prepulse intensity all affect the PPI levels mice exhibit. Murine strains exhibit different levels of basal PPI (Bullock et al., 1997; Paylor and Crawley, 1997; Dulawa and Geyer, 2000; Ralph et al., 2001). Although strains that do not exhibit robust PPI may not be suitable for studies assessing normal PPI or its restoration following PPI disruption (Paylor and Crawley, 1997), most inbred and outbred strains are suitable. Both male and female mice have been used in studies of startle reactivity and PPI, yielding comparable amounts of variability. Because sex differences in drug effects have not been examined systematically, gender should be matched between related studies. Selecting stimulus parameters appropriate for each strain can help ensure that intermediate levels of PPI are assessed, thereby enabling researchers to observe treatment-induced increases or decreases in PPI. For example, lower intensities of acoustic prepulse stimuli are optimal for studies of 129Sv-ter mice, which exhibit relatively high PPI levels, compared with levels appropriate for C57BL/6 mice, which exhibit relatively low PPI levels.

An extensive book, the *Handbook of Mouse Auditory Research: From Behavior to Molecular Biology* (Willott, 2001), is an excellent resource for the interested reader in topics such as audiology, auditory systems, hearing loss in mice, and other characteristics of the acoustic startle response. The findings by Willott et al. (1994, 1995) highlight concerns regarding the potential influence of hearing impairments when assessing levels of PPI. Across several inbred mouse lines there can be a relationship between PPI levels and hearing impairments. Several strains of mice develop high-frequency hearing loss as they mature (i.e., DBA/2 and C57BL/6). When pure tones or high-frequency stimuli are used, the influence of hearing impairments on PPI is most relevant. Hence, one must be cautious when choosing the type of acoustic stimuli for PPI studies. Importantly, the studies listed above used broadband white noise as the acoustic stimuli, and studied mice that were "young" (2–3 months of age). Therefore, their demonstrations of strain dependency are unlikely to be attributable to basal differences in hearing.

The effects of drugs on startle measures may be assessed after administering them systemically, centrally, or orally—either acutely or chronically. The appropriate dosages of many compounds necessary to induce behavioral effects in mice have not been reported; often, a similar behavioral effect will require higher doses in the mouse than in the rat. Less frequently, similar doses will produce equivalent effects. To date, studies reported have been limited regarding the influences of drugs and/or genetic modifications on startle measures in mice; these studies have been summarized previously (Geyer et al., 2002).

Prepulse Inhibition of Startle in Mice

As reviewed elsewhere (Varty et al., 2001; Geyer et al., 2002), many similarities have been reported between the effects of drugs on PPI in rats and mice. For example, both species exhibit reductions in PPI after treatment with one of several drugs: the D_1/D_2 dopamine agonist apomorphine, the indirect dopamine agonist amphetamine, the glutamate antagonist phencyclidine, and various serotonin ($5\text{-}HT_{1A}$) releasers and agonists. Nevertheless, some important differences have been found, such as the effect of the $5\text{-}HT_{1A}$ agonist 8-OH-DPAT to decrease PPI in rats and increase PPI in mice. Furthermore, although the effects of $5\text{-}HT_{1A}$ agonists on PPI are diametrically opposite in mice versus rats, the effects in both species appear to be receptor-specific. In addition, whereas dopamine D_2 receptors appear to mediate the effects of direct dopamine agonists on PPI in rats, dopamine D_1 receptors play a more important role in mice (Ralph-Williams et al., 2002, 2003). Murine strain differences have been observed regarding the effects of some compounds on PPI (Dulawa and Geyer, 1996, 2000; Ralph et al., 2001); these findings suggest the importance of characterizing the effects of a particular drug across several strains before attempting to test its underlying mechanisms of action. The similarities and differences in the pharmacology of PPI in rats and mice raise questions about the extent to which pharmacological studies in rodent models will best predict the drugs' respective effects in humans. Clearly, much more work is needed in this area. Fortunately, the practicality of conducting comparable tests of startle, as well as startle plasticity, across species should help resolve such questions.

In the context of assessing the efficacy of antipsychotic drugs used to treat schizophrenia, it is important to note that several antipsychotic drugs can readily increase PPI in mice, even in the absence of prior disruption of PPI (Olivier et al, 2001; Ouagazzal et al., 2001). In general, drugs that increase PPI in rats are most readily detected when they are used in combination with another manipulation that is known to impair PPI. For example, although data indicate that clozapine can increase PPI in rats when administered alone, most of clozapine's positive effects in rats are obtained when it is administered to animals in which PPI has been impaired. In contrast, in several strains of mice, clozapine and other antipsychotics by themselves clearly can increase PPI. Although it is not certain why rats and mice

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differ with respect to some drug effects, mouse models may be more useful than rat models for detecting the PPI-increasing effects of different drugs.

Concerns About Measurement

It is important to confirm that the startle response is being measured reliably and accurately. Some of the factors contributing to the validity of startle measures of rodents in general were discussed in a previous *Current Protocols in Neuroscience* presentation (Geyer and Swerdlow, 1998); more-specific details regarding the assessment of startle and startle plasticity were provided in a 2003 *Current Protocols* paper by Geyer and Dulawa. With respect to murine startle, a substantial issue is signal-to-noise ratio—that is, the ratio of the measured startle response to the values obtained from motor movements of the mouse in the absence of a stimulus—as recorded from trials in which no stimulus is applied and before substantial habituation has reduced the expected response. For startle measures to be deemed reliable, this ratio should be above 10 and is often above 100. If a low ratio is observed, the measurement system may be inappropriately responsive to slow movements or even body weight, rather than to the rapid dynamic movements associated with the motor response of startle. As in rats, signal-to-noise ratio is important for attaining a high degree of resolution in the measurement of startle, because the primary response exhibits an extremely wide range across different conditions, strains, and manipulations.

A second critical aspect of startle measurement relates to the dynamic nature of the startle response itself. Typically, a brief recording duration of 65 msec is optimal because the mouse startle response is completed within this time window. If the latency to the peak response is more than 35 msec, the measurement system is likely to be inaccurate.

A third aspect of startle measurement involves the characterization of the waveform of the startle response. In addition to providing an important means of validating the response measurement system and defining response latencies, waveform measures enable investigators to identify manipulation-specific alterations in the topography of the startle response. Hence, the startle measurement system should enable researchers to display and analyze individual response waveforms.

Fourth, it is essential that the background noise level to which the animal is exposed during startle testing be controlled, constant, and specifiable. Some ambient noise is always present and influences startle and PPI effects. Thus, it is advisable to provide an explicitly controlled level of background noise to standardize this important factor and to mask uncontrolled extraneous noises.

When initiating startle testing in a laboratory, it is important to confirm that startle and PPI are being measured reliably by testing the parametric effects of varied stimulus conditions in standard strains of mice that have been described in the literature. For example, using young C57BL/6 or 129SvEv mice, the first test of a PPI procedure is meant to confirm that the amount of PPI is clearly related to the intensity of the prepulse. Prepulses of 71–73 dB(A) against a constant background noise level of 70 dB(A) should produce low levels of PPI, on the order of 5–20%. As the prepulse intensity is increased to 76 or 82 dB(A), the amount of PPI should increase in an orderly fashion to 40–70%, depending upon the strain. If weak prepulses produce unexpectedly high levels of PPI, the acoustic delivery system is likely producing excessive onset spikes that are not detectable when sound levels are measured with continuous noise.

Potential Confounds

As discussed above, diminished auditory acuity in mice can lead to decreased levels of PPI (Willott et al., 1994; McCaughran et al., 1999). These effects are particularly problematic if pure tones are used as either prepulses or startle stimuli. The use of broadband noises for both prepulses and pulses largely obviates concerns about high-frequency hearing loss. Nevertheless, concerns regarding deafness must be addressed. Although some reports have examined age-related changes in hearing among multiple murine strains, the effects of genetic manipulations on hearing are often unknown. Caution is recommended when interpreting robust acoustic PPI deficits found in genetically manipulated mice that may have altered sensory systems. One approach is to reassess the PPI deficit using mixes of prepulse and startle stimuli of several modalities (acoustic, visual, tactile) to demonstrate that the effects are not limited to the auditory sphere (e.g., Brody et al., 2004a). Alternatively, the measurement of auditory evoked potentials provides a definitive gauge of auditory acuity.

The robust effects of a manipulation on startle reactivity can confound the interpretation of the effects on PPI. When large changes in startle are observed along with changes in PPI levels, potential dissociations between the two effects should be assessed. For studies in rats, this issue has been addressed extensively (Swerdlow et al., 2000). In brief, expressing PPI in rats as a percentage of the level of startle produced by the startle stimulus alone typically, but not always, corrects for manipulation-induced or strain-

related differences in startle. It is less clear, however, that such ratio transformations are adequate for use in mice, because there is a strong tendency for differences in startle reactivity to be inversely related to differences in percentage of PPI. Hence, other confirmatory approaches need to be examined, some of which were detailed by Swerdlow and colleagues (2000). For example, changes in PPI levels may be observed at some prepulse intensities but not others, suggesting that altered startle magnitude values do not necessitate significant changes in PPI values. Another approach is to use two different startle stimulus intensities in order to engender comparable levels of startle between two groups (e.g., two strains) and then to assess the relative effects of a given prepulse stimulus on PPI in the two groups once startle has been equated (e.g., Brody et al., 2004a).

It is particularly advantageous that startle and PPI are suitable for repeated tests of the same subject in rodents, as in humans. Thus, unless drug or other manipulations have substantial carryover effects, mice can be tested repeatedly. It should be noted, however, that in contrast to rats' stability of PPI across repeated tests, mice tend to exhibit improvements in PPI across repeated tests that are not attributable to changes in startle and do not depend on the age of the animals (Plappert et al., 2006). Progressive improvements in PPI are most prominent with lower prepulse intensities. Hence, across repeated tests, even given at intervals of multiple weeks, the function relating PPI to the prepulse intensity gradually becomes less steep. The processes contributing to these consistent and progressive changes in mice have yet to be elucidated.

Conclusion

The field of neuroscience is characterized by translational studies that are designed to elucidate the mechanisms subserving behavior. In the context of complex psychiatric disorders such as schizophrenia, the use of animal models having cross-species validity is essential to the identification of neurobiological mechanisms. The deficits in PPI and startle habituation seen in patients with schizophrenia provide an opportunity for studying clearly homologous phenomena in animal models. Appropriately designed studies of startle plasticity in mice combine the many advantages of murine models with an unusual degree of cross-species comparability; these features enable the detailed examination of the neurobiological substrates of specific behaviors having clear relevance to schizophrenia and other psychiatric gating disorders.

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