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Behavioral Evaluation of SOX10 Transgenic Mice

DATE

This study was conducted under the terms of an Order for Supplies and Services (#XXX) from the National Institutes of Health, dated XXX, to NeuroDetective Inc. as contractor

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1. Executive Summary

Title:	**Behavioral Evaluation of SOX10 Transgenic Mice**
Purpose:	A comprehensive sensorimotor and behavioral evaluation of SOX10 transgenic mice and wild type (WT) littermate controls was conducted in order to determine if there were significant group differences in neurologically defined functions between the mutant and WT mice.
Animals:	Six male and eight female SOX10 transgenic mice (designated Sox 10+), plus eleven male and nine female WT mice, were tested beginning at 5-6 months of age, 4 weeks after their arrival at the testing site.
Physiological Assays:	Body Mass General Assessment of Health
Behavioral Assays:	Sensorimotor and Reflex Tests Motor Skills • Latency to Move • Tail Hanging • Inclined Screen Test • Forepaw Grip • Food Reach Test Balance and Coordination • 2 cm Pole • 2 cm Plank • 4 cm Plank • Rotorod Performance Locomotor Abilities • Locomotor Activity (Open Field) Learning and Memory Assessment • Passive Avoidance Test of Cognition
	Emotional Behaviors • Social Interaction • Light-Dark Exploration Test
Results:	*Physiological Assays:* There was no significant difference between pre- and post-test body weights for either group, indicating that general mouse health remained stable during testing. The typical sexual dimorphism between male and female body mass was not observed in the Sox 10+ mice, but was seen in the WT mice. The Sox 10+ mice were faster to eat a cookie, but responded poorly to food restriction required for the reaching test—this may reflect a higher metabolic rate or digestive problems. Supra-normal muscle tone and higher body temperature in the Sox 10+ mice are consistent with the observed hyperactive circling behavior. The eyes of the mutant mice exhibited microphthalmia with iris hypoplasia, iris tears, and eccentric, irregularly

	shaped pupils. *Behavioral Assays:* Although the eyes of the Sox 10+ mice were malformed, visual placement results indicate that they retain some vision. Sox 10+ mice may also sustain some hearing loss, which is not surprising given the strong evidence of balance disorders in these mice; mice with inner ear defects often display the same hyperactive circling and head bobbing as the Sox 10+ mice. Although they were in excellent physical condition, Sox 10+ mice performed the wire hang grip test less well than the WT mice and they twisted wildly during the tail hang test during which their hindlimbs were hyperextended. Mutant mice invariably fell from the 2cm pole, the 2cm plank and the 4cm beam, and could not remain on the stationary RotaRod sufficiently long to reach criterion. Surprisingly, the mutant mice were easily able to right themselves, and were faster to turn in an alley than WT mice, but they did not rear. The Sox 10+ mice exhibited less exploratory behavior in the locomotor test. During the food reaching test, the Sox 10+ mice reached more than the WT mice and missed the food more often; this is consistent with these animals' impaired balance and coordination, greater expenditure of energy in hyperactive behavior, and visual deficits. *Assays for Cognitive Deficits:* Sox 10+ mice were slower to enter the shock chamber before and after the training shock was administered, suggesting that incipient motor or visual defects may be involved. The water maze test could not be performed because the mutants could not swim, further indicating a balance disorder.
	Emotional Assessment: Sox 10+ mice were slower than WT mice to cross the threshold of a lighted chamber to a dark chamber and reverse. They also spent less time in the dark and made fewer total entries than WT mice. This may be due to visual or motor defects. Both male and female WT mice spent more time digging, which may reflect a motor problem in the Sox 10+ mice.
Summary:	The most consistent result was that throughout tests requiring movement, balance, and coordination, Sox 10+ mice displayed a steady and high level of incoordination and lack of balance. Other senses are likely to be impaired as well; obvious eye defects were apparent and the hearing test suggested that Sox10+ mice have some degree of hearing loss, which is a common finding in other mutant mice exhibiting hyperactive circling behavior and head bobbing.

2. Introduction

The following report describes a functional evaluation of mice missing the gene for the transcription factor, Sry-Box 10 (Sox 10+). The mice and their controls were supplied by NHGRI.

The subjects were derived from the C57/Bl6 strain. The SOX gene family consists of genes related to the testis-determining gene SRY. The transcription factor Sry-Box10 (Sox10) is required for proper development of various neural crest-derived cell types, such as melanocytes, autonomic and enteric neurons, and subtypes of peripheral glia. Murine Sox10 is located on chromosome 15 in a region homologous to human chromosome 22q13.1. Mutations in SOX10 are associated with Waardenburg-Shah syndrome (WS type IV), and mouse mutants that do not express Sox10 display similar phenotypes.

In this study, the general health and sensorimotor abilities of the mice were assessed first, followed by specific tests of locomotor functions, strength, balance and coordination, then by tests of cognitive abilities to evaluate behavioral differences between the mutant mice compared with their wildtype (WT) controls.

3. Methods and Experimental Design

3.1. Design

After 13 separate assessments of the animals overall heath and sensory capabilities (i.e., body length, body mass, eye condition, muscle tone, vibrissae condition, body temperature, hearing, vision, touch, taste, olfaction, nail pinch, and toe pinch) the animals underwent 13 specific behavioral tests (i.e., tail-hanging, walking, inclined screen, wire-hang, 2 cm balance pole, 2 cm balance beam, 4 cm balance beam, turn in alley, locomotor activity, passive avoidance, light/dark preference, food reach, and social interaction) over a 6 week period. Assessment of performance in the Morris water maze was not possible as the mutant mice could not swim. Age was not considered as a factor in this analysis. All the animals survived the testing intact. At the end of behavioral testing, the animals were killed by CO_2 inhalation. The carcasses were put into plastic bags and placed on dry ice. Preserved animals and cage card identification were shipped overnight back to NIH for genotyping.

3.2. Animals

At the onset of the behavioral testing, the animals were ~5-6 months of age. There were 17 male and 17 female mice. According to the coding information provided by the source, there were 6 male mutants, 11 normal males, 8 female mutants and 9 normal females.

Animals were maintained in LD 14:10 photoperiods, with temperatures of $20^{o} \pm 2^{o}C$ and relative humidity of 60% ±10%. Food and filtered tap water were freely available throughout the study except during food restriction necessary to motivate food reaching behaviors.

All animal housing and testing occurred within the laboratories of The Ohio State University (Columbus, OH), which have been inspected by the United States Department of Agriculture (USDA) and are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The Ohio State University Institutional Animal Care and Use Committee approved the specific protocols for these behavioral tests.

3.3. Procedures

Each of the sensorimotor tests was repeated once and the scores for both test sessions were summed for an overall score, except for the chemosensory tests. Most circling mice responded poorly to food restriction, in that they weakened very quickly, presumably due to their high rate of activity (see Results). Accordingly, weight loss was monitored during food restriction prior to the food-reaching test.

The light/dark preference test was also repeated once, because the circling behavior of the mutants may have prevented them from entering or exiting either or both of the test chambers due to motor deficits. In addition all the mutant mice had obvious eye defects that might have resulted in an inability to see the doorway.

The rotorod test was not repeated because most mutants failed the pre-testing criterion, i.e. all but 2 were unable to remain on the stationary rod for any appreciable length of time, even after 6 attempts.

Locomotor, passive avoidance, food-reach and social interaction tests are typically not repeated, as responses from naïve animals more accurately reflect abilities in these tests.

All tests were performed during the dark cycle, when mice are more active, under dim red light (photographic darkroom) conditions.

3.3.1. Initial Physical Exam

Behavioral tests require that mice be able to function at a certain level of sensory and motor proficiency that allows them to make appropriate responses to different situations and stimuli. Genetically altered mice can exhibit physical defects that preclude the use of a given test. Therefore an initial assessment of general health was made prior to further behavioral testing.

3.3.1.1. ***Body Mass.*** Each animal was weighed before any testing began and again after the behavioral testing was completed.

3.3.1.2. ***Body Length***. Mice were measured from nose tip to base of tail, but tail length was not measured because some mice had been previously tail-clipped for genotyping.

3.3.1.3. *Tone*. General muscle tone was initially assessed by how strongly a mouse held onto a wire cage top when its tail was gently tugged. Scoring was as follows: 0=no tone (floppy), 1=subnormal, 2=normal. This scoring protocol was later changed given the unique condition of the Sox10 mutants (see Results).

3.3.1.4. *Eyes*. Each mouse's eyes were examined for abnormalities involving size, shape and general condition that might hinder vision. Eye scores were as follows: 0=normal, 1= mild abnormalities, 2=severe abnormalities.

3.3.1.5. *Vibrissae*. Mice were separately caged upon arrival, but barbering due to prior group housing can interfere with sense of touch. Vibrissae were examined and scored as follows for length and fullness: 0-4, with 0 = no whiskers and 4 = normal whiskers.

3.3.1.6 *Body Temperature*. Core body temperature was measured using the Thermitemp TH-5 unit (Physitemp, Inc., N J). The tail was elevated and the sensor was inserted into the rectum. It was necessary to partially immobilize the mutant mice during this procedure in order to prevent perforation of the rectum (see Results).

3.3.2 Sensory Panel

3.3.2.1. *Touch-Vibrissae Orientation*. The mouse was held by the scruff and slowly moved towards a black, non-reflective surface. When the vibrissae made initial contact with the surface, the mouse was expected to turn its head toward the stimulus. The intensity of the mouse's response to the vibrissae-touch stimulus was scored on a scale of 0-3, with 0=no response and 3=maximal response. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were summed and analyzed.

3.3.2.2-3. *Pain-Nail and Toe Pinch*. The mouse was held by the scruff, and a tweezers was used to gently squeeze the nail or the toe. The mouse either made no response or pulled the toe away. The intensity of the mouse's response to the stimulus was scored on a scale of 0-3, with 0=no response and 3=maximal response. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were summed and analyzed.

3.3.2.4. *Sight-Visual Placement*. The mouse was suspended by the tail approximately 30cm above a white mat on a black tabletop and was slowly lowered; the point at which the mouse extended its forepaws to touch the mat was observed. If the vibrissae touched the tabletop before the forepaws were extended, it was possible that the mouse had reduced vision. A score of 0 indicated no forepaw extension and 1 if the forepaws extended to the mat. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were summed and analyzed. This visual acuity test was slightly modified and repeated a second time with Sox10 mutants for verification (see Results).

3.3.2.5. *Hearing-Orientation to Cage Tapping.* Preyer's reflex, typically

done as an auditory screen, requires temporary immobility by the mouse, and could not be done with hyperactive Sox10 mutants (see Results). The mouse's home cage is placed away from the other cages during testing so that other mice will not habituate to the sound. A wooden dowel is gently tapped against the opposite side of the cage from where the mouse is located. This is done so that the motion of the dowel is not a visual cue. A normal mouse will orient towards the tapping sound. A score of 0 indicated negative response and 1= positive response. This test was repeated 24 hrs later. Results were summed and analyzed.

3.3.2.6-7. ***Smell and Taste-Chemosensory.*** The mice were given very small peanut butter cookie fragments 24 hrs before testing, since mice will avoid foreign food. The mouse was removed from its home cage and a cookie fragment (2 g) was hidden in the bedding; placement and size of cookie remained constant between mice. The mouse was replaced in its home cage. Latency to locate the cookie (sniff) was recorded as well as latency to dig out and nibble the cookie. Mice were tested in the satiated state (ad lib food), then deprived of food at the end of the dark cycle ~24hours prior to the second test. These tests were not summed or averaged, as the satiated state differs from that of the food-restricted state.

3.3.3. Motor Skills

3.3.3.1. ***Righting Reflex.*** The mouse was turned over onto its back. Normal mice will immediately right themselves onto all four feet. The quickness of the righting response was scored on a scale of 0-3, with 0=no response and 3=maximal response. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were summed and analyzed.

3.3.3.2. ***Tail-Hanging.*** A mouse with damage to pyramidal or extra-pyramidal motor control systems, including motor cortex and striatum, loses the ability to initiate normal extension of its limbs in response to suspension in space (inhibited flexor response). The forelimbs and/or hindlimbs are clasped together tightly, and 'hugged' close to the body. In the tail-hanging test, the mouse was suspended for approximately 10 sec. After a 10 sec rest in the home cage, the test was repeated. The degree of forelimb extension was rated as follows: 0=forelimbs apart and away from body, 1=forelimbs apart and near body, 2=forelimbs lightly clasped or nearby and near body. The degree of hindlimb extension was scored as follows: 0=abnormally hyperextended hindlimbs, 1=hindlimbs apart and away from body. Results of the two tests were summed separately for the forelimbs and hindlimbs. Scoring for hindlimbs differed from forelimb scoring because it was uniquely abnormal (see Results).

3.3.3.3. ***Latency to Move.*** The mouse was placed on a white mat within a circle equivalent to average body length. Latency to place all four paws outside the circle was recorded, with a 60 sec maximum. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.3.4. ***Inclined Screen.*** The mouse was placed on the center of a screen that was inclined at a 45^{o} angle, head facing downward. The latency for the mouse to turn completely around was recorded, i.e. when the mouse had turned head upward, with both paws

gripping the wire parallel to each other (60 sec maximum). The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.3.5. ***Wire-Hang Grip***. Balance and grip strength are required for a mouse to maintain its hold on small-diameter surfaces. The mouse was placed on a wire cage top and the cage top was slightly shaken so that the mouse instinctively held on tightly. The cage top was then slowly inverted until the mouse was suspended directly above the home cage at a height of approximately 1 ft for 60 sec. The mouse hung by its forepaws or fore and hindpaws before falling into the home cage. Latency to fall was recorded. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.3.6. ***Turning in Alley***. The mouse was placed in a blind alley, with the head facing the back. Latency to turn completely around toward the open end was recorded, as well as the animal reared on its hind legs prior to turning around. The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.4. Balance and Coordination

A mouse with significant motor deficits will typically fall or move more slowly than a normal mouse while beam walking. Similarly, a round surface will be more difficult to walk across than a flat surface, and a narrow beam more difficult to traverse than a wider beam.

3.3.4.1. ***Two Cm. Pole Walk***. The mouse was placed in the center of a wooden dowel (2 cm diameter) that was positioned 60 cm above a tabletop. A soft pad was placed below the animal. Latency to fall or reach a platform on either end of the dowel was recorded (120 sec maximum). The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.4.2. ***Two Cm. Plank Walk***. The mouse was placed in the center of a wooden plank (2 cm wide) that was positioned 60 cm from the floor. A soft pad was placed below the animal. Latency to fall or reach a platform on either end of the plank was recorded (120 sec maximum). The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.4.3. ***Four Cm. Plank Walk***. The mouse was placed in the center of a wooden plank (4 cm wide) that was positioned 60cm from the floor. A soft pad was placed below the animal. Latency to fall or reach a platform on either end of the plank was recorded (120 sec maximum). The mouse was returned to the home cage for 10-30 sec, and then was retested. Results were averaged and analyzed.

3.3.4.4. ***Rotarod***. This test assays the animal's ability to remain on a rotating rod (Med Associates, model ENV576M for single mouse) in successive testing sessions in which the speed of rotation increases. In general, a mouse with poor balance or less stamina will fall sooner than a normal mouse. The rotarod series was done in 3 separate pre-test sessions

followed by two test sessions. In the pre-test sessions the mice had to demonstrate a certain level of ability before progressing to the next, testing level. Each of the three pre-test sessions consisted of 3 trials lasting a maximum of 60 sec per trial, with a 10 sec interval between trials (3.5min total per session). In the first of these sessions, the mice had to remain on the stationary rod for a minimum of 60 sec total over the 3 trials. Mice with poor balance, coordination or nervous dispositions tend to fall or leap from the stationary rod. If the mouse reached criterion on this first session, it progressed to the second session, in which the rod rotated at a constant speed of 4rpm. The mouse thus has to learn to keep moving to remain on the rod. As in the first session, mice had to stay on the rotating rod for at least 60 sec over 3 trials. If the mouse reached criterion in this session, it progressed to the third session, in which the rotation of the rod increased at a steady rate from 4rpm to 40rpm. During this session, a normal mouse typically learns that it must face away from the trainer to avoid a fall. Mice with balance and coordination defects cannot remain on a rotating rod, and the difficulty of the task increases with the speed of rotation. If the mouse remained on the rod as its rotation speed increased from 4 to 40rpm for a minimum of 60 sec total over 3 trials, it progressed to the next (first test) session, in which the time for each trial is increased to 3 min. A normal, well-trained mouse is easily capable of completing 3 trials of 3 min each as the speed of the rotating rod increases from 4 to 40rpm with a 10 sec interval between trials, but mice with neuromuscular defects display diminished ability to complete these longer-duration trials. A second test session was performed 24 hours later and latencies to fall were averaged and analyzed.

3.3.4.5. ***Food Retrieval.*** The mouse was placed in a plastic cage containing a small attached tube, 20-25 mm above the floor. Metal bars (2 mm diameter) separated by 7 mm were placed in front of the tube. A plastic dish 2 cm wide containing cookie fragments was placed at the outer end of the tube, such that the mouse had to reach through the bar grid into the tube to obtain the food. A pre-test training session ensured that the mice reached asymptotic performance. Mice were food restricted for 3 days: reduced rations during training sessions, and no food for at least 24 hrs prior to the test session. Body weight was monitored to ensure all animals lost weight. On the first day of training, the mice were placed in the cage for 30 min and on the second day, for 15 min. On test day, each animal's performance was videotaped for 5-10min. Slow-motion videotape analysis was used to detect the frequency of forearm extensions in the test session.

3.3.5 Locomotor Abilities

Normal mice avoid the center of an open field, and mice with certain neuromuscular defects spend less cumulative time moving than do normal mice.

3.3.5.1. ***Activity Box (Open-Field test*)**. Mice were placed in a clear 36 cm x 36 cm acrylic box lined with bedding, inside a ventilated, darkened cabinet. A frame with 2 rows of infrared sensors surrounding the box (2 cm up from the base) detected horizontal movements (Open Field Photobeam Activity System, San Diego Instruments, Inc.). Total movement was tracked for 30 minutes in each of two separate sessions. Raw data was collected separately in four 7.5 min intervals. Besides total locomotor activity, total time spent in the center versus the periphery was also calculated.

3.3.6. Learning and Memory

3.3.6.1. ***Passive Avoidance***. Passive avoidance is a test of memory that does not require much movement, so mice with significant motor impairment can demonstrate normal learning performance. The apparatus consists of 2 darkened chambers with a wire grid floor, separated by a vertical metal wall with an automatic doorway (Gemini Avoidance System, San Diego Instruments, Inc). Mice were placed inside a clear acrylic box that was in turn placed inside one of the darkened chambers; one side of the clear box was open and was positioned against the closed doorway between the two chambers. After a 30 sec habituation, the door was opened and latency to enter the second chamber was recorded. When the mouse entered the chamber, an electrical current of 0.06 milliamps was delivered to the mouse via the grid floor. The duration of the shock was 2 sec; test time was 300 sec. The test was repeated 24-48 hrs later. If the mouse refrained from entering the second chamber for a longer time period in the second trial compared to the first, it suggested that the unconditioned negative stimulus (electric shock) was remembered and avoided.

3.3.6.2. ***Water Maze (Place-Learning Test plus Reversal Learning).*** The mouse learns to locate an unseen platform in a tank of water. This test measures the animal's ability to integrate and recall spatial information and is affected by dysfunction in the hippocampus, and to a lesser effect, general motor function.

The tank is 1 meter in diameter and 0.75 meter high. Water level is 15 cm below the rim of the tank and the hidden platform is approximately 7 mm below the surface. The tank and platform are white and the water is tinted white using non-toxic tempera paint to form a contrast with the black mouse for the automated tracking software to detect the mouse. Primary visual cues consist of three white panels approximately 1.75 meter in height, containing various black geometric patterns approximately 0.50-0.75 meter in size, that surround the tank. The temperature of the water is set at 23-25^{o} C, sufficiently cold to provide incentive to escape but not to induce hypothermia during a 60 sec swim trial. Mice underwent a preliminary free swim. Because none of the Sox 10+ mice could swim, this test was not conducted.

3.3.7 Emotional Behaviors

3.3.7.1 ***Social Interaction.*** The Sox 10+ and WT controls were separated into 2 groups: 17 males and 17 females. Each test mouse was placed in a neutral arena with one of eight normal male C57 mice from the in-house animal colony; these animals were previously known for their aggressive tendencies toward siblings and unrelated males. Social interaction of each Sox10+ and WT mouse with the C57male was videotaped. Latency, duration, frequency and average frequency of various interactions were scored: flee, fight (defensive), freeze/avoid, wrestle/spar, chase, attack (biting), aggressive groom, social groom, response to mounting, tail rattle, dig, face/body sniff, and ano-genital sniff. These interactions were defined as follows:

- 'Flee' = the mouse is being chased and is actively trying to escape the aggressor or exit the cage.
- 'Fighting' = the mouse rears up on its hind legs, threatens the aggressor with open mouth

and vocalizations, and twists to avoid bites. These behaviors were considered to be a defensive response to an attack by the aggressor.

- 'Sparring' = both mice upright and pushing each other.
- 'Wrestling' = both mice locked together and rolling around on the cage floor. Except for the initial attack, the distinction of 'aggressor' could not be made during sparring and wrestling.
- Freezing in place or avoiding the aggressor was considered to be a passive method of defense.
- 'Aggressive grooming' = the subordinate mouse is immobilized while the aggressor vigorously tugs and licks its fur; the subordinate mouse shows distress and discomfort. In contrast, normal social grooming was done to an aggressor, usually just before it attacked the subordinate mouse. It could therefore be an attempt to calm or placate the aggressor.
- 'Response to mounting' = this was not scored because none of the target mice performed mounting behavior.
- 'Tail rattling' = a rapid lateral thrashing of the tail.

3.3.7.2 . ***Light/Dark Preference.*** To assess dark preference, each animal was placed in a transparent box 1.35 m long, 60 cm wide and 15 cm high. A foam-board divider, black on one side and white on the other, separated the box into 2 chambers. A 10 x 10 cm^2 hole formed the entryway between chambers. Black cardboard was placed on the outer sides of one chamber and inside the lid. A photographic red bulb hanging outside of the box illuminated the other half of the box. The animal was placed in the darkened chamber and latency to enter the lighted chamber was recorded. Latencies and frequencies of entries were recorded for 5 min. Then, the test was repeated by placing the mouse in the lighted chamber of the box.

3.4 Statistical Analyses

Most statistical analyses were first conducted with the standard parametric 2-way analysis of variance (ANOVA) with gender as a factor. If the assumptions of normality or equal variance were not met, the data were transformed and re-analyzed, or a non-parametric 1-way analysis of variance such as the Kruskal-Wallis One Way Analysis of Variance on Ranks, was then employed. Either a Chi-square or Fisher Exact test was used when data were based on the degree of response to stimuli or based on a positive-negative result. In the case of social interaction, a *t*-test was used on two groups separated by sex. If normality failed for a *t*-test, then the non-parametric Mann-Whitney Rank Sum test was employed.

All data discussed below are means ± s.e.m.

4. Results

4.1. Initial Physical Exam

It was immediately apparent that a subset of the mice exhibited spotted coats in different

shades and displayed distinctive circling behavior, head bobbing, and hyperactivity. After all behavioral tests were completed, these mice were revealed to be Sox10 mutants.

4.1.1. ***Body Mass.*** Normally, age-matched males weigh more than females. In the overall ANOVA of the pre-test weights of these mice, males weighed significantly more than females (males=29.8 $\pm$1.05 g, females=23.49 $\pm$ 1.0 g, $p<0.001$), wildtypes weighed significantly more than mutants (wildtype=28.65 $\pm$0.93 g, mutant =24.65 $\pm$1.1g, $p<0.020$), and there was a significant interaction between Sex and Genotype. Specifically, wildtype males were heavier than wildtype females (34.18 $\pm$ 1.2 g verses 23.11 $\pm$1.37 g, $p<0.001$) but there was no significant difference in weight between male and female mutants ($p=0.49$). Wildtype males were heavier than mutant males (34.18 $\pm$ 1.2 g versus 25.42 $\pm$1.7 g, $p<0.001$), but there was no significant difference in weight between normal and mutant females ($p=0.705$). In general, wildtype mice were heavier than mutants, perhaps due to the high activity rate of the circling mutants. Alternatively, Sox10 mutations may cause digestive defects. Upon comparison of pre-test and post-test weights, it was found that both groups of females as well as the mutant males gained ~1gm of weight while wildtype males gained more (pre-test weight=34.18 $\pm$ 1.2 g verses post-test weight= 38.5 $\pm$ 1.5 g). Data analysis of post-test weight closely paralleled that for pre-test weight.

4.1.2. ***Body Length***. As expected, males were longer than females (9.61 ± 0.11 cm verses 9.13 ± 0.11 cm, $p<0.007$). There may be less of a difference in length between male and female Sox 10+ than between male and female WT mice ($H=10.42$, $p=0.015$).

4.1.3. ***Muscle Tone.*** A mouse with poor muscle tone has trouble maintaining a hold on the cage wires when the tail is gently tugged. Although the "spotted" circlers appeared to stagger across the top of the cage wire, they all displayed superior muscle tone (compared to wildtypes) during routine handling. They appeared lean and taut, presumably due to extended periods of high-speed circling/running. The scores for muscle tone had to be revised given the unique characteristics of the spotted circlers—they were supra-normal rather than sub-normal. Mice with normal muscle tone were therefore given a score of 2, supra-normal tone was scored as 3, and slightly sub-normal tone was scored as 1. All mice with a score of 3 were later identified as Sox10 mutants. There was a significant relationship between muscle tone and genotype (Chi square=34, $p<0.001$).

4.1.4. ***Eyes***. There was a strong relationship between the condition of the eyes and genotype (Chi-square=29.9, $p<0.001$). Specifically, the Sox 10+ eyes were smaller than the eyes of WT mice during the pre-test assessment. Directly after sacrifice, eyes were examined a second time. Microphthalmia with iris hypoplasia, iris tears, and eccentric, irregularly shaped pupils were apparent in all the Sox 10+ eyes, but not in the wildtype eyes. The ocular malformations of Sox10 mutants were reminiscent of those described in Pitx2 and Foxc1 mutants. Mutations in these transcription factors are associated with the ocular defects typically seen in Axenfeld-Rieger syndrome, a developmental disorder characterized by faulty neural crest cell migration.

4.1.5. ***Vibrissae***. There was no significant relationship between the length of vibrissae and genotype (Chi square=4.625, $p=0.099$).

4.1.6. ***Body Temperature.*** The mean body temperature of Sox 10+ mice was ~1 degree C higher than wildtype mice ($p<0.001$). There was also a significant interaction between sex and genotype: WT females had a higher temperature than WT males (36.47 ± 0.18^{o} verses 35.69 ± 0.16^{o}, $p<0.003$) but there was no significant difference in temperature between male and female knockouts ($p=0.207$). Mutant males had a higher temperature than wildtype males (37.15 ± 0.22^{o} verses $35.69 \pm 0.16\ ^{o}$, $p<0.001$), but there was no significant difference in mean temperature between Sox 10+ and WT females ($p=0.248$). Interestingly, these results correspond to a similar pattern in the significant interaction between sex and genotype in body weight (see above). Hyperactive circling behavior of mutants could account for their higher body temperature.

4.2 Sensory Panel

4.2.1. ***Touch-Vibrissae Orientation*****.** There was no significant relationship between genotype and the degree of response to vibrissae stimulation (Chi-square=3.88, $p=0.143$).

4.2.2-3. ***Pain-Nail and Toe Pinch.*** The proportion of withdrawal observations in both the nail and toe pinch categories was not significantly different between genotypes ($p=0.689$, Fisher Exact Test). There was also no significant genotype or sex difference in the intensity of reaction to toe pinch ($p=0.412$).

4.2.4. ***Sight-Visual Placement.*** All Sox10 transgenic mice received at least one positive score, and no statistically significant relationship was seen between the sum of the visual placement scores and genotype. This suggested that all of the Sox10 mice could see and react to the white mat as they approached it. However, the Sox 10 mice twisted frantically while being suspended by the tail, and so they invariably placed much closer to the white mat than non-circling mice. Thus interpretation of this test is problematic because late visual placement could involve use of the vibrissae to detect the mat, rather than just eyesight. Therefore in a follow-up test the Visual Placement test procedure was modified in an effort to assay better whether the spotted circlers were seeing the mat rather than detecting it by touch.

The muscle tone test (above) had suggested that the Sox10+ mice might have balance problems rather than muscular weakness. Balance anomalies would account for the wild twisting that made it difficult to monitor the vibrissae during visual placement. Therefore the mat was placed on a platform ~6 in. above the black tabletop. The spotted circlers were held so that all four feet were supported; they were then slowly lowered to the edge of the white platform. All spotted circlers oriented towards the edge of the approaching platform and reached out at least one forelimb towards the mat. The vibrissae did not make visible contact with the mat prior to visual placement. In this modification of the test, there was no significant genotype difference in visual placement. Together the results of both tests with different procedures suggested that the Dct-Sox10 mice do retain some degree of sight.

4.2.5. ***Hearing-Orientation to Cage Tapping***. The Sox10+ mice oriented less to the sound of cage tapping than their wildtype controls (Chi-square=26.775, $p<0.001$), suggesting that the mutants harbor some degree of hearing loss.

4.2.6-7. *Smell and Taste-Chemosensory.* There was no significant genotype difference in latency to find the cookie by olfactory cues when the mice were in either the satiated or food-restricted state (transformed data). There was also no significant effect of genotype on the time required to dig out and taste the cookie in satiated mice, but during food restriction the Sox10 transgenics were faster than wildtypes (average time for transgenics = 1.34 $\pm$ 0.1 sec verses 0.97 $\pm$ 0.12 sec for wildtypes, $p<0.03$). This may be due to increased appetite in the animals that exhibit hyperactive behavior.

4.3. Motor Skills

4.3.1. *Righting Reflex.* The time required for the Sox10+ animals to right themselves when placed on their backs was not significantly related to genotype (Chi-square=1.889, p=0.389). The righting ability of the Sox10 transgenics was almost indistinguishable from that of WT mice in terms of speed. Qualitatively, however, the Sox10 transgenics used a distinctive body twisting technique to right that was not apparent in WT mice.

4.3.2. *Tail-Hanging.* The general effect of tail hanging on Sox10 transgenics was that of excessive and hyper-extended twisting—they not only twisted to both sides, but straight upwards as well, with the nose almost level with the hindlinbs. Hindlimb placement by the mutants was especially distinctive: their limbs were extended at almost a 90 degree angle from the side of the body---effectively, they did 'splits'. Significantly greater extensions by the mutants were evident in both total forelimb score (Chi-square=29.997; df = 4, $p<0.001$), total hindlimb score (Chi-square=31.248; df = 1, $p<0.001$), and total limb score (Chi-square=31.248; df= 5, $p<0.001$). This extreme extension by the Sox10+ animals may be an extreme attempt to regain proprioception, which is lost when the animal is suspended in air.

4.3.3. *Latency to Move.* There was no effect of genotype (p=0.074) or sex (p=0.229) in latency to walk.

4.3.4. *Inclined Screen.* There were no effects of either genotype (p=0.056) or gender (p=0.779) on turning on the inclined screen.

4.3.5. *Wire-Hang Grip.* Latency to fall from the wire mesh was much sooner for Sox10 transgenics than wildtype mice (transgenic=8.99 $\pm$ 3.86 sec, wildtype=50.58 $\pm$ 3.2 sec, $p<0.001$). It is unclear whether this is due to a balance problem or muscle weakness.

4.3.6. *Turning in Alley*. There was a significant difference in latency to turn in an alley with the Sox 10+ mice turning faster than WT animals (2.03 $\pm$0.63 sec verses 5.55 $\pm$ 0.52 sec, $p<0.001$); the circling behavior of the mutants might be a contributing factor to this effect. ANOVA on ranks showed a significant genotype difference in their median time to rear before turning (H=15.3, p=0.002), because little rearing was observed in Sox 10+ mice, perhaps due to a balance problem brought on by their continual circling. No gender difference in rearing behavior was observed within genotypes ($p> 0.05$).

4.4. Balance and Coordination

The results of the following tests suggested that balance and not muscle strength was the most likely cause of the differences between genotypes seen in the muscle tone and wire-hanging grip assays (above).

4.4.1. Two cm Pole Walk. All mutants fell from the pole while all WT mice reached the platform (total falls, Chi-square=29.997, p=<0.001). There was no significant gender difference. There was a significant difference in latency to fall or reach the platform (transformed data); Sox 10+ mice fell from the pole faster than WT mice reached the platform (6.3 $\pm$ 4.3 sec versus 16.7 $\pm$ 3.6 sec, p<0.008).

4.4.2. Two cm Plank Walk. Once again all mutants fell and all wildtypes reached the platform (total falls, Chi-square=29.997, p<0.001), and again there were no significant gender differences. There was a significant difference in latency to fall or reach the platform (transformed data); mutants fell from the pole faster than WT mice reached the platform (2.6 $\pm$ 1.8 sec versus 21.3 $\pm$ 1.5 sec, p<0.001).

4.4.3. Four cm Plank Walk. All mutants fell while all WT mice reached the platform (total falls, Chi-square=29.997, p<0.001). There was a significant difference in average latency to fall or reach the platform (transformed data); mutants fell from the pole faster than wildtype mice reached the platform (6.1 $\pm$ 2.9 sec verses 14.2 $\pm$ 2.5 sec, p<0.001). There was a significant gender difference in performance on the 4 cm plank in that wildtype males took longer to reach the platform than mutant males took to fall (WT = 19.7 $\pm$ 3.3 sec; Sox 10+ = 5.0 $\pm$ 4.5 sec, p<0.001). This difference was not true for females.

4.4.4. *Rotarod.* During this test it was apparent that the spotted circlers (later decoded as Sox 10+ mice) were unable to remain on the stationary rod for the prerequisite 60 sec over the 3 pre-test trials, except for 2 mice. One of these two was able to remain on the rotating rod at 4 rpm for 60 sec in 3 trials, but failed to remain on the rod rotating at 4-40 rpm (ND 2.3). The other mutant (ND 2.27) did reach criterion for testing, but did poorly in both subsequent test sessions. A second round of pre-test (training) trials was then conducted 24 hrs later for all the mice that did not reach criterion, but performance did not improve for any of the mutants. As a result, no mutant except for ND2.27 met criterion for testing, while all the WT mice completed pre-testing as well as both test sessions. Analysis was therefore limited to the stationary rod pre-test. There was a statistically significant difference between Sox 10+ and WT mice on the average latency to fall from the stationary rod (8.73 $\pm$ 0.95 sec for the mutants versus 60 $\pm$ 0.79 sec for the wildtypes, p<0.001). ANOVA on ranks indicated that the Sox 10+ mice fell sooner from the stationary rod (H=30.3, p<0.001), a finding that is consistent with the beam walking findings above. These results further support the hypothesis that the Sox 10+ mice have impaired balance.

4.4.5. *Food Retrieval*. There was a strong interaction between gender and genotype in total number of reaches as well as number of unsuccessful reaches. Overall, Sox

10+ mice reached for food more often than WT animals (29.6 ± 4.4 reaches verses 14.0 ± 3.66 reaches, $p<0.02$) and males reached more often than females (28.57 ±4.13 reaches verses 15.04 ± 3.96, $p<0.03$). However the more frequent reaching by the mutants was due to the males; mutant males reached nearly four times as often as WT males (44.5 ±6.65 verses 12.64 ±4.9, $p<0.001$), but the mutant and wildtype females were not different ($p=0.918$). Within mutants, males reached significantly more than females (44.5 ±6.65 verses 14.63 ±5.76, $p<0.002$). Even though they reached more often, the mutants also missed retrieving the food pellets more often than wildtypes (18.35 ±3.1 misses verses 7.68 ±2.6 misses, $p<0.008$), and this effect was due to the males (30.3 ±4.7 misses by mutant males verses 6.36 ±3.5 misses by wildtype males, $p<0.001$). Females of each genotype missed retrieving the food pellets equally often. Because of the mutant males' poor success at this task, overall there was significant gender effect; males missed the food more often than females (18.4 ±2.9 verses 8.69 ±2.8, $p<0.03$). Within WT animals there was no significant gender difference, but within mutants males missed more often than females (30.3 ±4.7 verses 8.4 ±4.1, $p<0.001$). Less successful reaching probably reflects a combination of poor motor coordination in grasping the food plus visual defects in the mutants, especially the males, rather than increased appetite, because there was no significant genotype difference in latency to reach ($p=0.372$).

4.5. Locomotor Abilities

4.5.1. Activity Box (Open-Field test). With transformed data, there was a significantly higher mean rate of total locomotor activity by Sox 10 transgenic animals compared to WT mice (25,055 ± 1916 total photobeam crossings verses 8,505 ± 1595 total photobeam crossings, $p=<0.001$ [transformed data]); this result is consistent with the hyperactivity and circling behavior seen in the Sox 10+ mice. Overall there was no significant gender effect, which is usually observed in WT animals for this measure (males more active), nor a gender x genotype interaction. Also, normal mice tend to avoid the center of an open field and move around the periphery, and in this test male Sox 10+ mice spent even less time in the center than male WT mice ($H=16.276$, $p<0.001$), which may indicate greater anxiety by the male mutants compared to wildtype males, but, since there is no evidence of greater anxiety in Sox 10 animals in other tests, more likely reflects their greater circling tendency, which results in their spending more time around the periphery of the box.

4.6. Learning and Memory

4.6.1. ***Passive Avoidance***. The transgenic mice took significantly longer to enter the opposite chamber, both in the initial trial (before the electric shock was administered) -- transgenic=18.76 ±3.38 sec, WT=7.58 ±2.8 sec, $p<0.02$ -- and in the second (memory) trial -- transgenic=128.27 ±27.85 sec, WT=48.38 ±23.18 sec, $p<0.04$). Since the Sox 10+ mice hesitated more than the wildtypes both before and during the avoidance trial, this likely reflects a vision and/or a locomotor defect rather than evidence of learning to avoid shock. There was no gender effect or gender x genotype interaction.

4.7. Emotional Assessment

4.7.1. ***Light-Dark Preference***. Similar to their behavior in the two-chamber

passive avoidance test, the Sox 10+ mice took longer than the wildtype mice to cross into either the lighted chamber (mutant=187.77 ± 26.1 sec., wildtype=110.2 ± 21.7 sec, $p<0.04$) or the darkened chamber (mutant=148.56 ± 20.7 sec., wildtype=13.12 ± 17.25 sec., $p<0.001$ [transformed data]). There were no gender differences. The total time spent in the dark was higher in WT mice than in Sox 10+ animals (WT = 213.8 ±11.87 sec., Sox 10+ = 171.6 ±14.2 sec., $p<0.04$), indicating either less anxiety by the mutants, or (more likely) a lack of preference for light or dark due to decreased visual perception. WT animals showed more exploration behavior, reflected in their higher average number of chamber entries (WT = 9.23 ±0.7, Sox 10+ = 3.13 ±0.86, $p<0.001$). The mutants may have difficulty moving between the chambers due to their circling behavior.

4.7.2. ***Social Interaction***. In all cases except for one aggressive mutant male (ND2.3), the test mice (both WT and mutants) were subordinate to the C57 male. Therefore, aggressive behavior by the test mice, such as chasing, attack (biting), and aggressive grooming, was seldom observed.

Within the females, the following behaviors were seen: fleeing, defensive fighting, freeze/avoid, mounting response, face/body sniff, anogenital sniffing and digging. There were no significant genotype differences in fleeing, freezing, fighting, response to mounting, or sniffing. However the several measures of digging (a general stress behavior) were all significantly lower in mutant females compared to wildtype females: latency to dig ($t=108.0$, $p<0.001$), total duration of digging ($t=39$, $p<0.003$), frequency of digging behavior ($t=3.546$, $p<0.004$) and average duration of digging episode ($t=38.0$, $p<0.001$). The consistency of this difference suggests it reflects either the motor deficiencies of the mutant females, or their greater time spent in circling rather than engaging in digging behavior.

Within males, fleeing, defensive fighting, freeze/avoid, attack, aggressive grooming, social grooming, face/body sniff, anogenital sniffing and digging were seen. Results for digging matched those of females: latency ($T=84.0$, $p<0.004$), duration ($t=24.0$, $p<0.004$) frequency ($t=24.0$, $p<0.004$), and average duration of digging ($t=3.949$, $p<0.001$) were all lower in mutants compared to wildtypes. In apparent parallel to these lessened behaviors, latency to fight back ($t=5.195$, $p<0.001$), duration of fighting ($t=24.0$, $p<0.004$), frequency to fight back ($t=24.0$, $p<0.004$), and average duration of fighting ($t=24.0$, $p<0.004$) were all also decreased in mutant males compared to wildtypes. It is notable that these genotype differences in fighting behaviors were not observed in females, however it would be expected that (1) females, both WT and Sox 10+, would be more likely to avoid fighting altogether than males, and that (2) the C57s would be less likely to attack a female than a male. Thus the greater frequency of fighting behaviors by males creates a greater opportunity to detect genotype differences in fighting behavior by males.

The decreased tendency of Sox 10+males to fight back compared to wildtype males may reflect a general tendency in mutants to circle when stressed. Circling proved to be an effective mode of escape; the aggressive C57s could not catch the circlers fast enough to attack. It is possible that circling could be the mutant equivalent to fleeing.

The freeze/avoid category appeared to differ between male and female mice, irrespective of genotype. In WT and mutant males, freeze/avoid appeared to be a defensive mechanism to ward off or escape attack. In WT and Sox 10+ females, freeze/avoid was indicative of mating behavior, when the female approaches the male, freezes in place, and then darts away. Mutant females exhibited the same degree of interest as the WT mice, and when the C57 mice could catch the female Sox 10+ mice (difficult due to circling behavior), mounting response was

similar to that of WT females. Average duration of anogenital sniffing was elevated in males compared to females, as is typical ($t=2.3$, $p<0.04$), but there was no genotype difference.

5. Discussion

Generally, the Sox 10+ mice displayed sensory, balance, and coordination deficits. Because of these deficits, it was impossible to assess their cognitive abilities. In general the mutants did not appear to have altered levels of anxiety or other indicators of emotionality, and body mass and length appeared normal.

Physiological Assays: There was no significant difference in pre- and post-test body weights, indicating that general mouse health remained stable during testing. Normal sexual dimorphism between male and female body mass was not observed in the Sox 10+ mice, but was seen in the wildtype (WT) mice. The mutant mice were faster to eat a cookie, but responded poorly to food restriction—this may reflect a higher metabolic rate or digestive problems. Supra-normal muscle tone and higher body temperature in the Sox 10+ mice are consistent with the observed hyperactive circling behavior. The eyes of the mutant mice exhibited microphthalmia with iris hypoplasia, iris tears, and eccentric, irregularly shaped pupils.

Behavioral Assays: Although the eyes of the Sox 10+ mice were malformed, visual placement results indicate that they retain some vision. Sox 10+ mice may also sustain some hearing loss, which is not surprising because there is very strong evidence of balance disorders in these mice; mice with inner ear defects often display the same hyperactive circling and head bobbing as the Sox 10+ mice. Although they were in excellent physical condition, Sox 10+ mice performed the wirehang grip test less well than the WT mice and they twisted wildly during the tailhang test during which their hindlimbs were hyperextended. Mutant mice invariably fell from the 2 cm pole, the 2 cm plank and the 4 cm beam, and could not remain on the stationary RotaRod sufficiently long to reach the criterion required to proceed to the moving rod. Surprisingly, the mutant mice were easily able to right themselves, and were faster to turn in an alley than WT mice, but they did not rear. The Sox 10+ mice exhibited less exploratory behavior in the locomotor test. During the food reaching test, the Sox 10+ mice reached more than the WT mice and missed the food more often; this is consistent with the hypothesis that the mutants have impaired balance and coordination, expend more energy in hyperactive behavior, plus have some degree of visual deficit.

Assays for Cognitive Deficits: Sox 10+ mice were slower to enter the shock chamber before and after the training shock was administered, suggesting that incipient motor or visual defects may be involved rather than impaired memory. The water maze test of learning and memory could not be performed because the mutants cannot swim, which is further evidence of a balance disorder.

Emotional Assessment: Sox 10+ mice were slower than WT mice to cross the threshold of a lighted chamber to a dark chamber and reverse. They also spent less time in the dark and made fewer total entries than WT mice. These behaviors are more consistent with visual or motor defects rather than lessened anxiety. In the social interaction test, latency to freeze was lower in female mutants, indicating a possible visual problem. Both male and female Sox 10+ mice spent less time digging, which may reflect a motor problem.

6. Conclusion

The most consistent result throughout the tests that required movement, balance, and coordination was that the Sox 10+ mice sense of balance was impaired. Other sensory impairments were also likely. Obvious eye defects were apparent, but visual placement indicated that Sox 10+ mice probably possessed at least light perception and could see areas of high contrast (white mat against dark background in dim red light). Results of the light/dark preference test and the food-reaching test suggested some level of impaired vision, but the hyperactive circling behavior and lack of balance confounded the results of those tests. In addition, visual acuity could not be tested with the water maze because the Sox 10+ mice cannot swim. The hearing test suggests that Sox 10+ mice likely have some degree of hearing loss, which is common in mice that exhibit hyperactive circling behavior and head bobbing. Potential cognitive deficits could not be detected with the water maze, again, because the mice could not swim, and passive avoidance results may have been confounded by hyperactive circling behavior within the first chamber. Given their multiple defects, the Sox 10+ mice survive quite well; they appear to rely strongly on their remaining intact senses of smell, proprioception and touch (vibrissae) to navigate, feed, and interact with other mice.

Figure 1a. Turn in Alley Test (Latency to Turn)

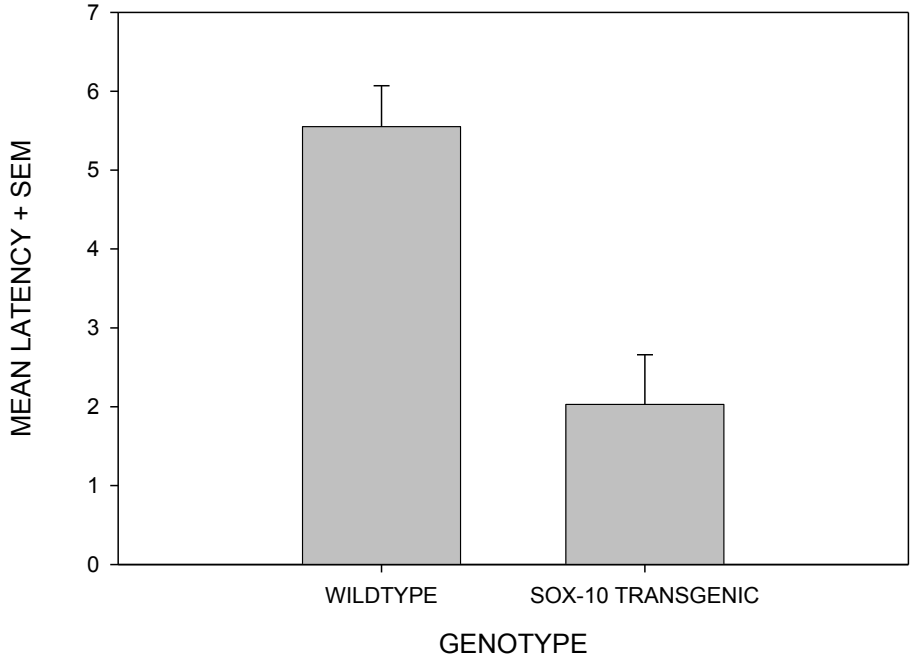

Figure 1b. Turn in Alley Test (Ttotal # of Rearings)

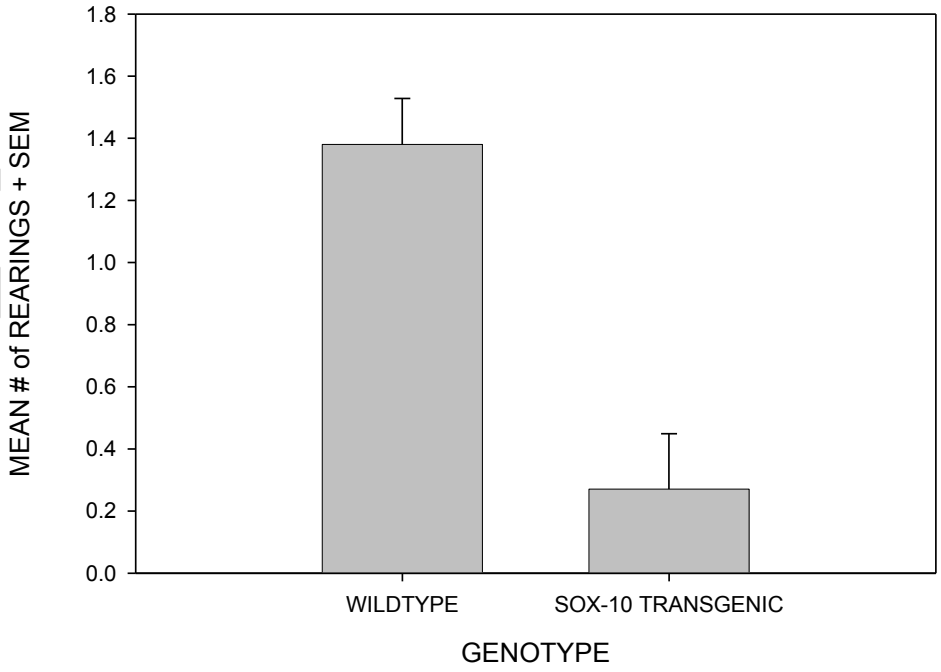

Figure 2. RotoRod Test (Stationary Rod)

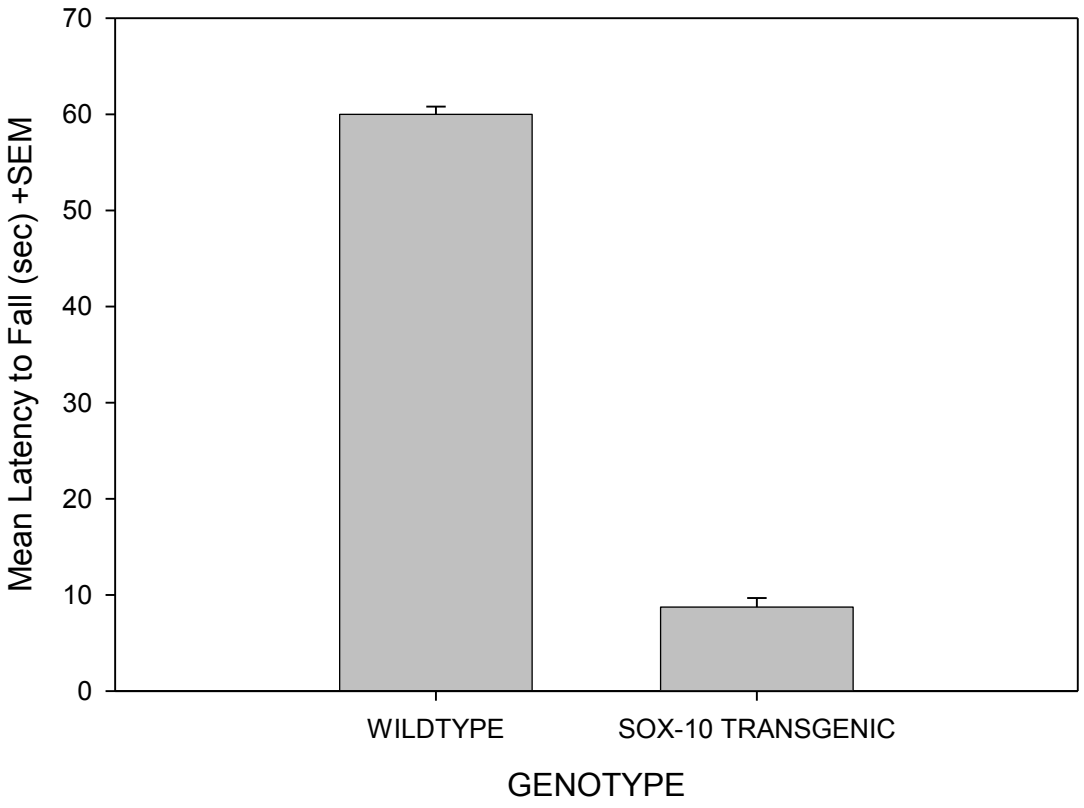

Figure 3. Food Reach Test

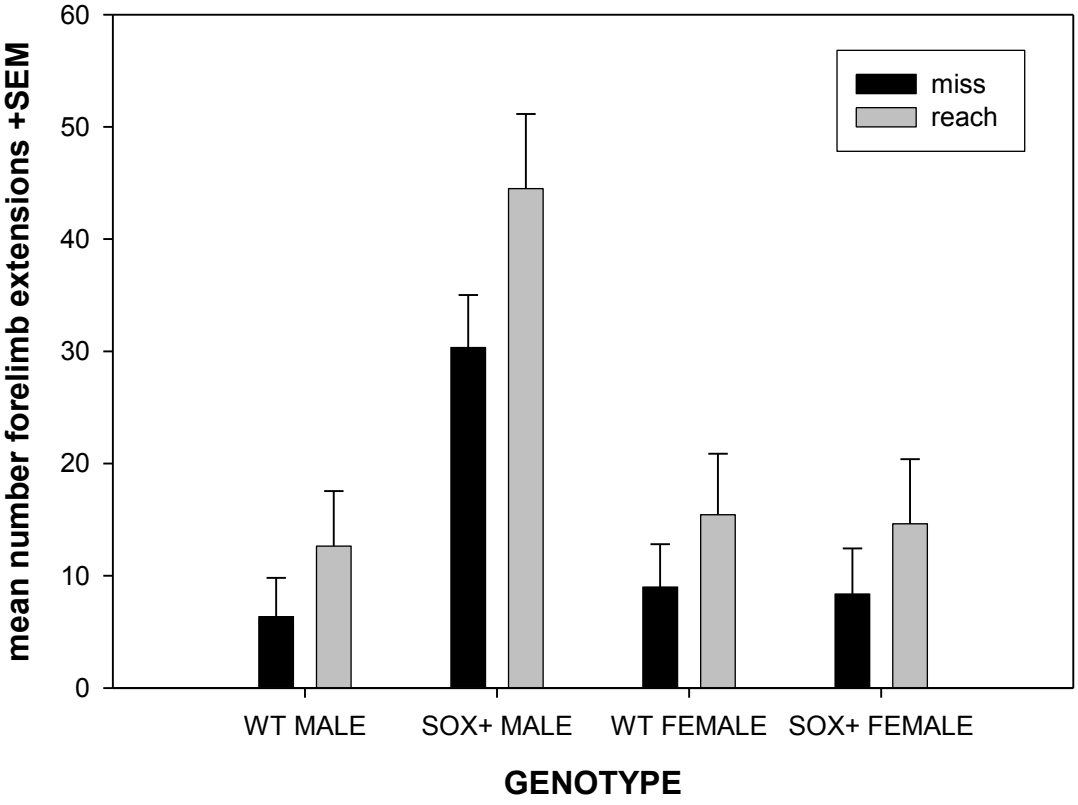

Figure 4. Open Field Test

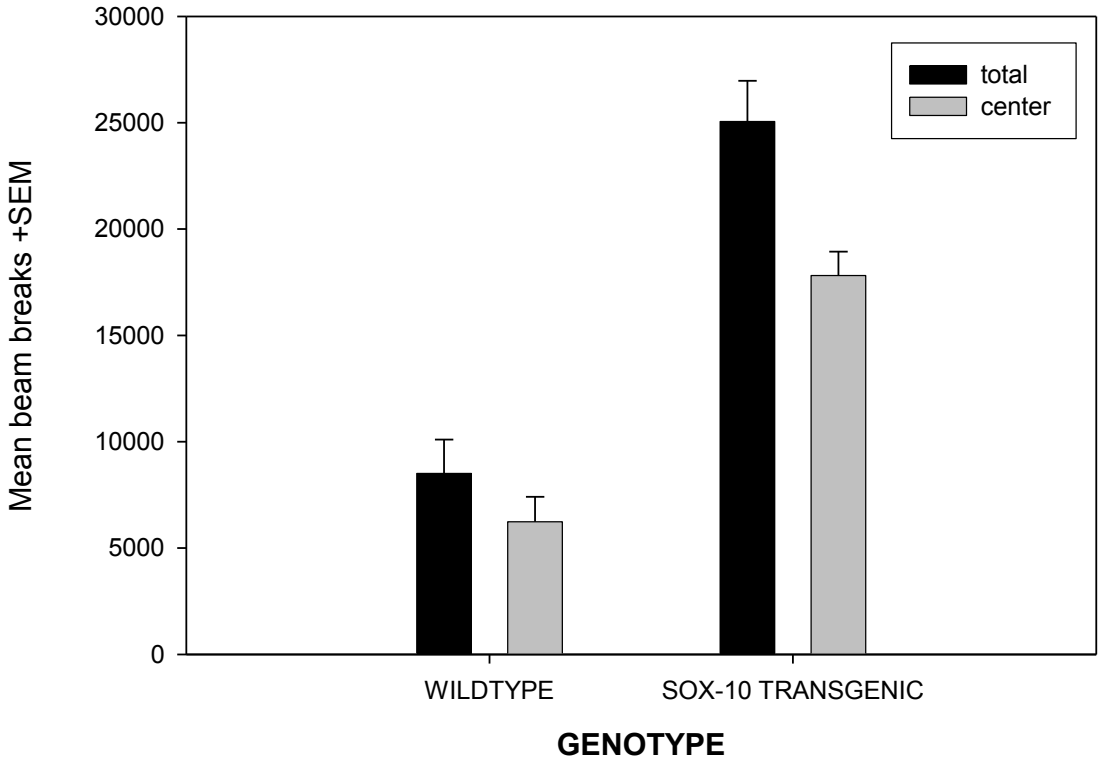

Figure 5. Passive Avoidance test

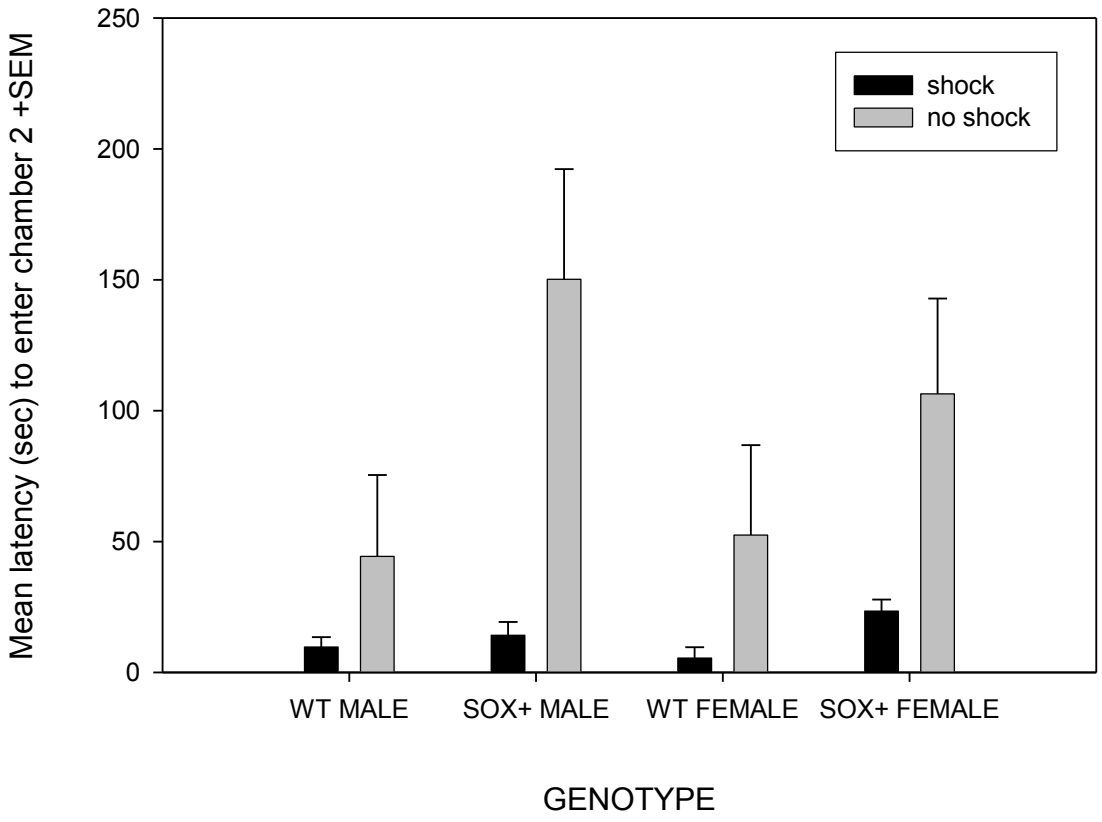

Figure 6a. Light/Dark Preference (Latency to Enter Chambe)r

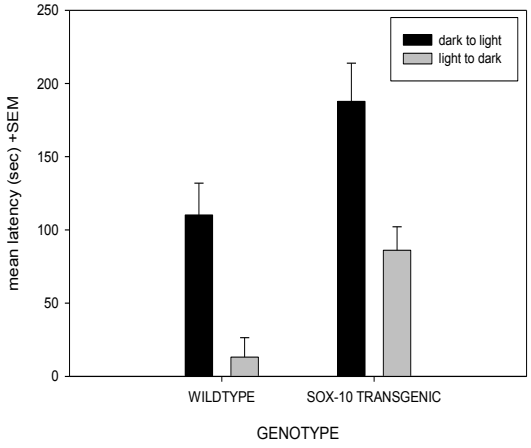

Figure 6b. Light/Dark Preference (Average Entries)

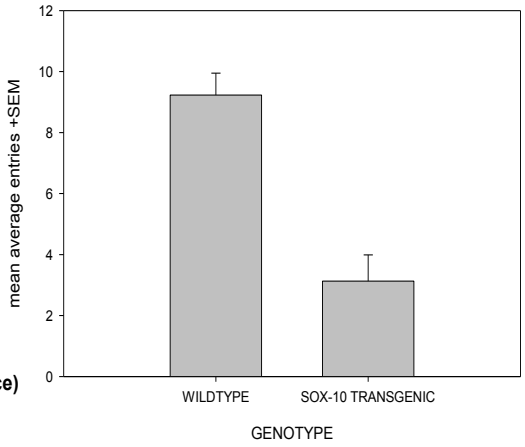

Figure 6c. Light/Dark (Dark Peference)

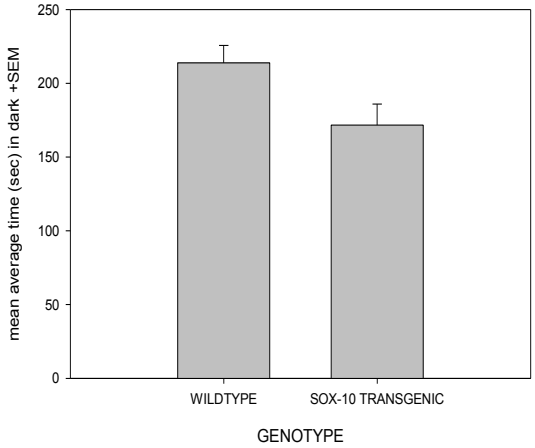

Figure 7a. Male Social Interaction (Latency to Fight)

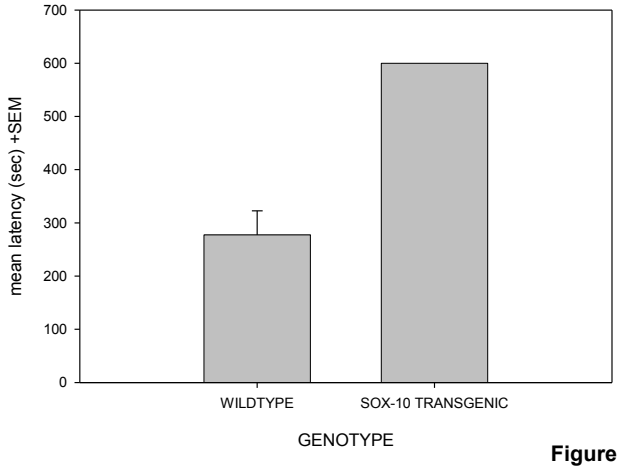

Figure 7b. Male Social Interaction (Wrestle/Spar Duration)

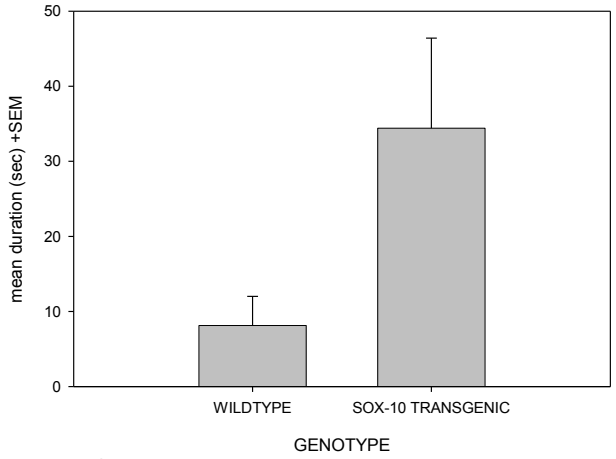

Figure 7c. Male Social Interaction (Frequency to Wrestle/Spa)