Appropriate End Points for the Characterization of Behavioral Changes in Developmental Toxicology

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The present paper is devoted to second- and higher-tier test methods for the characterization of behavioral changes produced in rodents by exposure to noxious agents during development. The paper analyzes a series of end points that are informative about specific processes and underlying regulatory mechanisms but require greater technical sophistication and larger investments than first-tier end points. This applies to ultrasonic emissions in successive postnatal periods; to mother–pup interactions, including appropriate cross-fostering controls; to social (including sexual) interaction tests from the infantile to the young adult stage; and to a variety of conditioning and learning tests using both positive and negative reinforcement. — Environ Health Perspect 104(Suppl 2):307–315 (1996)

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Introduction

Bignami (*1*) focused on first-tier end points that do not require large resources with respect to logistics and instrumentation. This applies to simple (but quite effective) reproductive success end points, to postnatal indicators of neural and behavioral development (particularly Fox-type scales), to economical tests that can reveal whether the typical developmental pattern of activity/exploration/habituation is affected (including the assessment of responses to selected drug challenges), and to the less burdensome of fostering procedures. This paper is devoted to end points that are informative about specific behavior processes and underlying regulatory mechanisms but require greater technical sophistication and larger investments than the previous ones.

Comparisons between humans and other animals are complicated by limitations in the direct evaluation of subjective states (i.e., unlike humans, animals do not have proactive direct communication of their self-perception). Nonhuman animals have no verbal language to express the subtleties of psychological states (emotional, motivational, cognitive, etc.). As a consequence, animal studies must be sensitive to the ways in which species can communicate their affective states. Studies of animal behavior must identify well-defined descriptive categories, avoiding redundancies and overlap, and monitor frequencies, intensities, sequences, patterns, and trends [see Martin and Bateson (*2*) for a general philosophy of how to score behavior productively]. Abnormalities may emerge by disruptions in sequences or by unpredictable fluctuations in intensity. It is equally critical to measure motivational levels and relate them to behavior; most of the time the intensity of motivation is defined as the latency to perform a given response. For example, maternal separation leading to the search for pups is a strong motivator and is measured by latency to retrieve a pup or by the proportion of pups retrieved within a given time. Within the obvious simplicity of this model, when transposing to the human experience of maternal separation, it is nevertheless useful in operationalizing complex psychological states in nonverbal animals.

Another evaluation issue stems from the fact that most laboratory animals are commonly social species. As a result, intrinsic to their behavioral repertoire is some direction toward a conspecific. From the diadic mother–pup relationship to the adult territorial interactions between males, rodents in particular are characteristically influenced by other conspecifics' signals. Accordingly, adequate measurement of any individual animal's responses must account for the animal's social context, an issue that is often ignored by standard laboratory procedures.

In the case of social and reproductive behaviors, the increasing availability of low-cost, high-performance videotape systems allowing single-frame evaluation makes ethotoxicological analyses the procedure of choice for careful quantitative and qualitative assessment of the behavioral alterations induced by a given treatment. In fact, these videotape systems allow both characterization of subtle behavioral change (e.g., by slow-motion scoring) and, by repeated analysis of the same tape, make it possible to measure behavioral items that were not planned at the beginning of the experiment. Moreover, these systems, supplemented by commercially available software [Observer, Noldus Information Technology b.v., Wageninpen, The Netherlands (*3*); Keybehaviour, Department of Zoology, University of Edinburgh, Edinburgh, Scotland, etc.], eliminate most of the biases due to inter- or intraobserver reliability while facilitating multicentric studies following standardized methodologies, which are compelling issues for regulatory purposes.

Emotional Reactivity

To evaluate the emotional behavior in laboratory animals, it should be possible to measure emotions directly, to classify types of emotions, and indeed, to identify emotions in animals that may have relevance to human emotional states. Recognizing the difficulty in specifying emotions, due to the fact that the complexities of overt behavior

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Abbreviations used: CSs, conditioned stimuli; UCSs, unconditioned stimuli; FR, fixed ratio; FI, fixed interval; VR, variable ratio schedule; VI, variable interval schedule; DRL, differential reinforcement of low rates of responding; DRH, differential reinforcement of high rates of responding; CNS, central nervous system.

must be ascribed to some underlying emotional state, the experimenter nevertheless attempts to classify emotions despite this limitation. Because of the subjective nature of emotional states, animal analogues of such states as anxiety have been difficult to design. Even though several models centering on particular aspects of the emotional behavior in rodents have been developed in recent years, none has been evaluated thoroughly for its efficacy in developmental behavioral toxicity testing. Most of these testing methods have been validated behaviorally and physiologically and appear to be useful for distinguishing anxiogenic and anxiolytic effects within several classes of drugs. A brief description of the most commonly used techniques for the assessment of emotional reactivity in infant, adolescent, and adult rodents is reported in this section.

Assessment of Emotional Reactivity in Infant Animals (Preweanlings)

Ultrasonic Vocalization Test. Rodent pup ultrasonic vocalizations provide a useful model for investigating the ontogeny of emotionality (*4*). This test, which can easily be included in routine developmental behavioral toxicity testing, seems to be more appropriate than traditional methods used for the assessment of emotional reactivity during early postnatal life, such as changes in arousal-locomotory levels or latency time to emergence from a nest box. One advantage of this technique is the possibility of recording the ultrasounds of newborn animals in a standardized experimental setup with minimal handling; moreover, calls represent one of the few response patterns emitted by very young rodents that are amenable to a rigorous quantitative analysis. Ultrasonic calls can be elicited by quantifiable stimuli and are produced with successive modifications of their pattern from birth throughout the lifespan, thus allowing a highly age-specific longitudinal analysis. Finally, ultrasonic vocalization may have more validity in cross-species comparisons than other end points because this response is part of the behavioral repertoire of most species (*5–7*).

The ultrasonic calls of infant rats are whistlelike sounds in the frequency range between 35 and 45 kHz. As these calls are associated with social isolation, they have been variously described as distress vocalizations or isolation calls. The rat pup ultrasonic calls, which are potent stimuli for maternal retrieval and prolactin release, are emitted during the first 2 postnatal weeks, with the rate of calling decreasing when the eyes open around day 14. Changes in temperature levels, odor cues, and tactile stimuli differentially affect neonatal ultrasounds (*8*).

Several findings have shown that ultrasonic calling is a valuable and sensitive indicator of the emotional state of newborn rats. Ultrasonic emission decreases during the acquisition of an operant task (crawling for nipple-suckling reinforcements) and increases during extinction, suggesting that the calls are indicative of stress and arousal (*9*). Moreover, ultrasonic vocalization in rat pups is a reliable test for detecting both anxiogenic and anxiolytic effects of several classes of compounds. Anxiolytic drugs, such as benzodiazepines, selectively reduce calling whereas anxiogenic agents, such as pentylentetrazol and ethylcarboline-3-carboxilate, increase the rate of vocalization (*10*).

The first behavioral teratogenicity experiments using ultrasonic calling have shown only small alterations in the rate and length of vocalizations of rat pups exposed prenatally to two positive control substances, such as vitamin A and methylmercuric chloride (*5,11*). Conversely, more recent findings analyzing time-sequence variables, modulations of intensity and frequency, and the responsiveness to pharmacological challenges have shown marked alterations in animals exposed to behavioral teratogens, such as methylmercury and carbon monoxide (*7,12*). It should be stressed, however, that nonvocal variables must be accurately monitored to determine whether the effects of a treatment are specific for ultrasonic behavior. Changes in both ambient and body temperature, motor activity, coordination (geotaxis), and respiratory rate are important covariants in studies of ultrasonic vocalization.

Ultrasonic calls can be recorded and analyzed by different systems and procedures. Signals can be recorded on tape, and the signal frequency is reduced by slow replay or by a bat detector for further analysis by equipment working in the audible range of humans. Other systems perform an on-line spectral analysis and store the output in digital form in a computer. To perform a microanalysis of ultrasonic vocalization, an on-line computerized system for the real-time recording of frequencies and amplitudes has been recently developed (*7*).

Assessment of Emotional Reactivity in Adolescent and Adult Animals

Open Field Test. One of the most traditional and widely used methods for the assessment of the emotional state in rodents is the open field test (*13*), of which many varieties exist. Computerized open field equipment have been recently developed (Image Motion Analyzer, Videotrack System, Biomedica Manponi, Pisa, Italy). Because this is a relatively simple technique and gives quantitative information on a broad range of responses, it has been used frequently in teratologic studies (*12,14–16*). A flat area bounded by walls is divided into squares, and several activities are scored (number of center and peripheral squares entered per unit time, latency to leave the center area, rearing, grooming, etc.).

In the open field situation, other responses such as defecation and urination can also be measured. Open field activity scores seem to reflect both emotional reactivity and exploratory behavior, whereas defecation primarily reflects emotional reactivity. Even though the results have not always been consistent, an inverse relationship between exploratory activity and the emotional state of the animal has been suggested, and activity has frequently been inversely correlated with defecation levels (*17*). However, according to Norton (*18*), the notion that the open field test can be used to measure general autonomic reactivity, or emotionality, is not substantiated by the evidence. In this regard, for example, different measurements of autonomic reactivity (i.e., cardiac rate and defecation) do not show parallel changes with habituation, and activity in the open field is not correlated with corticosterone levels (*19,20*).

Test Methods Using Conflict between Exploration and Aversion. Some testing methods used for the assessment of the emotional state in rodents are based on the conflict between exploration and aversion, that is, on the capacity of situational aversiveness to reduce or block exploratory responses. These methods include the elevated plus-maze test, the black–white transition test, and the emergence-from-cage test.

The elevated plus-maze apparatus consists of an elevated maze with intersecting arms of which two are open and two are closed. The animal is placed in the center of the maze and has free access to all arms. Entries into open and closed arms and time spent in open and closed arms are scored by incidence. Under nondrug conditions, rodents spend more time in the closed arms than in the open ones. This test has been validated behaviorally and pharmacologically (*21,22*). Anxiogenic compounds, such as pentylentetrazole and FG 7142, further decrease the percentage of entries

into and time spent in the open arms, whereas anxiolytic drugs, such as benzodiazepines, elicit opposite effects. The elevated plus-maze test has been frequently used for the assessment of emotional changes produced in rodents by developmental exposure to psychoactive compounds. Recent results obtained in rats exposed prenatally to a benzodiazepine derivative may be cited as an example (*23*). Adult male rats exposed *in utero* to diazepam spent significantly more time in the open arms than did rats exposed *in utero* to vehicle. The total amount of time spent in either the open or the closed arms, however, was not affected by prenatal drug treatment. Such data could be interpreted as indicating a decrease in the emotional reactivity of animals exposed to diazepam during gestation.

Another technique that is commonly used for the assessment of the emotional state in rodents is the black–white transition test. In this procedure, the number of transitions made by animals between brightly lit and dimly illuminated areas is measured (*24*). Rodents are confronted in this test with a conflict between their tendencies to explore a novel situation and their aversion to bright light. Rats and mice normally spend more time in the area with a low illumination level; the number of transitions into the brightly illuminated area is increased by anxiolytic drugs (i.e., benzodiazepines) at doses that do not modify locomotor activity. Amphetamine induces effects similar to those of benzodiazepines; however, unlike benzodiazepines, amphetamine also increases locomotor activity at dose levels that increase transitions (*25*).

In the emergence-from-cage test, the time required by animals to emerge from their home cage is recorded. The latency to emerge seems to be directly related to emotionality or timidity. Investigations dealing with the effects of prenatal handling on the emotionality of rat offspring have shown that cross-fostered male offspring of handled mothers emerged significantly sooner than controls, indicating that prenatal handling decreases emotional reactivity in male offspring (*26*).

Social Interaction Test. This test exploits the uncertainty and heightened emotionality elicited by placing rats in an unfamiliar or brightly lit environment. The dependent variable is the time that pairs of male rats spend in active social interaction, and both the familiarity and the lighting intensity of the test arena are varied. Specific interaction behaviors are scored: sniffing, following, pushing, jumping, wrestling, and grooming (of each other). This test has been validated behaviorally, physiologically, and pharmacologically (*27–29*). Under nondrug conditions, rats exhibit the highest social interaction when the test arena is familiar and dimly lit; conversely, unfamiliar or brightly lit environments decrease the level of social interaction. Measures indicative of increased emotional reactivity, such as defecation and self-grooming, are associated with the decrease in social interaction. The decline in social interaction induced by a novel environment or by high levels of illumination is prevented by anxiolytic drugs. Due to both the predominance of aggressive attacks in mice and their failure to respond to manipulations of the familiarity of the environment, this test does not seem to be applicable to this species (*30,31*). This procedure has frequently been used in developmental pharmacology and toxicology studies (*23,32,33*).

Assessment of Learning Abilities in Developing and Adult Animals

Numerous test methods are available for the assessment of learning abilities in adolescent and adult animals. However, age-specific tests are also necessary to reveal learning and/or retention deficits when the immaturity of sensory and motor systems does not allow easy screening. Particularly during the late prenatal and the early postnatal phase, the odor-aversion schedule is the most adequate test to evaluate learning capabilities and, more importantly, retention spans (*34–36*), because this test uses conditioned (CSs) and unconditioned stimuli (UCSs) fitting with the ecological requirements of a precocial pup. For example, an ecologically relevant context for suckling fosters associations based upon thermotactile and olfactory cues used for controlling milk consumption. Operant conditioning schedules, active- and passive-avoidance tasks, and mazes have proven to be among the most reliable and sensitive techniques for the assessment of learning changes in developmental toxicity studies.

Operant Conditioning Schedules

Operant conditioning methods typically use animals trained to give a specified response to obtain a reward of food or water. The schedule of reinforcement (i.e., the specific set or sets of response-reinforcement contingencies) determines the overall rate at which the animal responds as well as the response pattern. The rodent's response rate and pattern can be carefully controlled by type, size, and timing of reinforcement and can be brought under exteroceptive stimulus control, which can be quite useful in functional investigations of various sensory systems.

Two basic types of manipulations of schedules of reinforcement (one based on time and the other on frequency of responding) have been described, and the following four main schedules are commonly employed in operant conditioning studies of drug and toxicant effects: *a*) fixed ratio (FR) in which a fixed number of responses must be made before the reinforcement occurs; *b*) fixed interval (FI) in which reinforcement becomes available upon the first response after a specified time interval; *c*) variable-ratio schedule (VR) characterized by the delivery of reinforcements after a randomly varied number of responses with a specified average; and *d*) variable-interval schedule (VI) in which reinforcements become available upon the first response after randomly varied intervals of time with a specified average. These schedules result in characteristic response rates and patterns that can be affected by early treatments of teratologic interest (*37,38*).

In an excellent review of methods in behavioral teratology, Adams (*39*) pointed out that when operant-conditioning schedules are used to assess the influence of specific treatments (i.e., drugs or toxicants), a stable baseline response rate and pattern for each animal are established first and then the chemical is administered. The effects of the treatment are evaluated on the basis of the animal's behavioral change, which represents a sensitive indicator of responsiveness (before–after design). This design cannot be used in developmental toxicity studies because animals are treated during prenatal and/or early postnatal life. However, the before–after design can be used in experiments exploring the influence of developmental treatments on the behavioral responsiveness to drug challenges that can reveal changes in underlying regulatory mechanisms.

Other operant conditioning schedules that appear to be valuable and sensitive tools for the detection of subtle behavioral changes in rodents exposed to noxious agents during development are represented by differential reinforcement of low (DRL) or high (DRH) rates of responding tasks (*40,41*). In the DRL schedule the reinforcement is programmed to occur only if a response is delayed until a specified

period of time has elapsed since the previous response; that is, if the animal responds during this period of time, reinforcement is delayed. This schedule is characterized by low response rate and involves response inhibition. Conversely, high rates of responding are engendered by the DRH schedule in which more than a specified number of responses are required during the inter-reinforcement period. Both DRL and DRH schedules are not particularly more sensitive than other schedules of reinforcement. However, FI schedules appear to be sensitive to a wide variety of toxicants including (among others) metals, pesticides, and solvents.

More relevant information for human situations could be obtained by computer-assisted procedures allowing the simultaneous recording and microanalysis of several behavioral parameters in operant conditioning schedules (*42*).

Avoidance Tasks

In general, the results of tests requiring either activation or suppression of specified motor acts to avoid punishment can be strongly biased by any alteration in neuromotor and other functions, which can result in a confounding of associative effects (i.e., specific changes in learning and memory processes) and nonassociative effects. This is why active/passive ("go – no go") avoidance tests can provide an adequate control on such a bias: for a catalogue of caveats, including motor, sensory, and motivational confounders, see Bignami (*43*) and Bignami et al. (*44*). As concerns active locomotor avoidance, the most frequently used schedules require that an animal reenter the compartment in which it received punishment shortly before, an act that involves considerable stress; therefore, the assessment of genuine learning capability can be hindered by the development of coping responses, such as unconditioned and conditioned freezing, and by the predominance of strong passive avoidance tendencies that act as a brake on active avoidance responding. These phenomena can be attenuated, for example, by appropriate adjusting of intertrial intervals or by reducing shock intensity, which also meets the increasingly rigorous ethical requirements (*44*).

Active-Avoidance Tasks. Active locomotor-avoidance tasks require the animals to run from one compartment of a chamber to another to avoid an aversive stimulus (footshock) generally preceded by a discrete visual or acoustic stimulus. In the one-way avoidance tasks, the running response is unidirectional: animals typically are required to run from one side of the chamber to the other side and then are placed in the start compartment again for the next trial.

In two-way avoidance paradigms, the apparatus consists of a box with two compartments (i.e., a shuttle box) often separated by a hurdle. Unlike the one-way avoidance task in which one compartment always serves as the safe area and the other as the danger area, in the two-way task (*43,44*) the safe and dangerous sides alternate from trial to trial if intertrial responses are punished but not necessarily if intertrial responses are not punished. In the more frequent versions of the task, intertrial intervals are predetermined and the beginning of each trial is signaled by a warning stimulus (tone or noise or light), but operant versions in which each response postpones shock by a specified amount of time (with or without a superimposed warning signal) have also been used with considerable success. All other things being equal, performance in the two-way task progresses markedly slower than it does in the one-way task, and the average asymptotic level is often low with considerable variation between subjects.

Anisman (*45*) suggested that the assessment of rodent performance in both one-way and two-way paradigms can further elucidate the effects of various treatments. In fact, since both one-way and two-way performances are sensitive to associative manipulations, treatments that facilitate learning should improve performance in both tasks, while the opposite should be true for compounds that disrupt learning. Conversely, since two-way performance is influenced by nonassociative effects much more profoundly than one-way performance, differential changes should be observed in the two behaviors after effective treatments whose effects on learning or memory processes are either negligible or overshadowed by other effects, such as the attenuation of shock-induced response suppression (as in the well-known case of the apparently surprising facilitation of two-way avoidance by limbic lesions and muscarinic antagonists).

Adultlike learning of one-way active avoidance in rats is reached by 4 to 5 weeks postnatally (*46*). Two-way active avoidance fully develops at about the same age (*47*).

Passive-Avoidance Tasks. Generally, passive-avoidance tasks—probably the most widely used to evaluate long-term memory in rodents (*48*)—exploit the rodent's preference for darkness (step-through) or their tendency to step down from an elevated platform. In the step-through apparatus, the animal is placed on the lighted side of a two-compartment box and the latency to enter the dark compartment is recorded (approach latency). This is followed by a brief footshock immediately after entering the dark compartment. When the animal is placed again in the lighted compartment, the latency to reenter (avoidance latency) the dark side is measured (one-trial avoidance learning).

Passive-avoidance tasks in which the animal is required to withhold responding in all directions (such as step-down avoidance learning) should be preferred when testing animals as young as 10 days of age (*49,50*) because these tests reduce age differences in locomotor competence and do not necessarily involve the use of visual cues and spatial learning abilities. Moreover, the assessment of passive-avoidance learning in preweaning rodents should always take into account the known age differences in the unconditioned responses elicited by footshock and by exposure to a novel environment (*51*) as well as nonassociative interferences due to changes in locomotor and exploratory activity. On the other hand, the test lends itself to specific inferences on the nature of the effects since the appearance of the passive-avoidance learning capability precedes by several days the appearance of 24 hr retention capability. Furthermore, passive-avoidance learning normally vanishes in mice between postnatal days 15 and 18 when exploratory behaviors show a characteristic pattern of peak hyperactivity. Appropriate control groups for these age differences (yoked and nonreinforced groups, respectively) have been developed for this test (*52*). Effects of administration of several chemicals on passive-avoidance responding have been described, including postnatal and prenatal benzodiazepines (*53–55*) and cholinergic agonists and antagonists (*43,56,57*).

The extent of information is increased when the passive-avoidance task is used together with an active-avoidance paradigm. As already mentioned, comparable effects should be seen in both tasks after treatments affecting associative processes; conversely, differential task effects should be observed when treatment alters nonassociative processes (*45*). Finally, short-term and long-term retention of passive avoidance is not as good in 1-month-old rats as it is in 6- or 12-month-old rats, with a peak later than with active avoidance (*58,59*) that can also be exploited in

fine-grain analyses of the proactive effects of early treatments.

Mazes

Mazes with different shapes and sizes are often used to evaluate learning abilities in both adolescent and adult rodents exposed to noxious treatments during development (*17,39*). Moreover, the comparison of toxicant-induced learning deficits may greatly benefit from the use of different types of mazes (Hebb–Williams, radial, water, etc.).

T- and Y-shaped mazes are the more simple mazes used in appetitively and aversively motivated tasks. These mazes are also used for the differential assessment of discrimination learning abilities requiring the use of various types of cues; for example, the correct arm can be signaled by a discriminative stimulus, such as a light or a pattern of lines, or the discrimination can be on a positional basis.

Acquisition and reversal learning can be evaluated in the Biel water maze, which is characterized by a multiple T pattern with six choice points present in the correct pathway.

The Morris water maze is currently one of the most used tests for evaluating spatial learning deficits. Two groups have reported rather conflicting results about the onset of spatial memory in rats in the Morris maze, so more basic work is needed before exploiting this test to evaluate post-weaning alterations upon exposure to chemicals (*60*).

More complex mazes using food as the reinforcer include the Lashley III maze, the Hebb–Williams maze, and the radial arm maze.

The Lashley III maze is a rectangular chamber consisting of four parallel alleys, a start box, and a goal box (start and goal boxes are located on opposite external walls). The correct path from the start box to the goal box is characterized by a typical pattern through doorways in each of the walls of the four interior alleys. The ends of the four alleyways form eight cul-de-sacs.

The Hebb–Williams maze consists of a rectangular field with the start box and the goal box located on diagonally opposite ends of the apparatus. Different maze configurations (12 maze problems differing in complexity) can be obtained by placing barriers at different points of the field.

The radial arm maze consists of eight arms radiating from a central area. Access into an area is monitored, and animals obtain a food reward on the first entry into each arm. Subsequent entries into the same arm are errors and are not reinforced. Accuracy of selecting arms and activity (number of times each arm is entered) are obtained. This task requires that the rat use spatial cues and it also can be a test of recent versus reference (previous) memory. Spatial learning and memory in this test have been related to hippocampal function.

However, maze-type tests have their pitfalls. For example, working memory cannot be appropriately assessed in arm-baited mazes when they lack a central box in which the animal is confined at the beginning of each trial, since in this case what they exhibit is a range of individual strategies (clockwise or counterclockwise arm inspection, etc.) and the resulting score remains difficult to interpret. Dissociation in the use of olfactory and spatial cues, as well as strain-dependent locomotor biases, need also to be taken into account.

Sociosexual Interactions

Maternal Behavior and Effects of Fostering

The developmental effects of toxicants are sometimes misinterpreted by attributing them to direct and specific damage to the developing nervous system, when in fact they may depend, at least in part, on alteration in mother–pup dyadic relationships. An adequate analysis of maternal care (considering basic characteristics such as licking, crouching, nest building, retrieving, time budgeting of in/out nest periods) is therefore imperative (*61–63*). Pup responding [e.g., ultrasound emissions eliciting maternal licking (*64*)] should also be considered since impaired reactivity to maternal cues can be responsible for maturational deficits, which amplify direct toxicant effects. The cross-fostering procedure is commonly used, allowing a gross separation of direct effects on the pups from those mediated by changes in the mother (*65,66*). However, fostering per se may also play a detrimental role in maturation, as shown by studies comparing between-treatment (cross-fostering) to within-treatment (in-fostering) effects on mice receiving prenatal benzodiazepine treatment (*67*). Particularly in the case of delays in behavioral maturation occurring in altricial neonates [see Bignami (*1*) for the assessment of sensory–motor ontogeny in the early postnatal phase], it is necessary to exclude confounding due to procedural biases by identifying deficit components that can be ascribed to alteration in specific items of mother–pup growth regulation.

Developmental Changes in Sociosexual Patterns

The differences in aggressive behavior between the mouse and the rat species are particularly marked during ontogeny, with rats exhibiting a higher level and a wider spectrum of playful interactions than young mice (*68*). In rats, rough-and-tumble (including pinning) or crossover solicitation are good indicators of aggressive-like interactions. Rat play fighting is described by Meaney and Stewart (*69*), mouse play by Poole and Fish (*68*) and by Takahashi and Lore (*70*). Hood (*71*) provided an accurate description of the development of female aggressive behavior in rats.

Mouse locomotor-rotational and social play was initially described for wild subjects in physically complex environments (*72,73*). A more exhaustive description of play in laboratory strains appeared later in the literature (*74*). Very recently, Terranova et al. (*75*) characterized a complete mouse ethogram aimed at evaluating both normal maturation profiles and long-lasting effects upon developmental drug exposure (*76*). The effects of social isolation are highly age dependent in both species (*77–79*). The onset of sexual behavior depends on various factors, including social cues (*80–82*).

Adult Aggressive and Sexual Patterns

Intraspecific aggression can be evaluated using either fighting pairs or differently sized social groups, the latter reproducing a more natural social setting (*83,84*). Groups can be unisexed, but more often they include both genders (they are referred to as population cages) and can be maintained either in laboratory cages, arenas, or enclosures up to 4 m^2.

Placing a conspecific intruder into an established social setting is an easy way to produce territorial social behavior. The intruder is often selected according to its physiological/social condition to reduce the variability of responses (*85*). Subjects without previous sexual experience or belonging to socially stable groups are preferred. However, Dixon and Mackintosh (*86*) reported that young mice (4–6 weeks of age) barely induce aggressive behavior in adult conspecifics. Moreover, mice older than 10 weeks of age are often involved in social competition, and their status varies accordingly (*87*). It is essential to examine carefully the social role played by the intruder or the mate in its original social setting during the period immediately before its introduction into a new social group.

When evaluating social responses, a sound strategy is the use of intruders, mates, or standard opponents whose social history is known since birth, particularly during the preweaning period (*88*). Castrated subjects (*89*) or anosmic, and consequently less aggressive, opponents were fashionable in the past 2 decades [anosmia is produced by intranasal zinc sulfate irrigation or through bilateral removal of olfactory bulbs (*89,90*)]. Frischknecht and colleagues (*91*) preferred the use of an opponent from a genetically nonfighting strain [see Alleva (*92*) for interstrain differences].

The most widely used index of aggressive tendency is the Attack category, described by Grant and Mackintosh (*93*). Such a description is interchangeably valid for both mice and rats (*83,94–98*). Attacks are measured in terms of frequency, duration, latency time to first appearance, or a total time spent in attacking. Brain et al. (*85*) proposed intensity scales, ranging from rapid biting with short physical contact to deep biting with hemorrhage.

Other methodological studies have considered which parts of the opponent's body were targeted. In rats, wounds are mainly located on the head, back, and flanks (*99*), while life-threatening bites are directed at the ventral parts. Lactating mice tend to bite the head and the ventral region of the intruder. A detailed analysis of "wound maps" provides useful indications about the offensive as well as defensive attitudes of the confronting animal. In close association with the attacks are the offensive postures, either upright or lateral (*93*), which usually last for a few seconds with the two animals pushing each other with their forepaws (*94*). Dixon et al. (*100*) suggest that these postures are good indices of an ambivalent offensive tendency. Postures and social acts of four laboratory species (rat, mouse, guinea pig, and golden hamster) are compared by Grant and Mackintosh (*93*).

Sexual behavior can be assessed according to well-characterized scores, such as male mounts, penis intromissions, pelvic thrustings, ejaculations, and postejaculatory refractory periods following presentation of a receptive female in a mating arena. Lordosis is the usual score for females. Since sexual patterns are interchangeable, an adequate battery should include both male-type and female-type scores (e.g., male feminization after exposure to chemicals may be measured by the amount of lordosis). The development of external genitalia appears late in development, and usually at birth behavioral patterns are determined. However, which pattern becomes dominant greatly depends on hormonal secretion during critical prenatal and perinatal stages, which is also a function of intrauterine position and amount of maternal stress. Administration of chemicals may retard the normal appearance of sexual patterns: for example, in male rats prenatal alcohol exposure delays testosterone synthesis and release, markedly affecting sexual behavior (*101,102*).

Sociosexual roles can be easily assessed. Dominance is characterized by emission of aversion-inducing olfactory cues in the urine, which are different from those produced by subordinates (*103*) and release a female-attracting odor (*104*). The preputial gland of the male mouse is a known source of olfactory signals indicating social dominance (*105*). The acquisition of a social role (rank) is also reflected by changes in neuroendocrine status, particularly evident in the enlargement of the adrenal gland in subordinates (*106*).

Mice tend to arrange their social settings in hierarchies (*88*). Poole and Morgan (*87*) showed that, in laboratory cages, the stability of the hierarchical order depends on the number of caged subjects and that the social situation of groups of 9 to 12 individuals is highly unstable. Hierarchical roles were not found in mice belonging to the same litter (*87*), i.e., in the case of subjects with high familiarity during critical stages of behavioral development. Mice maintained in 1.8×1.8 m enclosures show pronounced territorial behaviors, with only a few adult males defending the borders of their own territories (*107*) and showing marked habituation to social stimuli (*108,109*). For naturalistic and testing conditions concerning the mouse species, see Alleva (*92*).

Usually, the intruder is attacked by the dominant animal while it attempts to escape or displays species-specific submissive postures aimed at inhibiting the attacking counterpart. The home cage effect characterizes the peculiar pattern of aggressive behavior displayed by the resident (*88,110,111*).

In rats, dominance hierarchies (threatening postures and biting attacks) do not appear before day 160 and depend on cage size. Grant (*112*) provided an ethological description of male rat social and agonistic behavior, which includes sequence and pathway analysis, displacement and ambivalence activities, and features of sociosexual behaviors. An updated version is in Miczek and Krsiak (*113*). Play fighting and actual aggressive behavior are often difficult to distinguish in this species (*70,114,115*). Unlike mice, the intruder rat often does not elicit increased fighting among colony members (*116,117*). In the case of colonies composed of individuals younger than 150 days of age, all males participate with the same role in the attack directed at the intruder (*115*).

During the course of agonistic interactions, male rats emit 22 to 48 kHz ultrasonic vocalizations (*8*). However, Takeuchi and Kawashima (*118*) found that rat ultrasonic signals do not inhibit the initiation of aggressive behavior and therefore dismissed their intraspecific communicative value.

Maternal aggression is a widely used methodology because the lactation period is associated with heightened levels of female aggressive behavior, barely observed in nonbreeding female rodents (*71,119*). Flannelly and Flannelly (*120*) analyzed the role of opponent's size in eliciting maternal aggression, while Svare et al. (*121*) characterized some situational and experiential determinants. Litter size influences maternal aggression (*122*). Aggressive behavior also increases in female rodents between week 2 of pregnancy and parturition (prepartum aggression) (*123*).

Treatment Effects on Social and Agonistic Behavior

Dixon and co-workers (*100,124,125*) provided exhaustive guidelines for an appropriate analysis of the effects of psychoactive drugs on rodent social and aggressive behavior. Earlier analyses are also valuable and indicate the different methods used in the past by psychopharmacologists (cerebral lesions, painful stimulation, selected hormonal or pharmacologic treatment, muricidal rats, or locusticidal mice) (*126–128*). Most of these tests, as well as automated devices recording audible vocalizations or producing aggressive reactions by repeated footshocks in five-rat batteries (*129*), are presently regarded as poorly complying with animal psychological welfare and, above all, of very little value in understanding treatment-dependent alterations in agonistic interactions (*100,130,131*). A catalogue of drug-induced modifications in rodent social and agonistic behaviors is reported by Miczek and Krsiak (*113*) and in Miczek et al. (*132*).

Natural Population of Rodents as Sentinels

Rodents are proverbial pests for humanity, gaining notoriety during the time of the Black Death and even before. They represent a commensal-type of parasite, living on

agricultural products such as harvested seeds, etc. Commensal species living on by-products are adapted to expand their population when food is available and to contract it when unavailable. Accordingly, they are highly prolific and attractive for laboratory breeding and investigation. Indeed, innumerable reagents have been devised solely from the products of laboratory investigations of rodents, and the neurobiology of the central nervous system (CNS) reflects to a considerable extent a dependency on these investigations.

We have historically treated rodents outside of the laboratory as pests to be controlled [the World Health Organization promotes guidelines for trapping them (*133*)]. Yet, with our laboratory-derived knowledge of rodents, it seems that we would all be well-served from studies of these natural populations by monitoring, e.g., CNS alterations caused by exposure to chemicals dispersed in the environment. Such an ecotoxicological approach using trapped rodents offers a very profitable direction for future research.

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