Video Article

Neurodevelopmental Reflex Testing in Neonatal Rat Pups

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Abstract

Neurodevelopmental reflex testing is commonly used in clinical practice to assess the maturation of the nervous system. Neurodevelopmental reflexes are also referred to as primitive reflexes. They are sensitive and consistent with later outcomes. Abnormal reflexes are described as an absence, persistence, reappearance, or latency of reflexes, which are predictive indices of infants that are at high risk for neurodevelopmental disorders. Animal models of neurodevelopmental disabilities, such as cerebral palsy, often display aberrant developmental reflexes, as would be observed in human infants. The techniques described assess a variety of neurodevelopmental reflexes in neonatal rats. Neurodevelopmental reflex testing offers the investigator a testing method that is not otherwise available in such young animals. The methodology presented here aims to assist investigators in examining developmental milestones in neonatal rats as a method of detecting early-onset brain injury and/or determining the effectiveness of therapeutic interventions. The methodology presented here aims to provide a general guideline for investigators.

Video Link

The video component of this article can be found at https://www.jove.com/video/55261/

Introduction

Neurodevelopmental reflexes, or developmental milestones, are one of the earliest assessments used on human newborns and infants. Neurological reflexes are involuntary and repetitive movements that demonstrate brain stem and spinal cord reflexes. Maturation of higher cortical networks characterized by evolving migration, myelination, and synaptogenesis promote voluntary control and cortical inhibition. Alterations in the normal progression of the central nervous system evolution can disrupt brain development, resulting in abnormal cortical wiring, functioning, and myelination, causing neurodevelopmental reflex delays or absences. Human infants at high risk for neurodevelopmental disability often display abnormal early reflexes. Abnormal reflexes can present as a delay in acquisition, absence, prolonged presence, or reappearance later in life, and are predictive of developmental disabilities.[1,2] Therefore, it is important to mimic reflex delays in experimental models of neurodevelopmental disabilities.

Rodents are commonly employed as experimental models. Rat pups are altricial when born, and therefore too immature to undertake specific or complex motor, sensory and/or cognitive behavioral tasks. In this regard, their developmental immaturity relates to both their physical and organ development. Rats are born hairless with an inability to thermoregulate, are blind, and unable to walk. With reference to brain development, substantial cortical maturation occurs postnatally. Newborn rat pups (day of birth referred to as postnatal day 1; PD1) have been suggested to reach a brain maturation level that is similar to a preterm human brain of 23 - 28 weeks gestation, whereas PD7-10 pups are equivalent to near-term human brain.[3,4,5,6] This correlation is based on gross anatomical analyses, however, other measures of brain maturation such as myelination and amplitude integrated electroencephalograms have also been described.[5,7] For example, pre-oligodendrocytes are the predominant cells in the developing human fetal brain from 23 - 32 weeks *in utero*, and this maturational stage corresponds to a PD1-3 rodent.[5,8,9,10] Moreover, myelination begins *in utero* in humans whereas in rat pups it appears in the forebrain around PD7-10; the newborn rodent brain remains largely un-myelinated.[11,12] Tucker *et al.* found that the amplitude integrated electroencephalogram pattern of a P1 rat to be similar to a 23-week gestation human fetus, whereas a PD7 and PD10 pup is akin to a 30 - 32 week and term infant, respectively.[7] For these reasons, newborn reflex testing in neonatal rat pups provides an opportunity for capturing the ontogeny and/or disruption of brain development.

The battery of reflexes described below are adapted from studies by W.M. Fox and A. Lubics[13,14] W.M. Fox was one of the earliest investigators with respect to the ontogeny of reflexes in the mouse.[13] These reflexes include, but are not limited to, limb grasping and placing, cliff avoidance, righting, accelerated righting, gait, auditory startle, posture, and eye opening. Both forelimb and hindlimb grasp (referred to as palmar and plantar grasp in humans, respectively) are facilitated by spinal reflexes and corticospinal inhibition from non-primary motor areas.[15,16] Hindlimb placing (plantar reflex) reflects maturation of the corticospinal tract.[16,17,18] Cliff avoidance (protective responses), righting (labyrinth), and accelerated righting involve integration and communication between sensory input and motor output (such as those involved with the vibrissae and vestibular systems).[19,20,21] Gait reflects locomotion.[14] Auditory startle assesses acoustic stimulation and synaptic connections of giant neurons in the nucleus reticularis pontis caudalis.[21] Posture involves appropriate cortical-spinal/spinal-cortical projections, muscle strength, and neuromuscular innervation.[22,23] Maturation of the *gamma*-aminobutyric acid receptors may correlate with eye opening.[24] It is important to keep in mind that the

reflexes reflect a much more complicated network and provided here is a general correlation. Moreover, these reflexes provide a quick and easy method of assessing neurological development at very young ages where more complex behavioral testing is not feasible.

The objective of this paper is to provide a general guideline for neurodevelopmental reflex testing that can be easily incorporated into experimental neonatal rat studies. The methodology described was carried out in Long-Evans neonatal rat pups and quantification of the results was based on first day of appearance. The day that reflex testing is initiated and the equipment utilized may be modified to better suit a different experimental model (such as for different strains and species). By establishing the normal physiological progression of reflex maturation in a specific animal model, investigators can evaluate the effects of external stressors, endogenous manipulations, and/or therapeutic interventions on neurodevelopment in neonatal rat models. Overall, the use of reflexes as a determination of brain maturity is advantageous in predicting perinatal brain injury, and is reflective of later neurodevelopmental outcomes.

Protocol

The Animal Care and Use Committee, Health Sciences at the University of Alberta approved all animal studies.

Note: While this protocol may be adapted to other species and strains, this protocol is written for Long-Evans rats. These rats have been shown to have superior motor performances and visual acuity compared to other rodent strains. [25,26] The protocol for timed pregnancies, dietary supplementation, maternal inflammation, and neurodevelopmental reflexes is as follows.

1. Experimental Animals

1. When rats arrive to the animal housing facility, refrain from any interaction for a day or two to allow them to acclimate to their new environment. Following acclimatization, handle the rats every day for approximately five days, or when the rats are no longer showing signs of stress. Human-induced stress can alter test results and lead to pregnancy loss.
2. Once the rats are comfortable, breeding begins. Place two females and one male rat in a double decker breeding cage (h= 38 cm, 1,800 cm^2 floor area) overnight (3 PM - 8 AM). These cages prevent stress and overcrowding. In the morning, using a transfer pipette, flush the vagina of the female rats with approximately 0.25 mL of saline. Transfer the solution into a microcentrifuge tube.
3. Place the two females and male back into their respective conventional cages.
4. View the solution collected in section 1.2 under a light microscope (100X) to determine which stage of the estrous cycle the rat is experiencing and whether or not sperm are present. If there are sperm, record the date as embryonic day (E1) (**Figure 1**). If pregnant, give the female her own cage on E14.
   NOTE: The cells in the vaginal smear are clear, therefore use a low light setting. Ensure that the condenser is adjusted to its lowest setting. Adjust the brightness control to dim the light. If a brightness dial is available, set it to 1.

2. Dietary Supplementation

NOTE: This protocol is intended to assess the therapeutic effects of dietary broccoli sprout consumption during the last week of pregnancy. Grow the sprouts according to Wu *et al.* [27].

1. Soak the broccoli sprouts seeds for 2 - 3 h. Spread the seeds out on a countertop seed sprouter box and set on a warm surface for germination.
2. Water the seeds twice daily, once in the AM (8 - 9 AM) and once in the PM (3 - 4 PM).
3. On day five, place the sprouts by a window for the day (sprouts should have two leaves each and will become green over the day).
4. At the end of the day, harvest the sprouts by gently pulling the broccoli sprouts out of the sifted box and lay on a flat surface to dry.
5. Once the sprouts are completely dried, weigh out 200 mg and place in a sealed plastic bag.
6. Beginning on E14, feed each pregnant rat 200 mg of dried broccoli sprouts daily until PD21 (the day pups are weaned).

3. Inflammation

1. To induce inflammation and reproduce white matter injury in offspring, inject pregnant rats with lipopolysaccharide (LPS) every 12 h on E19 and E20. On the day of injection, bring the cage into the lab from the housing facility. Allow the pregnant rat to acclimate and become calm for at least 1 h. If a rat shows clinical signs of permanent systemic or kidney damage, it will be euthanized immediately and removed from the study.
2. Weigh the pregnant dam.
3. Dissolve 200 µg/kg of LPS (serotype 0127:B8) in 100 µL of saline. Aspirate the solution into a 1 mL syringe equipped with a 30 G ½ needle.
   1. Use a new needle for each injection. Allow the solution to warm up for a few min to minimize discomfort.
4. Record the time and inject the pregnant dam intraperitoneally. Alternate injections between sides to ensure equal distribution.
   NOTE: Machholz *et al.* describes proper restraint and injection procedures. [28]

4. Developmental Reflex Tests

1. On PD1 (day of birth), remove pups from the dam and record birth weights. Place the pups back into their respective cages and move them to the animal facility until PD3. This ensures maternal bonding between dam and pup.
   1. Record the reflex testing for reliable assessment. Adjust the video camera so that it is at maximum shutter speed for frame-by-frame analyses (1/1,000 s). Position the video camera so that it is directly above or beside the rat pup and all materials used for each reflex. For example, for hindlimb placing, place the camera beside the rat pup to ensure the lifting and placing of the hindlimb is captured.

2. For accelerated righting, place the camera above the rat pup and landing pad in order to record the ability of the pup to right in midair.

2. Record weights daily until the end of the experiment. On PD3, begin neurodevelopmental reflex testing (**Figure 4**). Move cages into a quiet room for a minimum of 1 h prior to testing to allow for acclimatization to the environment.
3. Perform neurodevelopmental reflex testing at a consistent time each day as rats are nocturnal. For example, test between 9 AM - 12 PM daily. Keep the pups directly under a heating lamp or on a heat pad at all times, to maintain a stable body temperature of 36.5 °C; rat pups of this age lose heat easily. Measure with a rectal temperature probe. If done efficiently, pups body temperatures do not change because they are not removed from the dam and litter long enough. NOTE: Monitor the temperature of heat lamp to ensure it does not exceed 37 °C.
    1. Record the score of each reflex each day until a positive response is observed. A positive reflex response occurs when the pup is able to perform a task on two consecutive days. The date of the first occurrence of a positive response is used for quantification. No further testing is required following the positive response. Hence the end date for each reflex is variable.

4. **Forelimb Grasping**
    1. Beginning on PD3, conduct the forelimb grasp reflex test. Place a blunt rod against the palm of each forepaw and apply light pressure manually. The light pressure should slightly displace the forepaw to ensure that contact is made and the pups can feel the rod. Grasping will appear as flexion of all digits around the rod. Successful acquisition of this reflex occurs when both forepaws grasp the rod for two days in a row.
    2. Score the forelimb grasp:
    0 for no grasping
    1 for successful grasping by one forepaw (left or right forepaw can be specified here)
    2 for successful grasping by both forepaws

5. **Hindlimb Grasping**
    1. Beginning on PD3, conduct the hindlimb grasp reflex test. Place a blunt rod against the sole of each hindpaw and apply light pressure manually. The light pressure should slightly displace the hindpaw to ensure that contact is made and the pups can feel the rod. Successful grasping of the rod appears as flexion of the digits around the rod. Successful acquisition of this reflex occurs when both hindpaws grasp the rod for two days in a row.
    2. Score the hindlimb grasp:
    0 for no grasping
    1 for successful grasping by one hindpaw (left or right hindpaw can be specified here)
    2 for successful grasping by both hindpaws

6. **Righting**
    1. Beginning on PD3, begin the righting reflex test. Firmly hold the pup in a supine position, with all four paws upright. Let go of the pup and immediately start the timer. Righting is achieved when the pup is able to flip/roll over onto all four paws, and each paw is perpendicular to the body. Give each pup a maximum of 15 s to achieve this goal.
    2. Score the righting:
    0 for lying on the back (or 15 s for the maximum allocated time)
    1 for lying on the side (left or right side can be specified here) or the ability of the pup to right but in the wrong posture (or 15 s for the maximum allocated time)
    2 for successful righting and appropriate posture

7. **Hindlimb placing**
    1. Beginning on PD4, begin the hindlimb placing reflex test. Hold the pup vertically in the air, by the torso. Gently stroke the dorsum of the hindpaw with a blunt surface (such as the edge of a table). A correct placing reflex is when the rat pup withdraws the stimulated hindlimb, followed by placement of the hindlimb down on that surface.
    2. Score the hindlimb placing reflex:
    0 for no successes
    1 for placing of one hindpaw (left or right hindpaw can be specified here)
    2 for successful hindlimb placing for both hindpaws

8. **Cliff Avoidance**
    1. Beginning on PD4, begin the cliff avoidance testing. Place the rat pup at the edge of a flat surface, such that the forepaws and snout of the pup are over the edge. The correct outcome is a protective response, where the rat pup turns away from the edge of the cliff. The experimenter's hand or a foam landing is placed beneath the cliff to catch the pup from the fall.
    2. Score the cliff avoidance:
    0 for no movement or falling off the edge
    1 for attempts to move away from the cliff but with hanging limbs
    2 for successful movement away from the cliff

9. **Gait**
    1. Begin the evaluation of gait on PD6. Place pups in the center of a 15 cm diameter circle and allow the pup 30 s to complete the task.[14] A successful gait is performed when the rat pup is able to move both forepaws outside the circle in less than 30 s.
    2. Score the gait as the time in seconds it takes the rat pup to move outside the circle. Record the time it takes the pup to move both forepaws outside the circle. Record 30 s if the pup is unable to complete the task within the given time

10. **Auditory Startle**
    1. Begin the analysis of auditory startle on PD10. Present a loud noise (bell) directly over the pup to assess whether or not a startle response is present. A positive startle response is observed when the pup displays a 'jerking' movement, away from the sound.
    2. Score the auditory startle:
    (or no) for no startle response

(or yes) for a positive startle response

11. **Posture**
    1. Begin posture analysis on PD12. Place pups on a non-slippery open surface, and observe the posture the pup has when moving. An immature posture is reflected by dragging of the abdomen when moving, and perpendicular pointing of both forepaws and hindpaws relative to the body. A mature posture is acquired when the pup can lift the abdomen from the surface and both forepaws and hindpaws are pointed straight, or parallel to the body, when moving.
    2. Score the posture:
       0 for no movement
       1 for immature posture when moving
       2 for mature posture while moving

12. **Eye Opening**
    1. Begin eye-opening analysis on PD12. Record the day both eyelids open:
       0 for no visible eyes
       1 for one visible eye (left or right side can be specified here)
       2 for two visible eyes

13. **Accelerated Righting**
    1. Beginning on PD14, begin accelerated righting testing. Place the pup in a supine position 30.48 centimeters above a foam landing. Drop the pup and observe its ability to right itself. Righting refers to when the pup is able to turn over to the prone position and land on its paws.
    2. Score the accelerated righting:
       0 for falling on its back
       1 for falling on its side (left or right side can be specified here)
       2 for landing on its paws

Note: Different types of quantification of the reflexes are available depending on the question of the investigators. Quantification of the reflexes can be done as initial observation of a reflex, first day of appearance up until the disappearance of the reflex, time it takes to successfully perform the task, speed of performance, and/or improvement of performance over time. [13, 14, 29,30,31] For the current study, the first day of appearance was used as a score. When using multiparous models, pups from the same litter typically behave similarly due to their genetic makeup, *in utero* and postpartum environment, and nutritional availability.[32] Therefore, a single pup cannot be accounted for as an $n = 1$ due to litter bias. Pups examined within a litter can be averaged so that each litter represents an $n = 1$. [32, 33] Alternatively, several pups within the same litter can be analyzed using the mixed effects model, which takes into account pups within the same litter. [32, 33]

Representative Results

The timeline of this experimental design is presented in **Figure 2**. [30] The methods and results have previously been published. [30] The objective of the study was to assess whether dietary supplementation with broccoli sprouts during gestation and the preweaning period protected the offspring from neurodevelopmental delay induced by *in utero* exposure to LPS. Timed pregnant rats were given intraperitoneal injections of saline (control, 100 µL) or LPS (200 µg/kg diluted in 100 µL of sterile saline) on E19 and E20 every 12 h. Randomly selected pregnant dams were also given dietary supplementation of dried broccoli sprouts (200 mg/day) from E14 to PD21. LPS was used to mimic maternal inflammation and broccoli sprouts were given as a potential therapeutic intervention to protect the offspring. Pups were born naturally on E23 (*i.e.*, PD1) and birth weights were recorded (**Table 1**). Beginning on PD3, pups underwent a battery of neurodevelopmental reflexes and acquisition of the reflex was defined as day the reflex appeared (**Figure 3**, **Table 1**). Please refer to the referenced article for further detail. [30]

Birth Weights

Analysis of birth weights revealed a main effect of treatment ($F(1,23) = 18.5$, 0.0003), diet ($F(1,23) = 6.5$, $p = 0.02$), and an interaction effect ($F(1,23) = 7.4$, $p = 0.01$). LPS, as a reflection of maternal inflammation, resulted in pups (5.1 ± 0.2 g) born significantly smaller compared to Saline pups (6.3 ± 0.2 g, Tukey's $p < 0.001$), Saline + Broccoli Sprouts pups (6.2 ± 0.2 g, Tukey's $p < 0.001$), and LPS + Broccoli Sprouts pups (6.0 ± 0.1 g, Tukey's $p < 0.01$). Birth weights of the LPS + Broccoli sprouts pups were not different from controls, suggesting that the Broccoli Sprouts spared the LPS pups from being growth restricted.

Neurodevelopmental Reflex Testing

Grasping Reflex

No effects of treatment or diet were observed for both forelimb and hindlimb grasping. All pups were able to perform these tasks.

Hindlimb Placing

A main effect of treatment ($F(1,23) = 6.8$, $p = 0.02$) was detected for hindlimb placing. Compared to Saline (4.3 ± 0.1 days, Tukey's $p < 0.05$) and Saline + Broccoli Sprouts (4.3 ± 0.0 days, Tukey's $p < 0.05$), LPS pups were significantly delayed (5.1 ± 0.3 days). Although the performance of LPS + Broccoli Sprouts pups did not differ from LPS pups, they no longer differed from controls, suggesting an improvement in this reflex task from those pups receiving LPS and no treatment.

Cliff Avoidance

A main effect of treatment ($F(1,25) = 6.0$, $p = 0.02$) following cliff avoidance analysis was detected. LPS pups (5.8 ± 0.4 days) were significantly compromised in obtaining this reflex compared to Saline (4.4 ± 0.2 days, Tukey's $p < 0.05$). LPS + Broccoli Sprouts pups did not perform differently from any of the groups, suggesting an improvement in performance.

Gait

A significant effect of treatment ($F(1,24) = 15.1$, $p = 0.0007$), diet ($F(1,24) = 6.3$, $p = 0.02$), and an interaction between treatment and diet ($F(1,24) = 9.5$, $p = 0.005$) was found following gait analyses. A delayed acquisition of gait was detected in LPS pups (9.7 ± 0.4 days) compared to Saline (7.5 ± 0.3 days, Tukey's $p < 0.001$), Saline + Broccoli Sprouts (7.7 ± 0.3 days, Tukey's $p < 0.001$), and LPS + Broccoli Sprouts pups (7.9 ± 0.2 days, Tukey's $p < 0.01$).

Auditory Startle

No differences were observed.

Righting

A sex effect was detected ($F(1,43) = 16.3$, $p = 0.0002$) for righting, therefore, male and female pups were examined separately. No differences were detected for males. In females, a main effect of Diet ($F(1,21) = 11.8$, $p = 0.002$) and an interaction effect ($F(1,21) = 15.6$, $p = 0.0007$) was found. LPS + Broccoli Sprouts pups (4.9 ± 0.4 days) were able to right earlier compared to LPS pups (6.7 ± 0.6 days, Tukey's $p < 0.05$).

Accelerated Righting

The accelerated righting test revealed a significant main effect of treatment ($F(1,25) = 4.51$, $p = 0.04$). LPS (17.4 ± 0.6 days) and LPS + Broccoli Sprouts pups (16.9 ± 0.2 days) were delayed in performing accelerated righting compared to Saline (16.0 ± 0.4 days) and Saline + Broccoli Sprouts (16.4 ± 0.5 days).

Posture

A significant interaction effect of treatment and diet ($F(1,24) = 5.8$, $p = 0.02$) was detected following analysis of posture. LPS + Broccoli Sprouts pups (14.9 ± 0.4 days) had a mature posture earlier than LPS (17.0 ± 0.4 days, Tukey's $p < 0.05$).

Eye Opening

A significant main effect of diet ($F(1,23) = 4.71$, $p = 0.04$) was observed in regards to eye opening. Both Saline + BrSp (15.9 ± 0.1 days) and LPS + Broccoli Sprouts pups (15.4 ± 0.2 days) attained this reflex earlier than Saline (16.0 ± 0.2 days) and LPS pups (16.0 ± 0.1 days).

Overall, broccoli sprout consumption during late gestation and the preweaning period was able to afford protection against LPS-induced developmental delays in several of the reflexes investigated. Please refer to the referenced article for further detail. [30]

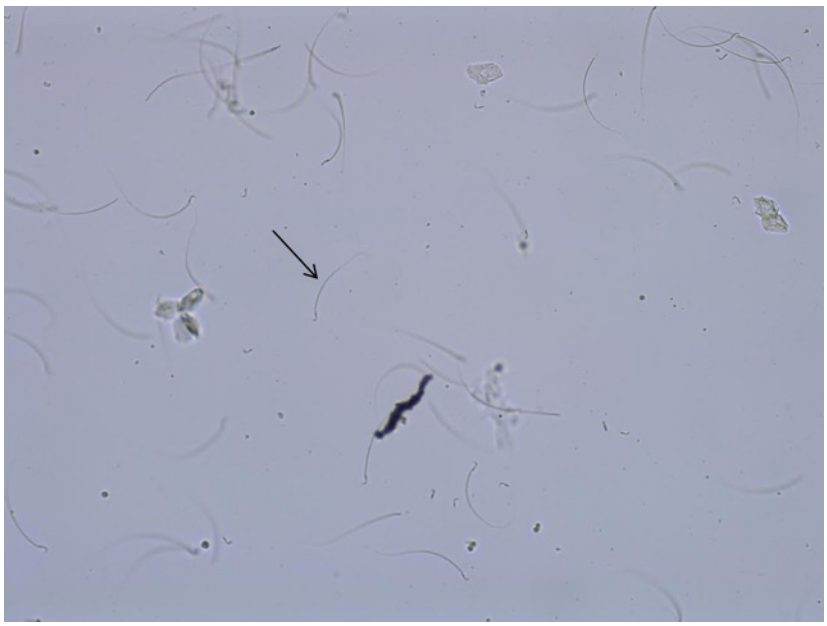

Figure 1. Positive Vaginal Smear. An example of a vaginal smear imaged with a 10X objective lens on a light microscope. This slide represents a positive slide indicated by the presence of sperm (arrow). When performing a vaginal smear, the estrous cycle of the rat can be assessed as either proestrus, estrus, metestrus, or diestrus. This assists in accurately timing breeding for a higher pregnancy success rate.

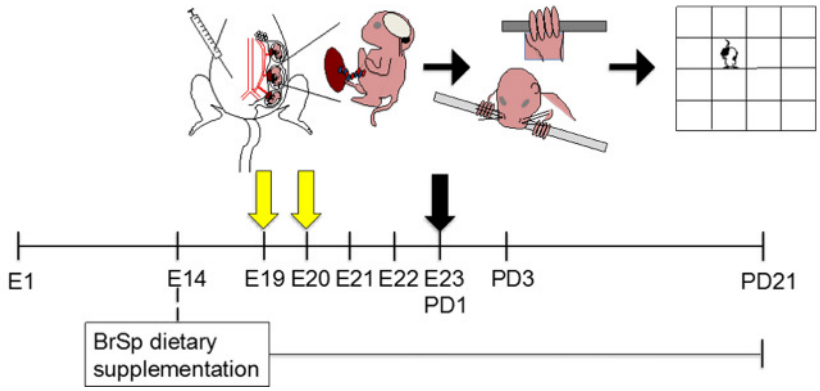

Figure 2. Experimental Design Timeline. The experimental timeline for evaluating offspring born to dams given intraperitoneal injections of LPS. Dietary supplementation with broccoli sprouts begins on E14 up to PD21. Dams are injected on E19 and E20 every 12 h (denoted as yellow arrows). Maternal weights are recorded with a weight scale and body temperature with a rectal temperature probe covered with lubricant. Pups are born naturally on E23 (denoted as a black arrow). Beginning on PD3, pups undergo several reflex testing. On PD21, pups undergo the open field test to evaluate behavioral responses.

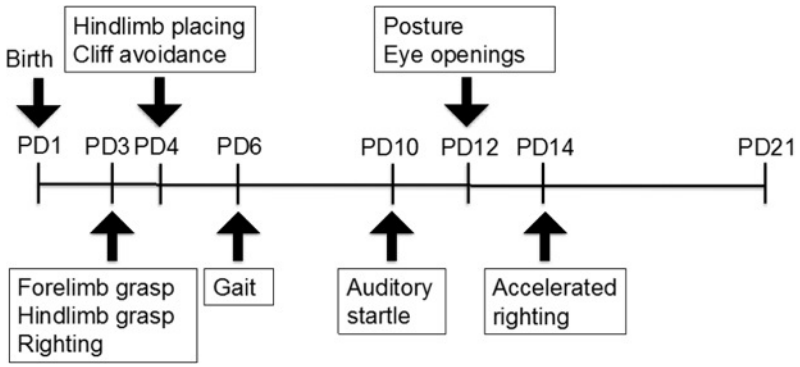

Figure 3. Example of a Timeline for Assessment of Neurodevelopmental Reflexes. Reflexes appear at different stages of development, thus, capturing the acquisition of these reflexes requires that testing begin a few days prior. This study used Long-Evans rat pups, but differences between strains and species exist, therefore a preliminary experiment should be performed using these dates as a general guide. Forelimb grasp, hindlimb grasp, and righting are assessed on PD3, hindlimb placing and cliff avoidance on PD4, and gait on PD6. Auditory startle is evaluated on PD10, posture and eye openings on PD12, and accelerated righting on PD14.

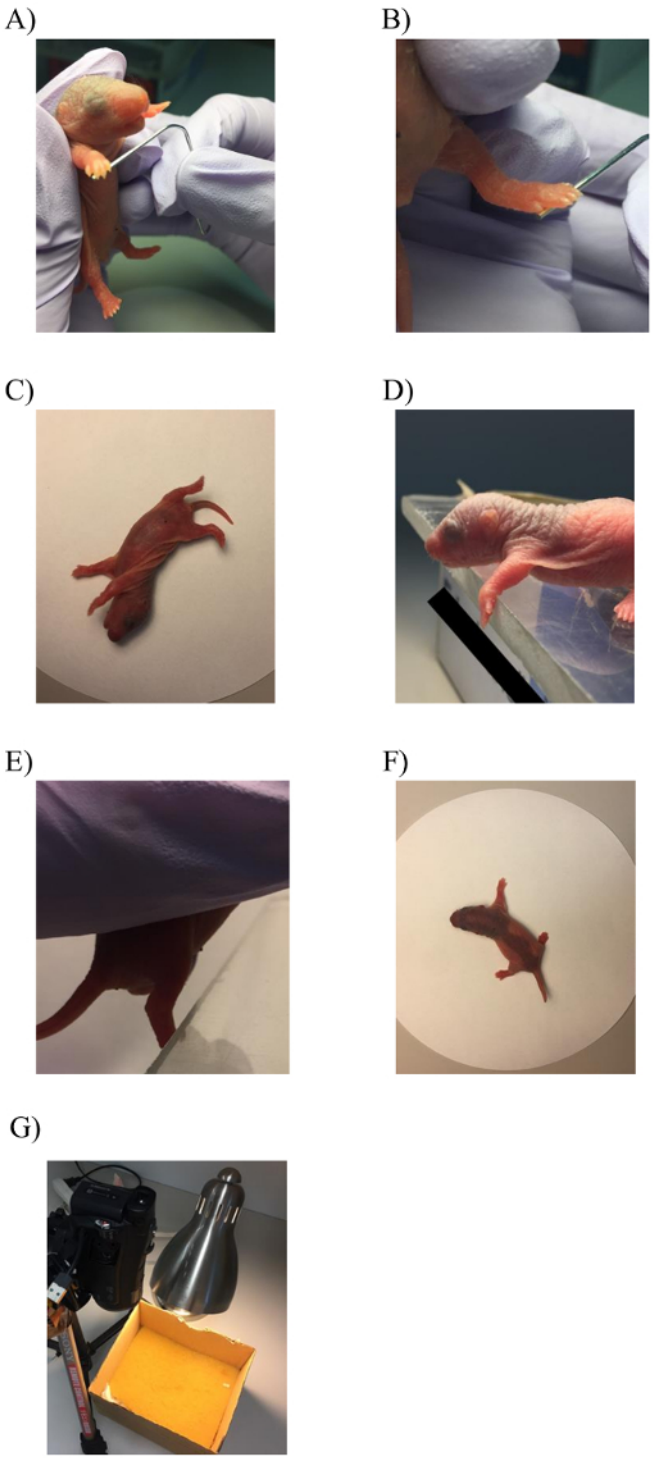

Figure 4. Images of Developmental Reflexes. Images of reflex testing performed on PD3 in Long-Evans rat pups. Forelimb grasp **(A)**, hindlimb grasp **(B)**, and righting **(C)** examination begins on PD3. Cliff avoidance **(D)** and hindlimb placing **(E)** testing begins on PD4. Gait **(F)** analyses begins on PD6. The apparatus for accelerated righting is depicted **(G)**. Please click here to view a larger version of this figure.

Examination Parameter	Groups (mean ± SEM)				Main effects (p-value)		
Litter Size and Weights	**Saline**	**Saline + Broccoli Sprouts**	**LPS**	**LPS + Broccoli Sprouts**	**Treatment**	**Diet**	**Interaction**
Litter Size (number of pups)	13.3±1.0	13.1±1.0	10.3±1.5	11±1.2	0.03	0.8	0.7
Birth Weights[*#d]	6.3±0.2	6.2±0.2	5.1±0.2	6.0±0.1	0.0003	0.02	0.01
PD7 Weights[*d]	15.0±0.5	15.5±0.4	11.4±1.1	13.6±0.3	0.0006	0.09	0.2
PD21 Weights[d]	50.0±1.4	48.7±1.6	46.4±1.2	48.2±1.4	0.2	0.9	0.3
Neurodevelop-mental Reflexes							
Forelimb Grasp[a]	3.0±0.0	3.0±0.0	3.0±0.0	3.0±0.0	n/a	n/a	n/a
Hindlimb Grasp[a]	3.6±0.3	3.5±0.3	4.6±0.6	3.3±0.1	0.3	0.1	0.1
Hindlimb Placing*[a]	4.3±0.1	4.3±0.0	5.1±0.3	4.5±0.2	0.02	0.07	0.2
Cliff Avoidance*[a]	4.4±0.2	4.7±0.2	5.8±0.4	5.0±0.4	0.02	0.4	0.1
Gait[*#a]	7.5±0.3	7.7±0.3	9.7±0.4	7.9±0.2	0.0007	0.02	0.005
Auditory Startle[a]	11.5±0.3	11.5±0.1	12.0±0.5	11.8±0.2	0.2	0.8	0.9
Posture[#a]	15.6±0.5	15.9±0.6	17.0±0.4	14.9±0.4	0.8	0.08	0.02
Eye Openings[a]	16.0±0.2	15.9±0.1	16.0±0.1	15.4±0.2	0.08	0.04	0.2
Accelerated Righting[a]	16.0±0.4	16.4±0.5	17.4±0.6	16.9±0.2	0.04	1	0.3
Righting[#a] (females)	4.9±0.5	5.1±0.4	6.7±0.6	4.6±0.4	0.09	0.003	0.0007
Righting[a] (males)	3.9±0.2	4.6±0.3	5.9±1.9	4.2±0.6	0.4	0.6	0.2
[a]Day of appearance	[*]Significantly different between LPS and Saline						
[b]Number of times activity was performed	[#]Significantly different between LPS and LPS + BrSp						
[c]Seconds							
[d]Grams							

Table 1. Weights and Neurodevelopmental Reflex Outcomes. Results obtained from offspring exposed to *in utero* inflammation and/or dietary broccoli sprouts supplementation. [30] The weights presented here are from birth, PD7, and PD21. The time points were selected to represent a preterm, term, and 1-2-year-old infant. The data are presented as mean ± SEM. Statistical analyses used in the study was a two-way ANOVA followed by Tukey's post hoc test. a = day of appearance, b = number of times the activity was performed, and c = the time (s) the activity was performed, * = significant difference between LPS and control, # = significant difference between LPS and LPS + broccoli sprouts. *n* = 5 - 7 litters per group (values from four pups, two males and two females, within a litter were averaged to represent the litter).

Discussion

Neurodevelopmental reflex testing is a predictive measure of abnormal cortical development and maturation, which may be of significance under circumstances where overt neuropathology is not evident. During neurodevelopmental testing, it is critical to ensure that pups are examined at the same time daily. Rats are nocturnal and therefore, their circadian rhythm may alter performance if testing is performed at different times during the day.[34] Testing should be completed in a quiet room as loud noises can add stress to the pups.[35] The pups require a minimum of 1 h to acclimate to new environments, since the transport, human handling, and novel settings can induce stress in the pups.[35] Pups also need to be tested directly underneath a heated lamp or on a heated pad. The younger the pups, the less able they are able to thermoregulate.[36] For this reason, pups are given 15 s and 30 s for performing the righting on PD3 and gait on PD6, respectively.[36] In addition, control pups should not be

growth restricted when born, as weight represents a confounding factor. Litters should also be culled to 8 - 10 to provide equal nourishment and representation of sex. Once the pups are born, it is important to allow a day for the rat to form a bond with the dam to prevent maternal neglect.

Pups must be tested a day or two prior to the day they acquire the reflex in order to capture the onset of appearance. Depending on the experimental model, some modifications may be required. Newborn mice are much smaller than rats. Therefore, the size of the equipment used may have to be adjusted to the size of the newborn mice. Some troubleshooting may also be required. Different strains and species may have slightly different timing of the onset of reflexes.[13] The timing detailed in this report is specific to Long-Evans pups, with testing for new reflexes beginning on PD3, 4, 6, 10, 12, and 14 (**Figure 3**). The investigator may need to perform reflex tests on 2 - 3 litters to establish the natural reflex progression prior to experimental testing. Moreover, when performing statistical analyses on the data collected, the experimenter must use appropriate techniques to prevent litter bias. Pups from the same litter tend to perform similarly to one another, therefore the litter represents an *n = 1*, not the individual pup.[32,33]

Testing neurodevelopmental reflexes is important because it is the only testing available for newborn rodents, since more complex behavioral testing is not possible at these ages. No other measures are available in this early period besides weight and physical appearance. The methods provided here have been used by several investigators, thus supporting the use of neurological reflex testing, especially in young rodents where other behavioral tests are not feasible.[13,14,29,30,31,37,38] These tests parallel to reflex testing conducted in human infants, are predictive of neurodevelopmental disorders such as cerebral palsy, and provide additional information regarding the ontogeny of neural networks. The emergence of reflexes occurs during specific times during the preweaning period, reflecting the maturation of the nervous system.[39] A limitation of this protocol is that not all experimental models can be subjected to reflex testing. Rodents are born immature, such that their postnatal development offers the advantage of capturing the onset of reflex development. Other animal models such as the lamb are born mature and develop differently from rodents, so that some of the reflex testing is not applicable to this model. Also, reflex testing is not a direct measure of cognitive or behavioral abnormalities, and it is uncertain whether any observed abnormalities are transient or persistent.

In addition to reflexes, behavioral tests can be performed for complementary analyses at older ages. This is of importance because aberrant reflex delays do not always lead to neurodevelopmental disorders.[2,40] The tests are typically administered beginning on PD21 (the day of weaning) when rodents are able to perform cognitive and motor tasks. These tests include bot not limited to the rotarod, Morris water maze, and elevated plus maze. Briefly, the rotarod test is conducted by placing the rodent on a rotating rod, and recording its performance on the rod for a period of time or until the rodent falls off. This test evaluates motor learning and strength, as well as the integrity of brain structures such as basal ganglia and cerebellum.[41] The Morris water maze consists of placing the rodent in a pool of milky white water, and recording the time it takes the rodent to find a submerged platform. Cues can be placed above each quadrant of the pool with the same cue always above the platform. This examines egocentric vs allocentric forms of hippocampal mediated spatial learning and memory.[42] The elevated plus maze test measures fear and anxiety, as well as inherent exploratory behavior. Rodents are placed in the center of four arms, two enclosed and two open, elevated above the ground.[43] The avoidance and exploratory behavior (assessed as time spent in each arm) of the rodents are recorded.[43] These additional tests examine sensorimotor and cognitive functioning, allowing the investigator to assess whether the insult lasts into adulthood, or whether it is transient.

Developmental delays are predictive, and supplemental behavioral testing is required to provide complementary evidence for the presence of a disorder later in life. In addition, control responses must be optimized prior to experimenting with strain and species differences, genetic alterations, drug administration, *etc.* Despite these conditions, the described techniques are quickly learned, easily mastered, inexpensive, and provide important information regarding the development of the nervous system. They offer an opportunity to evaluate the ontogeny and maturation of the nervous system in pups at an age when they are unable to perform mature behavioral tasks. Furthermore, human infants often undergo developmental reflex testing, and thus, rodent reflex testing provides a means of translatability. In conclusion, this is an excellent method for preliminary screening of abnormal neurological development in the newborn that can lead to later investigation of cognition and behavior.

Disclosures

The authors have nothing to disclose.

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