ARTICLE
A Rat Model of Fetal Alcohol Syndrome: A Series of Undergraduate Laboratory Exercises for Biopsychology Courses

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Many undergraduate students are aware that consuming alcohol during pregnancy can result in many serious physical and behavioral deficits. Student interest in this clinical syndrome allows instructors to provide engaging laboratory exercises that relate topics covered in most biopsychology courses to Fetal Alcohol Syndrome (FAS). Through this series of experiments, students will use rodents to study the behavioral deficits that can be caused by developmental alcohol exposure, including impaired ultrasonic vocalizations, hyperactivity, balance, and spatial learning. Other possible exercises include analyzing blood alcohol concentrations (BACs), completing histological studies of anatomical effects, and/or discussing the societal implications of developmental alcohol exposure. The instructor has the flexibility to determine which of the exercises fit into the class schedule and budget since he or she may choose to complete all of the behavioral tests or only one of them. Students will also learn about the benefits and drawbacks of animal models for human disorders, important considerations in research design such as reliability and validity, while also gaining experience in statistical analyses and writing empirical research papers. The application of these important concepts to a human syndrome and the use of small, easy-to-handle rodent pups make these exercises an accessible, stimulating introduction to animal research for most undergraduate students.

Key words: animal research; fetal alcohol spectrum disorders (FASD); blood alcohol concentration (BAC); ultrasonic vocalizations (USV); open field; balance; Morris water maze

Preparing engaging laboratory exercises that span all of the concepts covered in an introductory biopsychology course can be difficult and time-consuming. This series of exercises can be tailored to fit most lab courses in biopsychology, physiological psychology, or neurobiology, while providing experience with an animal model that most undergraduate students find both relevant and approachable.

Using a rat model of Fetal Alcohol Syndrome (FAS), students can learn about the usefulness, and limitations, of animal models for human disorders and diseases. Understanding animal models is critical to most areas of neuroscience, but not an area of research that is intuitive or appreciated by many undergraduate students (Metzger, 2014). These exercises introduce students to animal research by using rat pups, which tend to be easier to handle and less intimidating than adult rats. In some classes, the students may be responsible for learning basic animal care by providing food and water daily to the rats, breeding, culling and/or weaning pups. For all students, these exercises can provide an opportunity to discuss the importance and the ethics of animal research, especially with rodents.

These lab exercises also provide instructors an opportunity to provide hands-on experience with many of the concepts typically covered in an introductory biopsychology course including neuroanatomy, neurodevelopment, motor behaviors, learning and memory, language, and social interactions. Students can also observe considerable developmental behavioral changes over the 15 weeks of a semester-long course.

Furthermore, students can gain experience with experimental design. Instructors should have students consider the value of using both injected and suckle control groups in these studies. Topics such as inter-rater reliability, the need for operational definitions, and standard operating procedures will become obvious to the students as they conduct these experiments. Some instructors may choose to allow students to write and submit the required institutional animal care and use committee (IACUC) protocol in order to further discuss ethical animal research. Finally, for some of the behaviors, one-way ANOVA tests should achieve statistical significance with a few litters of animals, although even non-significant findings are a useful way to introduce statistical power to students.

FEASIBILITY

Any institution with a rodent facility should be able to perform these experiments. Instructors may choose to do any behavioral experiments that they think would be beneficial for their class. Thus, there is considerable flexibility for performing the behavioral experiments; however, the breeding aspect of the experiment will take some advanced preparation. It is recommended that the instructor start the breeding process prior to the beginning of the semester to ensure that the students can begin experiments near the beginning of the semester.

Once students are trained on the intraperitoneal injection technique (see below), most should be able to perform these injections without supervision from the instructor. Other instructors may choose to perform the injections themselves to ensure reliability and/or to complete the alcohol exposure before the beginning of the semester, which would allow the students to start behavioral tests immediately.

Beyond the intraperitoneal injections, which will occur daily during the first postnatal week, most of the behavioral experiments could be completed within a standard three-hour laboratory period. However, depending on the number of animals and the number of students, behavioral and statistical analyses will often need to be done separately, either outside of scheduled class time or during the laboratory period the following week.

LEARNING OBJECTIVES

Following completion of this series of laboratory exercises, the student should be able to:

1) explain the importance of animal research, including the benefits as well as the limitations.
2) define and appropriately apply experimental design vocabulary words such as reliability, validity, statistical power.
3) summarize the effects of alcohol on the developing brain.
4) hypothesize behavioral outcomes in alcohol-exposed rat pups based on previous literature.
5) design protocols that are double-blind, use operational definitions, and exhibit high inter-rater reliability.
6) apply statistical knowledge to behavioral data analyses.
7) graph results using appropriate software.
8) write a full research paper summarizing the semester's results and conclusions.

FETAL ALCOHOL SYNDROME

Although many women abstain from alcohol completely during pregnancy, over 7% of pregnant women in the United States self-report drinking alcohol and 1.4% report binge drinking during pregnancy (CDC, 2012). Fetal Alcohol Syndrome (FAS) describes those who have permanent birth defects and often life-long behavioral and psychological deficits due to the maternal consumption of alcohol during pregnancy (for review, see Mattson et al., 2011; Dorrie et al., 2014; Senturias, 2014). The precise prevalence of FAS is difficult to identify for many reasons, but has been estimated to occur in 1 to 2 of every 1000 live births in the United States, while less severe Fetal Alcohol Spectrum Disorders (FASD) occur more often, up to 48 per 1000 live births (Riley et al., 2011; May et al., 2014).

To gain a better understanding of the teratogenic effects of alcohol, researchers often employ the use of animal models (for review, see Patten et al., 2014). The most common models are rodents, particularly mice and rats. In rodents, the first two weeks of life after birth are a period of rapid central nervous system growth and proliferation that is comparable to the third trimester of pregnancy in humans (Clancy et al., 2007). The third trimester of human pregnancy marks a critical time, because mothers who cease drinking alcohol prior to the third trimester have improved infant outcomes (Rossett and Weiner, 1982). With the knowledge of the correlation between the first two postnatal weeks in rats and the third trimester in humans, most rat models involve administering alcohol during this window of time to best mimic human consumption effects. Specifically, alcohol generally has the most severe effects in rats if administered early during those first two postnatal weeks (Hamre and West, 1993).

After alcohol has been administered to rat pups, a variety of experiments can be done to study the effects of alcohol on behaviors associated with FASD. For example, most children with FAS have problems with social communication (Kelly et al., 2000), are hyperactive (Fryer et al., 2007), exhibit some balance problems (Roebuck et al., 1998), and have spatial learning deficits (Uecker and Nadal, 1998). Rat models of FAS have related deficits in ultrasonic vocalizations (USVs), increased locomotion, balance beam traversal, and Morris water maze (MWM) performance as outlined below.

Currently, the majority of rat models used to model the effects of fetal alcohol exposure have administered alcohol via oral intubation. However, this method requires considerable practice to perform correctly in order to minimize stress to the pups. An easier method for undergraduates to learn and perform effectively is intraperitoneal (i.p.) injection. Research in mice has shown similar blood alcohol content (BAC) and neuronal loss following i.p. injection as is observed following oral intubation of ethanol (Bonthius et al., 2002).

PROCEDURES

Advanced Planning and Considerations

As mentioned above, some preparations need to be completed prior to the beginning of the semester. It is recommended that the instructor start breeding the rats prior to the start of the semester, especially if students will only be performing behavioral experiments. In addition, the instructor may want to consider using the first laboratory period as a way to introduce animal testing to students. Some recommendations for this laboratory period include explaining the importance of animal testing to students, debunking any myths students may have heard about animal research, and showing students how to properly handle rodents. This should help make laboratory periods devoted to behavioral testing run more smoothly.

Due to the number of students who will be involved in the behavioral testing part of the experiment, the instructor should take into consideration how to properly interpret the data gathered at the end of the semester. This would be a good opportunity for the instructor to have a discussion with students about interpretation of data, inter-rater reliability, internal validity, etc.

Animals

Rats can be bred on campus, or pregnant females can be ordered for delivery a few days before birth. Our lab uses Sprague-Dawley rats from Envigo (Indianapolis, IN). Food and water should be available *ad libitum* throughout all experiments.

The day of birth is considered postnatal day (PD) 0. On PD 1, litters should be culled to 10 pups, with 5 males and 5 females when possible. The animals should be permanently identified, usually by ear punch (Dickie, 1968). Generally, each pup is assigned a number 1-10 corresponding to their treatment, with males being odd numbered and females

being even numbered. Rat pups should remain in standard plastic cages with the dams until weaning on PD 21. Any time the pups need to be removed from the dam (e.g., during injections), the pups should be kept in a plastic cage with bedding, and the cage should be placed on top of a heating pad. Even though these periods away from the dam are brief (less than 30 minutes at a time), the heating pad ensures the pups maintain a normal body temperature.

Intraperitoneal Alcohol Injection

Rat pups should be randomly assigned to one of three treatment groups on PD 1. On PD 1-7, two males and two females from each litter should receive 4.5 g/kg of ethanol via intraperitoneal (i.p.) injection. Four pups receive phosphate buffered saline to serve as injected controls (IC). The remaining two "suckle control" (SC) pups receive no treatment, but should be handled as if they were receiving an injection.

Injections should use 30 gauge, ½ inch needles with 1mL syringes. These i.p. injections should be performed with the needle entering laterally (from the pup's left side), rather than entering caudally as is done in adult rats. Both solutions should be warmed to approximately 37˚C prior to injections. Over the course of these injections, the control groups should continue to gain weight rapidly, while the alcohol exposed pups should weigh significantly less than the control groups (Fig. 1). These data should produce statistically significant findings with small numbers of rat pups, especially if they are allowed to develop at least until weaning (PD 21).

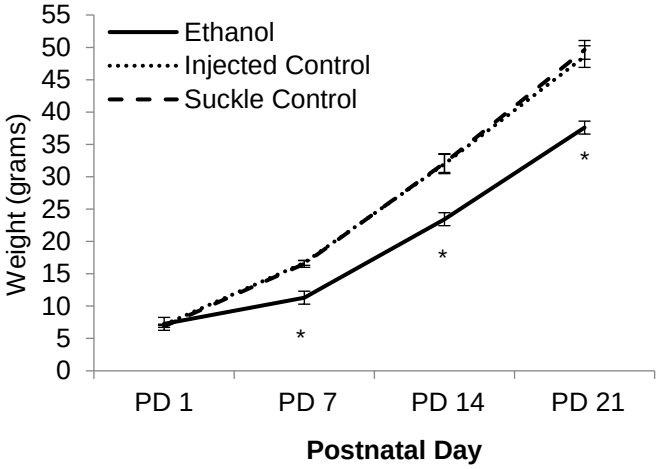

Figure 1. Mean weight (±SEM) in rat pups over the first three postnatal weeks. The ethanol-exposed rats (n = 17) weighed significantly less than both the injected control (n = 20) and suckle control (n = 15) by PD 7, *p < 0.05.

Blood Alcohol Concentration Analysis

Tail blood samples may be collected on PD 7 to be frozen for later BAC analysis. The technique has been described elsewhere (NIH, 2012). Briefly, 2 millimeters of the pup's tail is cut with a sharp scalpel blade. Approximately 20 microliters of blood are collected in heparinized capillary tubes. The samples should then be transferred to Eppendorf tubes to be frozen for later analysis. The BAC analyses can be completed with a spectrophotometer using a reagent and protocol available from Sigma-Aldrich (N7160).

When the class is discussing neurotransmitters, and how drugs affect neurotransmitter signaling, instructors can use that week's laboratory period to analyze BACs from the rat pups. Alcohol acts as both a glutamate antagonist and GABA agonist, which may underlie some of the most damaging neurological effects (Ikonomidou et al., 2001). Discussion may also include how the method of alcohol administration affects BACs, as students compare the i.p. injection in rats to the ingestion method humans use to administer alcohol.

The i.p. method of alcohol administration described above mimics a binge pattern of alcohol consumption by human mothers and should result in relatively high blood alcohol concentrations. A previous study that used the same protocol to expose PD 4-9 mouse pups to a similar dose of alcohol resulted in average peak BACs around 375 mg/dL (Bonthius et al., 2002). These BACs are higher than achieved by most casual drinkers, however, the high levels are useful here for two reasons. Most importantly, women who have developed a tolerance to alcohol regularly reach BACs well beyond the legal limit of 80 mg/dL, including some reports of estimated BACs over 450 mg/dL (Handmaker et al., 1999). From a more practical standpoint, these high BACs cause greater behavioral changes than lower doses, which increases statistical power, thus keeping the number of animals required to lower numbers.

Isolation-Induced Ultrasonic Vocalizations

Rat pups use USVs to communicate their location to the dam if they are separated from the litter. When pups are removed from their litter and dam, prior to the opening of their eyes, the pups produce isolation calls in the form of 40 kHz USVs that trigger seeking behaviors from the dam. Instructors may want to use the study of the effects of developmental alcohol exposure on this form of communication as an introduction to the benefits and complexities of human language compared to other species' forms of communication. Other chapter topics in which this USV task might fit into the discussion would be in the regulation of body temperature (Allin and Banks, 1971) and/or to the need for communication in the formation of social groups (Hofer and Shair, 1978).

This behavioral test requires an ultrasonic detector set to 40 kHz. We recorded USVs on cassette tapes (Fig. 2) although a digital recorder would be preferable for data analysis and storage, because if one is available, students can easily record these vocalizations for later analysis using freely available software such as Audacity (audacity.sourceforge.net). Testing should be done by PD 16 to ensure that the pups have not yet opened their eyes. Pups exposed to developmental alcohol produce significantly fewer USVs than control pups and show a longer latency to the first vocalization (Rubin et al., 2009). Each pup should be individually transported to the testing site. USVs should be recorded for at least 5 minutes. Audiotapes can be analyzed for the number of USVs emitted, although the latency to first USV (Fig. 3) is easier for undergraduates to detect without requiring much training.

These analyses provide excellent opportunities for discussion of double-blind scoring and inter-rater reliability.

Figure 2. USV testing chamber. The ultrasonic bat detector is set up directly above a small enclosed area in which the pup should be placed, to ensure the pup stays directly under the detector. A heating pad should be placed under the cage to ensure that the pups will not become hypothermic while separated from the dam and litter.

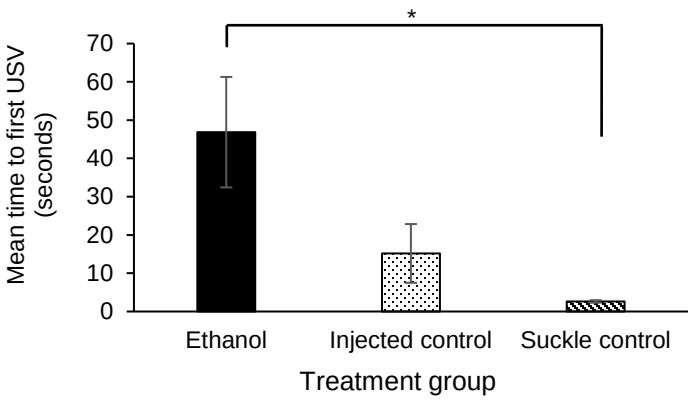

Figure 3. Mean (±SEM) latency to first USV on PD 14. Alcohol-exposed rats (n = 17) were significantly delayed in producing their first USV, compared to the suckle control rats (n = 12), *p < 0.05, but not the injected control rats (n = 18), p > 0.05.

Locomotor Activity in an Open Field

Developmental alcohol exposure also causes hyperactivity in rats (Melcer et al., 1994). Instructors can link this open field activity task with their discussion of motor behaviors.

Typically, activity levels are recorded around the age of weaning (PD 21). Locomotor activity can be observed in an open field arena, preferably a circular arena so that anxious animals do not simply stay in one corner of the arena (Walsh and Cummins, 1976). If an automated system is not available, the arena should be divided into a number of even spaces so that students can record the number of line crosses completed by each animal (Fig. 4). If possible, a video camera should be positioned directly over the open field arena. All students can then review the videotapes, after operationally defining a "line cross" and allowing for discussion of the need for inter-rater reliability.

Two weeks after the last alcohol injection, the long-lasting effects of alcohol result in significantly higher activity levels (Fig. 5). Although the data may not reach statistical significance with only a few litters of pups, it is usually obvious to students which animals were in the alcohol-exposed group. Instructors can use this as another opportunity to stress the importance of double-blind scoring.

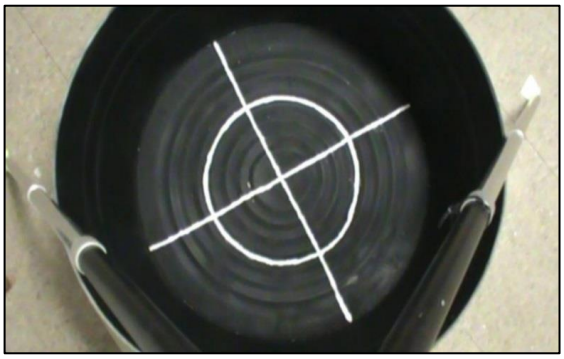

Figure 4. Open field arena. Students should count the number of times a rat crosses one of these lines. A video camera on a tripod over the arena allows for later data analysis by the entire class.

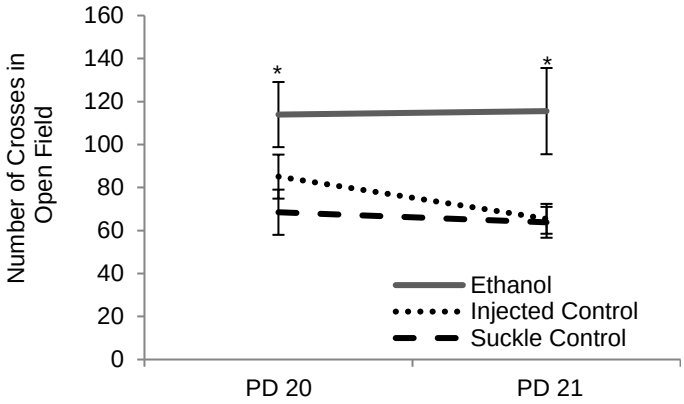

Figure 5. Mean (±SEM) activity levels in the open field task. The ethanol-exposed rats (n = 14) performed significantly more line crosses (indicating increased activity) than the injected control (n = 19) and suckle control rats (n = 14) on both days of testing, *p < 0.05.

Balance

The discussion of motor systems in a Biopsychology course allows instructors to tie together all of the sensory systems while introducing other areas of the brain such as the basal ganglia and the cerebellum. The cerebellum is particularly affected by developmental alcohol exposure in both rats (Goodlett et al., 1991) and humans (Norman et al., 2009).

These effects on the cerebellum cause behavioral deficits that are well-documented following developmental alcohol exposure in rats, including impaired ability to balance on either a rotating rod (Goodlett et al., 1991) or a balance beam (Lewis et al., 2007). Motor deficits, including impaired balance, also occur in children diagnosed with FAS (Lucas et al., 2014).

If your university has access to a rotarod, the appropriate age for testing will depend on the size of the rod, however at most ages the alcohol exposed rats should walk on the rod for a shorter period of time than the control rats. If an automated rotarod is not available, a single balance beam apparatus can easily be constructed with a thin dowel rod.

Previous tests have shown alcohol-induced balance deficits at PD 31 on such a balance beam (Lewis et al., 2007). We tested on PD 29 by placing pups 10 cm from an enclosed platform on a 9.5 mm diameter metal dowel rod (Fig. 6). All rats completed four trials, moving 10 cm farther from the platform after each successful traversal. The alcohol-exposed pups traveled significantly shorter distances than the suckle control pups, $p < 0.05$ (Fig. 7).

Figure 6. Balance beam. The beam is marked by 10 cm segments from the enclosed platform.

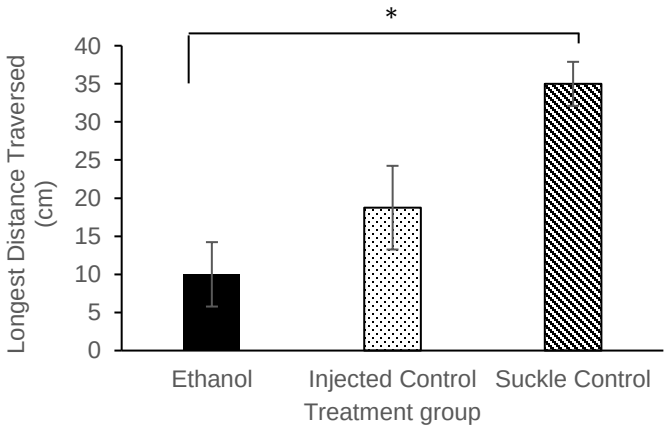

Figure 7. Mean (±SEM) longest distance traveled on the balance beam. The ethanol-exposed pups (n = 8) sucessfully traveled significantly less distance than suckle control rats (n = 4), *$p < 0.05$, but not the injected control rats (n = 8), $p > 0.05$.

Morris Water Maze

Most biopsychology labs have access to a MWM, allowing students to actually observe a classic learning and memory behavioral test they have read about in textbooks. Chase and Barney (2009) provide suggested methods and tips for using the MWM in an undergraduate lab. Some research examining spatial learning and memory following developmental alcohol exposure has shown spatial learning impairments in rats (Tomlinson et al., 1998; Johnson and Goodlett, 2002). One study even found place learning impairments in children with FAS who completed a virtual MWM task (Hamilton et al., 2003).

Students seem to find this behavioral test to be both intuitive and straight-forward to conduct, albeit more time-consuming than the previous tasks. Most MWM experiments studying developmental alcohol exposure use adult rats that are at least PD 70 (Johnson and Goodlett, 2002). Due to the time constraints of a semester, our pups were trained in a 1.5 m diameter MWM on PD 36 and tested for retention on PD 43, which showed significant alcohol-induced impairment compared to suckle control pups, $p < 0.05$ (Fig. 8).

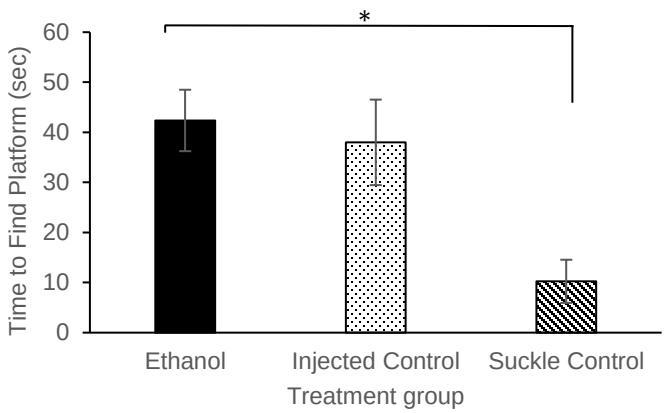

Figure 8. Mean (±SEM) time to find the platform in the Morris Water Maze. The ethanol-exposed rats (n = 8) had a significantly longer latency to find the platform on the first retention trial than the suckle control rats (n = 4), *$p < 0.05$, but not the injected control rats (n = 8), $p > 0.05$.

Additional Exercises

Due to the numerous effects of developmental alcohol exposure throughout the brain, there are other behavioral tasks that might be included in some Biopsychology laboratory courses. Visuospatial tasks have been shown to be impaired both in rats (Thomas et al., 2000) and in humans (Mattson et al., 2011) following developmental alcohol exposure. Executive functioning is disrupted, as measured by perseveration errors, in alcohol-exposed rats (Hamilton et al., 2014) and humans (Mattson et al., 2011). Finally, eyeblink conditioning is a classical conditioning task that is impaired in both rats (Green et al., 2006) and humans (Coffin et al., 2005).

Other instructors may choose to follow-up these behavioral exercises with a section on histology. Students may find significant differences in brain weights across the three treatment groups (Bonthius et al., 2002), but it is unlikely that more specific anatomical differences will be obvious without stereological analyses. Still, the introduction of histological techniques and hands-on practice with neuroanatomy, without the use of additional animals, may be a useful addition for some courses.

Finally, some instructors may choose to discuss the public health and sociological implications of these studies. Bhuvaneswar et al. (2007) review some of the concerns and practical tips for physicians working with pregnant women who drink alcohol. The National Organization on Fetal Alcohol Syndrome (www.nofas.org) provides a large number of resources that undergraduate students could use to consider how they will interact with children affected by FAS in their future careers in education, law enforcement, social work, counseling, etc.

CONCLUSION

In summary, these exercises provide considerable flexibility for the instructor to choose as many, or as few, of these

exercises to complete with their class, depending on resources and time available to them. Even those instructors who choose to complete all of the exercises will only need a few litters of animals during the course of the entire semester, thereby reducing the costs of purchasing new animals for multiple lab exercises. Furthermore, housing costs for ten rat pups and the dam in one cage are less expensive for the first three weeks of life than the adult male rats traditionally used in biopsychology lab courses.

Students find these exercises appealing for a number of reasons. First, the connection to a syndrome that affects a number of human children, including many that will be future students or clients of the education and counseling majors in the class, is a useful way to draw students into animal research. Furthermore, the rat pups are very easy to handle and few students are intimidated by them, as can occur with large adult male rats. In one semester's class, 100% of students (n = 11) agreed that "*the rodent behavioral testing experience was an integral part of this college course,"* that they "*better understand the ethical responsibilities required when conducting animal research,"* and that they had "*an increased understanding of the importance of animal research"* (survey questions modified from Gallup and Beckstead, 1988 and Moely and Ilustre, 2014).

Finally, of importance to both educators and students, these exercises allow many biopsychology lecture topics to be applied to behavioral tests in the lab. Therefore, students can make connections more easily between the separate sessions and better understand the usefulness of having laboratory experiences tied to a biopsychology course.

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