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AN INVESTIGATION OF ATTENTION IN DOPAMINE β-HYDROXYLASE KNOCKOUT MICE

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An abstract of
A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
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Abstract

An Investigation of Attention in Dopamine β-Hydroxylase Knockout Mice

By Aman Barkat Ali

The aim of this research was to test the hypothesis that norepinephrine signaling is critical for attention tasks in mice. To do this we used homozygous (DBH -/-) norepinephrine knockout mice and heterozygous (DBH +/-) mice and predict that the KO mice will perform lower on an attention test when compared to heterozygous (DBH +/-) mice, which are capable of synthesizing norepinephrine. Seven DBH -/- and seven DBH +/- mice were trained to nose poke on a Fixed-Ratio 1 (FR1) schedule for a palatable food reward without food deprivation. Twelve mice (6 of each genotype) were subsequently tested on the 3CSRTT test of attention. The results showed that DBH -/- mice, compared to the DBH +/- controls, were greatly impaired in 3CSRTT performance. Their poor performance was primarily manifested as more errors of omission. Only 2 of 6 DBH -/- mice learned the 3CSRTT with a 32 sec stimulus duration; while 5 of 6 DBH +/- controls learned the task. In tests at successively shorter stimulus durations (16, 8, 4, 2 sec), significantly more DBH +/- mice met criterion (less than 60% errors of omission and more than 80% correct responses) than DBH -/- mice. The poor performance of the DBH -/- mice was not due to an inability to nose poke, an inability to detect the light stimulus, or lower motivation to respond. Preliminary evidence showed that transient restoration of brain norepinephrine in the DBH -/- mice via DOPS administration tended to reverse their performance deficit. These data are consistent with a powerful role of norepinephrine in attention, and may be suggestive of a role of norepinephrine in Attention Deficit Disorder in humans.

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INTRODUCTION

Greater demands, heavier workload, and more competition have motivated many individuals to use *Adderall*. The use of *Adderall* is continuously increasing on college campuses and in the workforce to enhance productivity (DeSantis et al., 2008). Officially, *Adderall* (constructed of three salts of amphetamine in a single tablet) is a medication prescribed to treat Attention Deficit Hyperactivity Disorder (ADHD). ADHD is an early-onset disorder of inattention, hyperactivity, and impulsivity (Dopheide et al., 2009). It has been proposed that ADHD patients have a deficiency in monoamine neurotransmission, such as dopamine and norepinephrine (Xu et al., 2007). The pharmacological action of *Adderall* is to increase monoamine concentration in the synaptic cleft between neurons; presumably, this increase mediates the attentional enhancement in ADHD patients.

Amphetamine, which is the active ingredient in *Adderall*, increases dopamine, norepinephrine, and serotonin transmission. Amphetamines inhibit the dopamine, norepinephrine, and serotonin reuptake transporters, inhibit monoamine oxidase (which breaks down dopamine, norepinephrine, and serotonin), and lead to dopamine efflux via reverse transport of the dopamine transporter (Dopheide et al., 2009)

In recent years, evidence has suggested that norepinephrine may be a critical neurotransmitter influencing enhanced attention. Systemic injections of idazoxan, which increases norepinephrine release, to Sprague-Dawley rats showed increased amount of time spent around novel and unexpected objects in a familiar hole board (Devauges and Sara, 1990). Devauges and Sara suggested that activation of the noradrenergic system facilitates an attentional shift in rats. Similar conclusions were reached in earlier research which concluded that depleted norepinephrine levels in the hippocampal-cortical region

affected the distractibility of rats when performing a learned response (Roberts et al., 1976). Both of these studies suggest that increased synaptic norepinephrine levels may lead to enhanced attention abilities.

The locus ceruleus is the primary norepinephrine-producing nucleus in the brain. Neurons in the locus ceruleus have axons that penetrate into various parts of the brain, such as the hippocampus, cerebellum, and cerebral cortex (Moore and Bloom, 1979). Decreased norepinephrine transmission will not only affect forebrain areas, but will also decrease excitation of dopaminergic neurons in the ventral midbrain because adrenergic receptors located on the dopaminergic neurons will not be activated (Liprando et al., 2004; Dopheide et al., 2009).

It is important to note that the neurons in the locus ceruleus show two modes of activity (Aston-Jones et al., 2005). In the phasic mode, the neurons fire rapidly in bursts during a brief period of time. In the tonic mode, the neurons are typically constantly active and fire slowly and steadily. Extracellular recordings have shown the phasic mode is associated with good performance in attentional tasks. In addition to the locus ceruleus norepinephrine is also produced in nuclei located within the pons and medulla, which form the ventral adrenergic bundle.

Currently there are medications available that selectively target norepinephrine to treat ADHD. Atomoxetine, also known as *Strattera*, is a selective norepinephrine reuptake inhibitor which has high selective affinity for the norepinephrine transporter but not receptors for norepinephrine or other transmitters. Presumably because of its lack of action on dopamine, it lacks the behavioral activating aspect of amphetamine and is not

categorized as a psychostimulant (Garnock-Jones & Keating, 2009). Atomoxetine was shown effective in treating ADHD in various randomized, double-blind, placebo-controlled, clinical trials (Spencer et al., 1998; Michelson et al., 2002). Atomoxetine does not have a high abuse potential (Heal et al., 2009), again presumably because of the lack of action on dopamine. Although the mechanism of action of atomoxetine appears to enhance norepinephrine transmission, the site(s) of action for the treatment of ADHD is not yet determined; the prefrontal cortex has been proposed (Garnock-Jones & Keating, 2009).

If atomoxetine is effective in treating ADD/ADHD because it facilitates noradrenergic transmission in the brain, does a deficit in noradrenergic transmission manifest symptoms of ADD/ADHD? The experiments reported herein were designed to examine this question. Mice lacking norepinephrine are created in Dr. David Weinshenker's lab at Emory University. These mice lack Dopamine β-Hydroxylase (DBH), which is necessary to convert the precursor dopamine into norepinephrine. DBH -/- mice lack the ability to synthesize norepinephrine but their ability to synthesize dopamine and other transmitters is unaffected. If norepinephrine is important in attention then these DBH "knockout" mice will not perform well on attentional tasks. Based on this knowledge, the specific aim of my experiments is to test the hypothesis that norepinephrine signaling is critical for attention tasks in mice. DBH -/-mice will perform lower on an attentional test when compared to the controls, which are capable of synthesizing norepinephrine.

If one took control adult mice or rats and blocked NE transmission in their brains via an acutely administered drug and examined their behavior, the results would be

valuable but would only partially model ADHD. This is because ADHD is a developmental disorder. The DBH-/- knockout, on the other hand, alters the developmental trajectory of the knockout mouse's brain. This, of course, is exactly what is thought to be the case with behavioral developmental disorders such as ADHD.

METHODS

Animals

Professor David Weinshenker of the Department of Human Genetics, School of Medicine, Emory University, has generated "knockout" mice which do not synthesize norepinephrine in their bodies (Weinshenker et al., 2002). These mutants (DBH-/- mice) do not make norepinephrine because they lack the enzyme dopamine β-hydroxylase necessary to convert the precursor dopamine into the neurotransmitter norepinephrine. The absence of norepinephrine during gestation has fatal consequences. For this reason, L-3,4-dihydroxyphenylserine (DOPS) was added to the maternal drinking water until birth, providing sufficient norepinephrine to the gestating pups. After birth, the pups are no longer exposed to DOPS and survive, but cannot produce norepinephrine or epinephrine (primarily in the body; there is little epinephrine in the brain) on their own. Heterozygotic littermates (DBH +/-) have control levels of norepinephrine and in previous work have exhibited no behavioral differences from wild type strains (Weinshenker et al., 2002). These DBH +/- littermates were used as controls.

In the following experiments, the DBH -/- knockout mice behavior was directly compared to DBH+/- heterozygous mice. A total of fourteen mice were initially tested, of which seven were mutants (DBH-/- mice) and seven were DBH+/- heterozygous mice. Of the initial mice used, one mutant mouse was eliminated from testing because he failed to meet response criteria for FR1. Also, one control was eliminated from testing because he was accidentally exposed to an erroneous computer program that altered his subsequent behavior.

All animals were singly housed in a temperature and humidity-controlled environment on a 12-hr lighting cycle and had unrestricted access to food and water. Except for the 12-hr overnight nose-poke training, all behavioral procedures through 3CSRTT training were conducted between 0900 and 1500h with colony lighting on 0700 – 1900 hrs. 3CSRTT testing was conducted between 1500 – 2100 hrs with colony lighting off 1400- 0200 hrs. The 3CSRTT testing was conducted during the dark phase of the lighting cycle to encourage responding. All procedures were approved by the Emory University Animal Care and Use Committee.

Apparatus

The mice were tested in an aluminum and Plexiglas chamber (Med Associates, Georgia, VT) measuring 15.5 cm x 16.5 cm x 12.7**.** This chamber was enclosed in a larger sound-attenuating chamber which was located in a room separate from the programming and recording equipment. Nose-poke responses were made into 1 cm-diameter holes in the chamber wall; each hole was equipped with an infrared sensor and a stimulus light. The centers of the holes for nose pokes were 1.5 cm above the bottom of the chamber; the numbers of holes and their locations varied with the phase of the experiment (see information below). Nutritionally-balanced 14 mg food pellets (BioServe, Frenchtown, NJ) were delivered to a hopper in one wall 0.5 cm above the bottom of the chamber.

24-Hour Food Intake

All fourteen mice were individually housed in their control cages in the colony. Each mouse was given a weighed amount of laboratory rodent diet. Each day, a mouse

and its laboratory rodent diet were weighed on a Mettler PM3000 electronic toploading balance. For each mouse, the body weight and the amount of food eaten were recorded for five days. During this time, the mice did not leave their respective boxes and they were not trained or tested for any task.

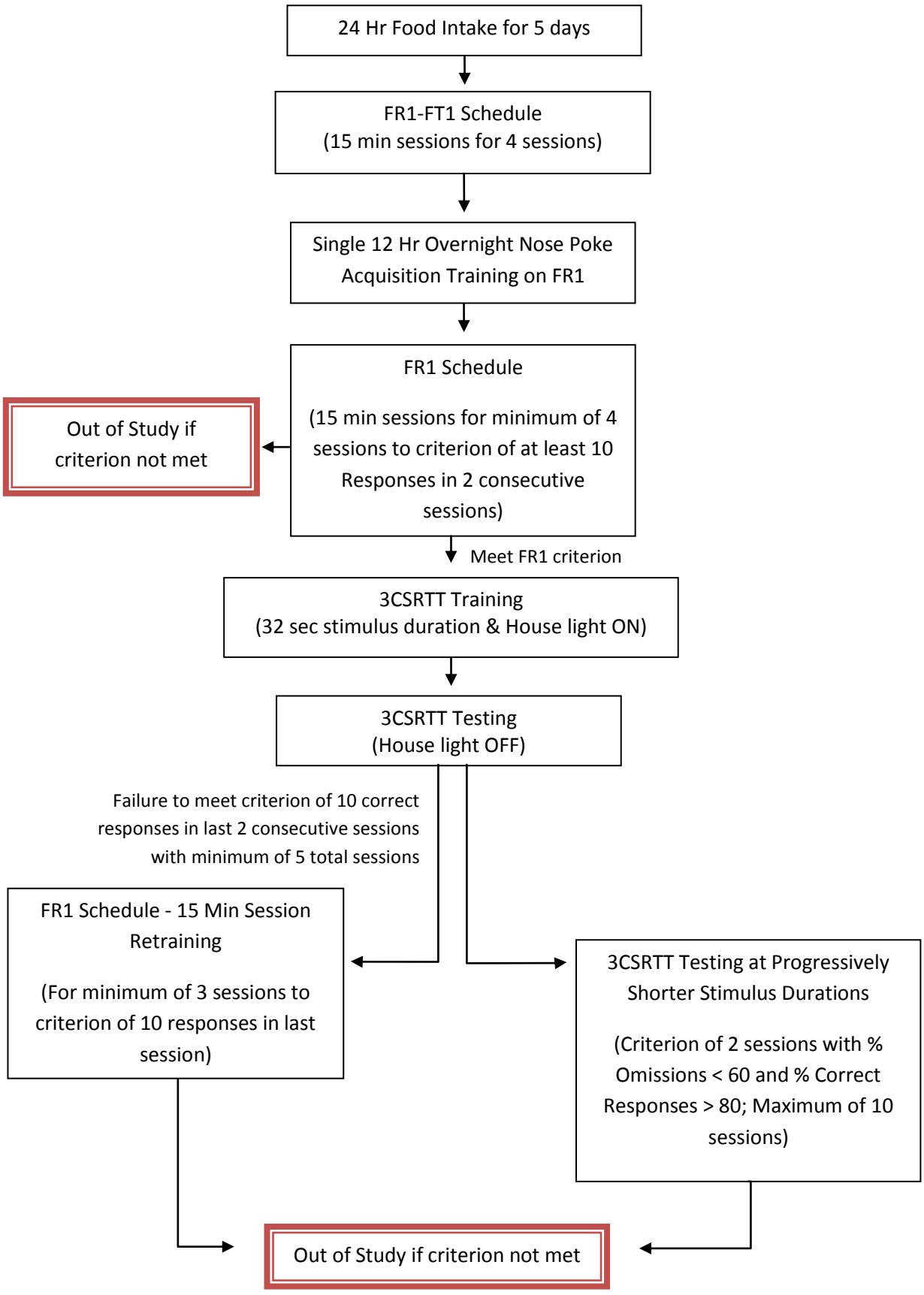

Figure 1: A flow chart of the sequence of experimental procedures.

Training

Training the mice for the experiment involved several steps, shown in Fig. 1. Next, the mice were trained to acquire the food pellet by nose poking in any of the three holes on the chamber wall opposite the food hopper on an FR1 schedule; the hole next to the food hopper was removed. After this basic training, the mice were ready to be trained for the 3CSRTT.

FR1-FT1 Schedule

All fourteen mice were first trained on the FR1-FT1 schedule to acquaint them with the chamber, the nose-poke response, and the reward. Food pellets were automatically delivered once a minute without a required response (FT1). In addition, if the mouse responded at the single hole next to the food hopper, a pellet was also delivered (FR1); thus the schedule was a FR1-FT1.The FR1-FT1 program was set to run for 15 minutes, constituting one session. The house light was programmed to be on during the entire session. One session was conducted each day for 4 days. In the fourth session, because most of the animals did not acquire responding, the mice were food deprived for 24 hours before running the FR1-FT1 schedule.

Overnight 12-Hour FR1

As mentioned above, many of the mice, both knockout and control, did not acquire robust responding on the FR1-FT1 schedule. Therefore, all fourteen mice received one overnight nose poke acquisition training on a FR1 schedule for 12 hours. In the testing chamber, there was only one nose poke entry hole present, and it was next to the food hopper; a water bottle was also installed. Under the FR1 schedule, a food pellet

was delivered only via nose poke; no "free" pellets were delivered. The house light remained on during the entire 12 hours, which lasted 2100 hrs – 0900 hrs.

FR1 Schedule Training

After completing 12-hr FR1 training, all fourteen mice were further trained on the FR1 schedule for 15 minutes per session for a total of four sessions. The house light was programmed to remain on for the session duration. All of the FR1 schedule testing was conducted between 1200 to 1700 hours. A mouse must have met the criterion of at least 10 responses (nose pokes) in two consecutive sessions to advance to the Three Choice Serial Reaction Time Task (3CSRTT) training. If a mouse failed to meet the criterion then it was removed from the study (see Fig. 1).

3CSRTT Training

Thirteen mice met the FR1 criterion and were trained on the 3CSRTT, which measured the attentional abilities of the mouse. For this behavioral test, a mouse was placed in a chamber identical to those used for earlier FR1-FT1 and FR1 response training, except the single hole adjacent to the food hopper was absent and replaced by 3 holes horizontally spaced at equal distances on the wall opposite of the hopper. In the 3CSRTT, the animal must respond during the time one of these holes is illuminated, and only in the illuminated hole, to receive a food pellet. 3CSRTT Sessions were 20 min in duration, conducted at 1500 hr – 2100 hr during the dark phase of a 1400 hr – 0200 hr "reversed" colony lighting cycle. Mice were transferred to and from the testing chamber in their home cages, which were covered by black cloth.

The sequence of possible events in the 3CSRTT procedure is shown in Fig. 2. In initial training/testing, a light was illuminated for 32 sec in one of the holes. If the mouse poked in that hole (correct response) during the 32 sec, a food pellet was delivered, the chamber stayed lit, and 5 sec later a new trial began with the illumination of another hole. Choice of hole to illuminate was decided by the computer using a random number generator. If the mouse poked the incorrect hole (incorrect response), then the mouse was punished by not receiving a food pellet and the house light was switched off for 5 sec (time-out), after which another trial began. Finally, if the mouse did not respond at all (error of omission) during the 32 sec, the house light was switched off at the end of the 32 sec period for a 5 sec time-out, and another trial began. The computer was programmed to end the 3CSRTT test at either a maximum of 60 trials or at 20 minutes, whichever came first.

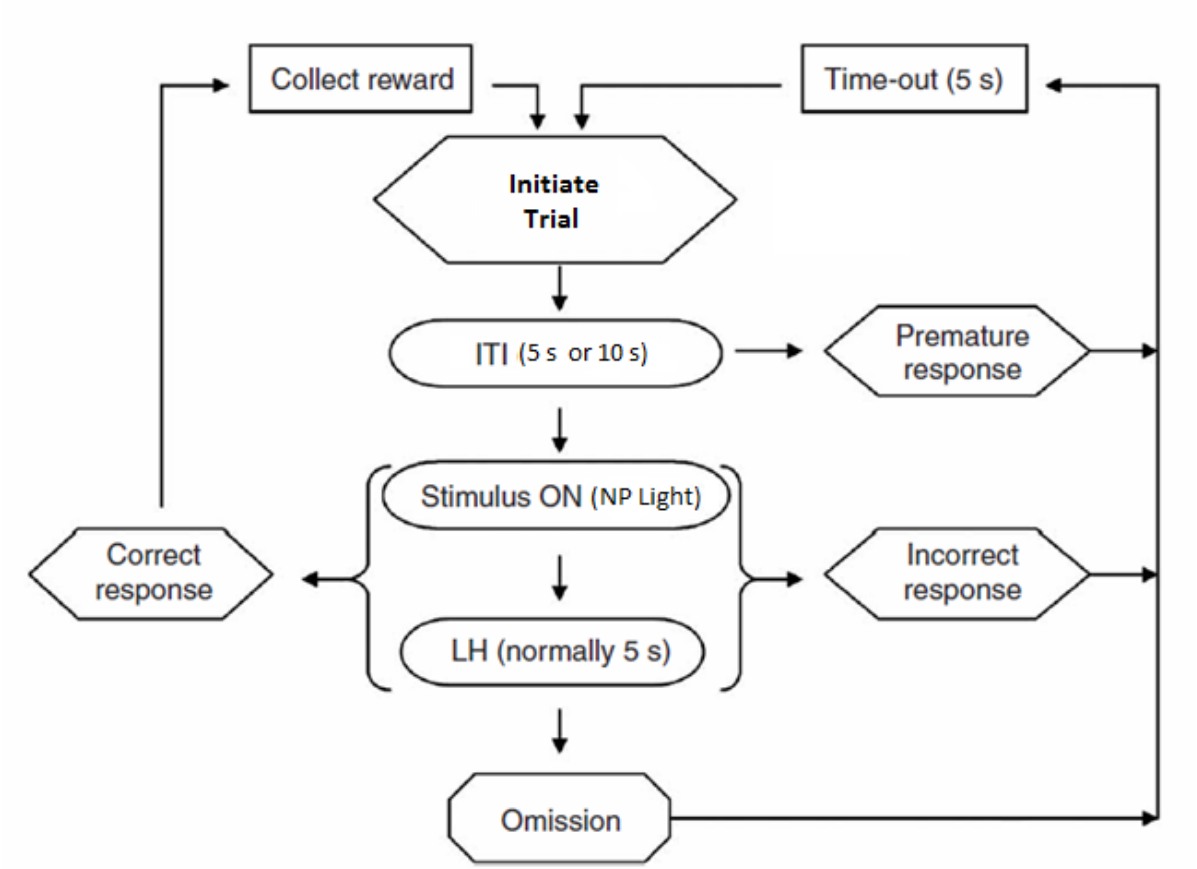

Figure 2: Flowchart of possible trial sequences in the 3CSRTT program (modified from Bari et al., 2008).

3CSRTT Testing

Twelve mice (6 mutants and 6 controls) were tested on the 3CSRTT test. One DBH -/- mouse was excluded from the study because the use of an erroneously programmed 3CSRTT program resulted in a lasting impairment in performance.

The performance criterion was 80% correct responses with less than 60% omissions for two consecutive sessions. For example, if a mouse met this criterion with a 32 sec stimulus duration, it proceeded to tests with a 16 sec duration, then an 8 sec duration, etc. If a mouse failed to meet the criterion after 10 sessions at a given stimulus duration, it was removed from 3CSRTT testing. If the mouse failed to make a total of ten

correct responses for the last two sessions in a minimum of 5 sessions of the initial 32 sec stimulus duration training, and was in danger of stopping all responding, it was removed from 3CSRTT training and returned to the FR1 schedule for retraining.

Modifications were made to the program based on experimenter observations. The program for the house light was reversed from 3CSRTT training, meaning that house light was switched off during the 3CSRTT testing and was only illuminated in the 5 sec time-out. After the initiation of a trial, the presentation of the stimulus occurred after 5s (inter-trial interval, ITI) for stimulus durations of 64, 32, and 16 sec. For all shorter stimulus durations (8, 4, 2, and 1 sec), the ITI was set at 10s to allow the mouse to have ample time to eat the food pellet from the food dispenser before another trial began. The duration of time-out after an incorrect response or an error of omission was kept at 5s. The limited-hold time (LH) of 5s, implemented to allow a mouse approaching but not yet responding to an illuminated hole to make a response, was used with all stimulus durations.

If a mouse at 32 sec stimulus duration failed to make a total of 10 correct responses in 2 consecutive sessions with a minimum of 5 sessions, he was returned to a FR1 schedule with the house light off to hopefully re-acquire responding.

If, at any stimulus duration, a mouse failed to meet criterion within 10 sessions and thus progress to the next shorter stimulus duration, it was removed from 3CSRTT testing.

FR1 Schedule – Retraining

The FR1 schedule for retraining followed the same rules as the previous FR1 schedule except the program code for the house light was altered to conduct retraining in the dark. The stimulus hole was constantly illuminated for the mouse to poke for the duration of the retraining. All retraining was conducted between 1800 to 0100 hours during the dark cycle. Mice were retrained for a minimum of 3 sessions to a criterion of 10 responses in the last session. If the mouse failed to meet the criterion during retraining, then it was removed from the study. Once a mouse met retraining criterion, he was tested on a 64 sec stimulus duration 3CSRTT. If he met criterion on the 64 sec duration, he was tested at 32 sec duration. If criterion was met at 32 sec, testing was conducted at 16 sec, etc.

DOPS Administration & 3CSRTT Test

Mice that were out of the study due to a failure to meet a criterion were excellent candidates for subcutaneous DOPS administration. DOPS was administered at 1200 hours and the mice were placed back in their home cages with lighting cycles at 0200 to 1400 hours. 3CSRTT testing was conducted between 1600 to 2000 hours, because norepinephrine levels peak in the brain after four to six hours after DOPS injection. Baseline 3CSRTT tests were conducted two days after DOPS injections. Vehicle injections were administered 3 days after DOPS injection. Vehicle injections were also given at 1200 hours and the mice were placed back in their home cages. After vehicle injections, 3CSRTT testing was conducted at 1600 to 2000 hours.

PREDICTED EXPERIMENTAL OUTCOMES

The mutants (DBH-/- mice) will show lower attention abilities when compared to the DBH+/- heterozygous mice. In the 3CSRTT, mutants may show higher errors of commission and/or errors of omission. Error of commission will occur when the mouse nose pokes in the absence of a stimulus. Error of omission will occur when the mouse fails to nose poke after a stimulus. The heterozygous mice are expected to perform fewer errors and display a greater attention in the 3CSRTT.

Subcutaneous DOPS injections will enhance the KO mouse's performance on the 3CSRTT by lowering omissions and increasing the number of correct responses. DOPS injections in control mice will not alter their 3CSRTT performance because NE is already present in their brain.

RESULTS

Qualitative Measurements of DBH KO Mice

As shown in Table 1, Knockout mice were generally smaller in size compared to the heterozygous (control) mice. They also showed ptosis (droopy eyelids). In their home cages and the testing chamber, knockout mice were often observed doing repetitive grooming of fur and nails. When startled, knockout mice had a tendency to freeze and vibrate their bodies.

24-Hour Food Intake

All fourteen mice were measured for their body weight and the amount of food consumed for five days (Table 1). The data for the mice that were excluded (3420[KO] and 3586[Control]) from the 3CSRTT study are included. The statistical t-test and graphical representation of the food intake data does not include the two mice that were excluded from the 3CSRTT test. Knockout mice had a lower body weight than the controls; however, average food consumption per day by the knockouts was higher than the controls

Food Weight - KO

Mouse	2/2/2010	2/3/2010	2/5/2010	2/6/2010	2/7/2010	Average
3422	4.2	3.8	4.8	4.7	3.5	4.2
3420	4.7	4.6	5.7	5.7	4.4	5.02
3421	4.6	4.8	4.9	5.1	4.6	4.8
3396	4.7	5.7	4.5	5.3	4.3	4.9
3330	3.2	3.8	4.1	4.3	3.5	3.78
3329	4	4.1	4	4.4	3.7	4.04
3587	4.2	4.9	5.6	5.5	3.9	4.82
					Total Avg	4.508571

Food Weight - Control

Mouse	2/2/2010	2/3/2010	2/5/2010	2/6/2010	2/7/2010	Average
3419	4.2	4.3	5	5.5	4.2	4.64
3395	4.2	4.7	5.3	4.6	4.1	4.58
3327	3.8	4	3.8	5	4.2	4.16
3586	4	3.4	3.4	4.8	2.4	3.6
3585	3.1	3.3	3.3	3.6	3.3	3.32
3584	3.3	3.6	4.1	5.2	3.7	3.98
3583	3.4	3.8	4.1	5	3.3	3.92
					Total Avg	4.028571

Body Weight - KO

Mouse	2/2/2010	2/3/2010	2/5/2010	2/6/2010	2/7/2010	Average
3422	28.5	28.2	29	28.9	28.6	28.64
3420	27.7	28.4	28.8	28.6	28.5	28.4
3421	30.8	31	31.6	31.8	31.4	31.32
3396	27	27.5	27.6	27.6	27.2	27.38
3330	27.8	28	27.6	28	27.6	27.8
3329	27.8	28.4	28	28	27.7	27.98
3587	23.6	23.9	24.5	23.9	24.1	24
					Total Avg	27.93143

Body Weight - Control

Mouse	2/2/2010	2/3/2010	2/5/2010	2/6/2010	2/7/2010	Average
3419	35.7	36.1	35.6	35.7	35.2	35.66
3395	30.9	31.1	31.4	31	30.8	31.04
3327	30.9	31.3	30.9	31.3	31.2	31.12
3586	32.9	32.3	32.5	33	32.6	32.66
3585	29.3	28.8	28.5	28.6	28.6	28.76
3584	29.7	29.3	29.5	30.2	30.1	29.76
3583	33.2	33	33.1	34.4	33.2	33.38
					Total Avg	31.76857

Table 1: Body weight and food weight data for 5 days. Mice 3420(KO) and 3586 (Control) which were excluded from the 3CSRTT testing are included in the tables.

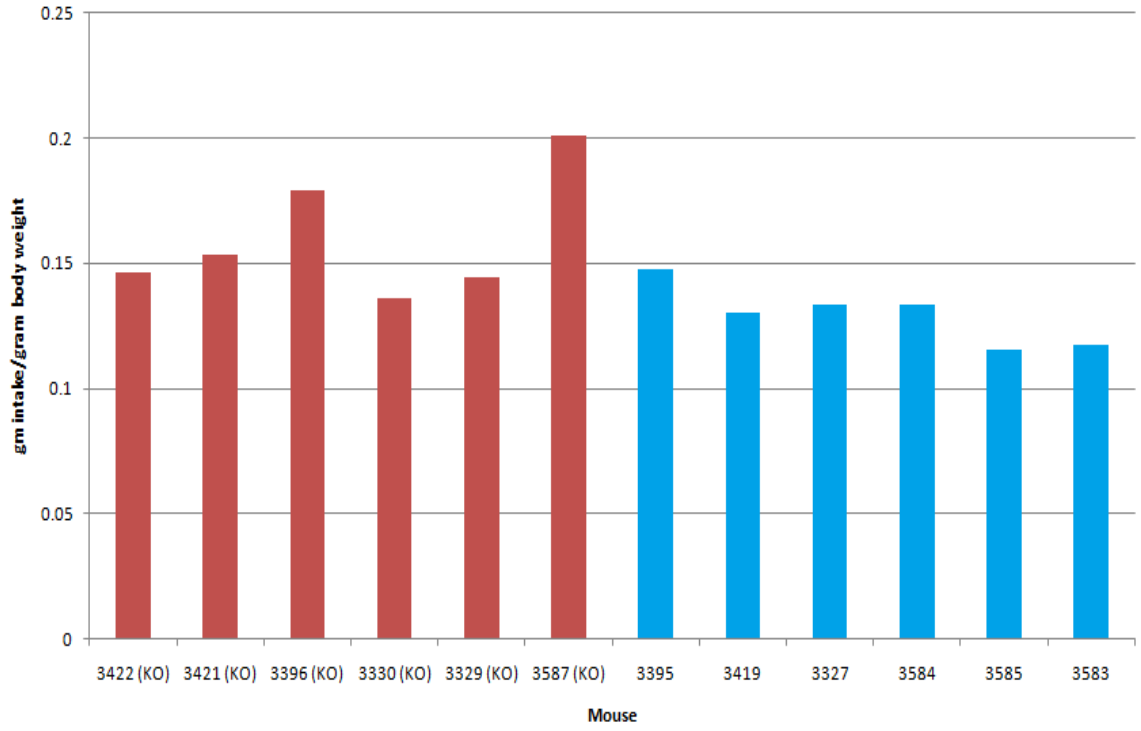

Figure 3: Food intake data measured in grams of food intake per gram of body weight (t=2.71 df =10, p=0.02).

Fig. 3 shows grams food intake per gram body weight for each mouse, averaged across the 5 days of 24-hr measurements. Knockout mice statistically (p = 0.02) consumed more food than the control mice with three knockouts averaging above 0.15 in grams intake per gram body weight; no control mice were able to average above 0.15. The general trend observed in the knockout mice was that a lower body weight was related to more food consumption per day. This trend was not observed in the control mice. Even if the two mice subsequently excluded from 3CSRTT testing were included in the t-test for food intake/body weight, the p-value remained at 0.02.

FR1-FT1 Schedule/15 min Sessions

All fourteen mice were trained on the FR1-FT1 schedule for four days. Each mouse received at least 15 food pellets per session, but most mice did not consume each pellet. Many of the mice failed to nose poke to receive additional food pellets. On the fourth day, all fourteen mice were food deprived for 24 hours before being run on the FR1-FT1 schedule. Similar results were observed on the fourth day with no remarkable increase in the number of food pellets eaten or the number of nose pokes. All mice were subsequently moved to overnight nose-poke training.

Single 12-Hr Overnight FR1

All fourteen mice were trained on the single 12-hr overnight FR1 schedule. In the graphical representation (Fig. 4), data from the two mice are not present because they were excluded from the 3CSRTT test.

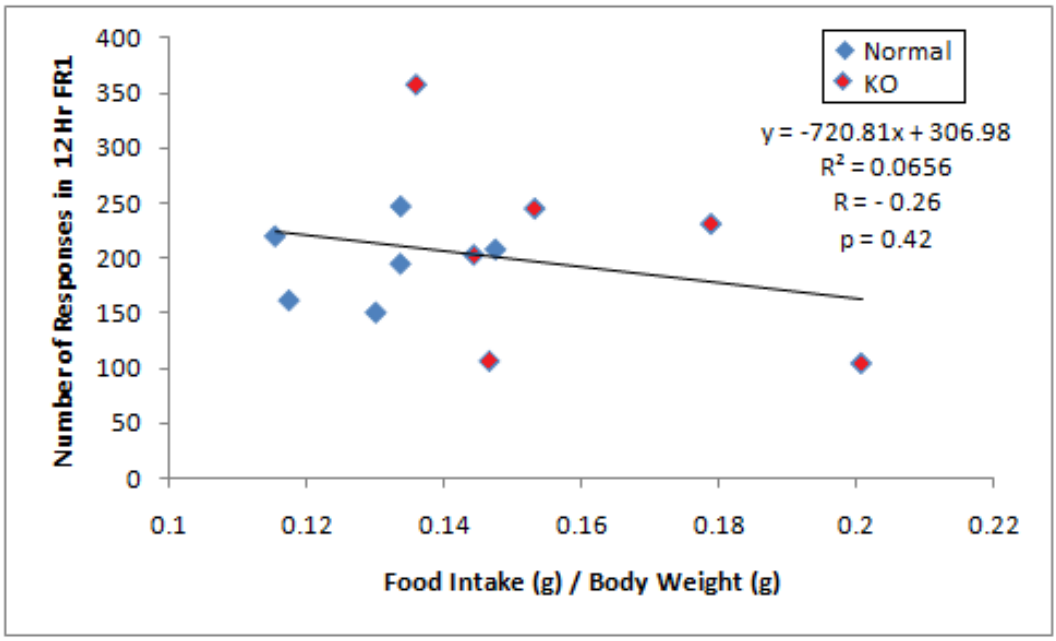

Figure 4: Number of responses in a single 12-hr overnight FR1(r=-0.26, p=0.42, n=12).

For the 12-hr FR1 schedule, the knockout mice had a greater variance in the number of nose pokes compared to the control mice. Because the knockout mice ate more per gram body weight in their home cages, one might expect them to make more responses in the 12 training session. However, Fig. 4 shows that food intake per body weight was not statistically related to the number of nose pokes on the FR1 schedule and that there was no significant difference in average responding by the two groups ($t = 0.26$, $df = 10$, $p = .80$).

FR1 Schedule/ 15 min Sessions

After the 12-hr FR1 schedule, all mice were run on the 15 minute FR1 schedule for 4 sessions to check that nose-poke acquisition had occurred. Data from all fourteen mice are recorded below (Table 2). Knockout mouse 3420 failed to meet the criterion of at least 10 total nose pokes in two consecutive sessions and was excluded from progressing to the 3CSRTT test.

Mouse	12-hr FR1	1 *	2 *	3*	4*	Avg 15min
3395	208	18	25	6	8	14.25
3419	151	14	8	12	19	13.25
3586	160	2	8	10	5	6.25
3327	195	19	6	17	18	15
3584	247	15	23	27	24	22.25
3585	220	18	18	19	19	18.5
3583	162	11	12	9	13	11.25
3422 (KO)	107	32	15	26	17	22.5
3421 (KO)	245	0	1	16	11	7
3396 (KO)	231	16	3	4	8	7.75
3330 (KO)	357	28	21	19	25	23.25
3329 (KO)	203	19	11	4	13	11.75
3587 (KO)	105	0	13	9	10	8
3420 (KO)	6	0	0	2	0	0.5

*Table 2: Number of nose pokes for 12-hr FR1 and 15 minute FR1 schedules. (*Numbers 1-4 represent four daily 15 min. FR1 training)*

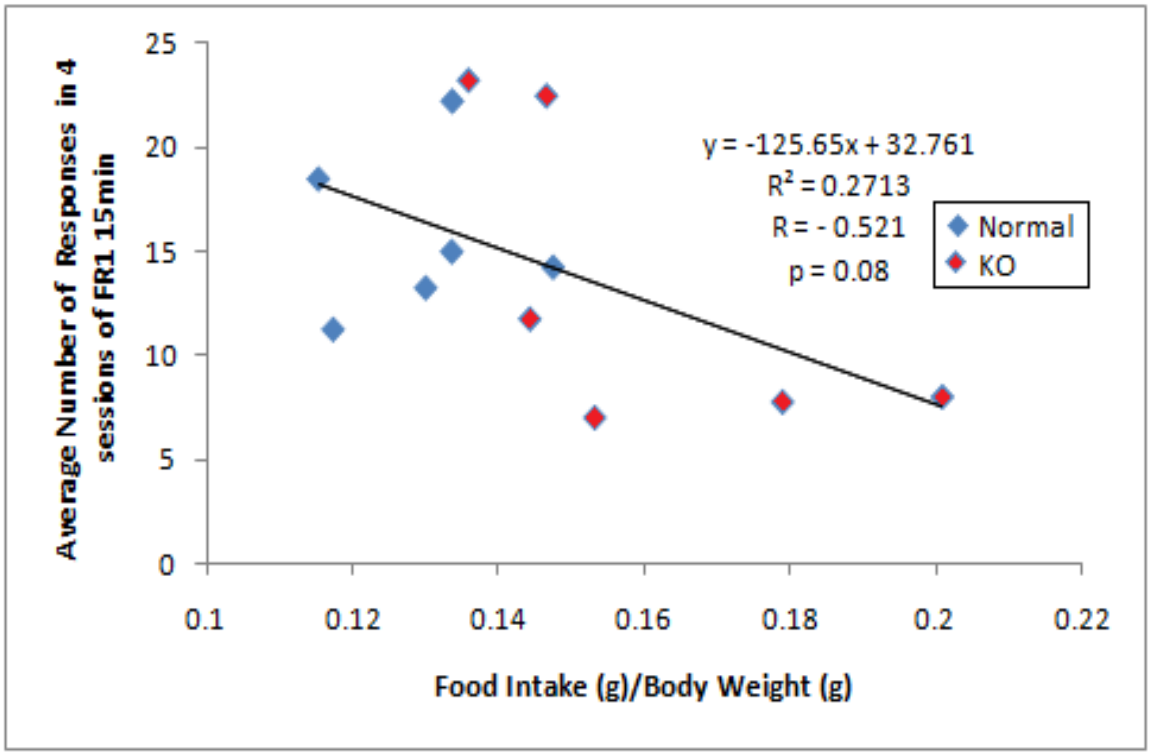

Figure 5: Average number of nose pokes on 15 min. FR1 schedule (r=-0.5, p=0.08, n=12).

In Fig. 5, mouse 3420(KO) and 3586 are not included because they were excluded from the 3CSRTT test. The figure shows that average number of nose pokes on the 15 min FR1 schedule tended, although without statistical significance (p = 0.08), to be inversely related to food intake/body weight. Once again, the knockouts had a greater variance in the average number of nose pokes compared to the controls.

3CSRTT Training

All FR1 trained mice, a total of thirteen, were started on the 3CSRTT training. The only mouse excluded from the 3CSRTT training was knockout mouse 3420. During the 3CSRTT training, numerous complications were encountered and solved. The food hopper did not have an infrared sensor to initiate a new trial as commonly observed in the

rat version of the 3CSRTT, so the program was modified to initiate the next trial after five or ten seconds depending on the stimulus duration. Mice are nocturnal and are observed to be less active during their light cycle when compared to their dark cycle, so the 3CSRTT program was altered to run in the dark and the mice were tested during the dark phase of their colony lighting cycle.

3CSRTT Testing

As shown in Fig. 6, control mice progressed to lower stimulus durations on the 3CSRTT test in greater numbers than the knockout mice. The data were analyzed in the following manner. For each group of animals, the number progressing to 64 sec, 32 sec, etc., stimulus durations were recorded. These numbers, after passing a test for equal variance, were then compared between the two groups using a two-sample t-test. There was a significant difference between the groups ($t = 2.83$, $df = 10$, $p = .04$). All mice were initially tested at 32 sec stimulus duration. Therefore mice that progressed to a stimulus duration below 32 sec were assumed to have successfully completed the 64 sec stimulus duration 3CSRTT. Mice who failed to progress below 32 sec were tested on the 64 sec stimulus duration.

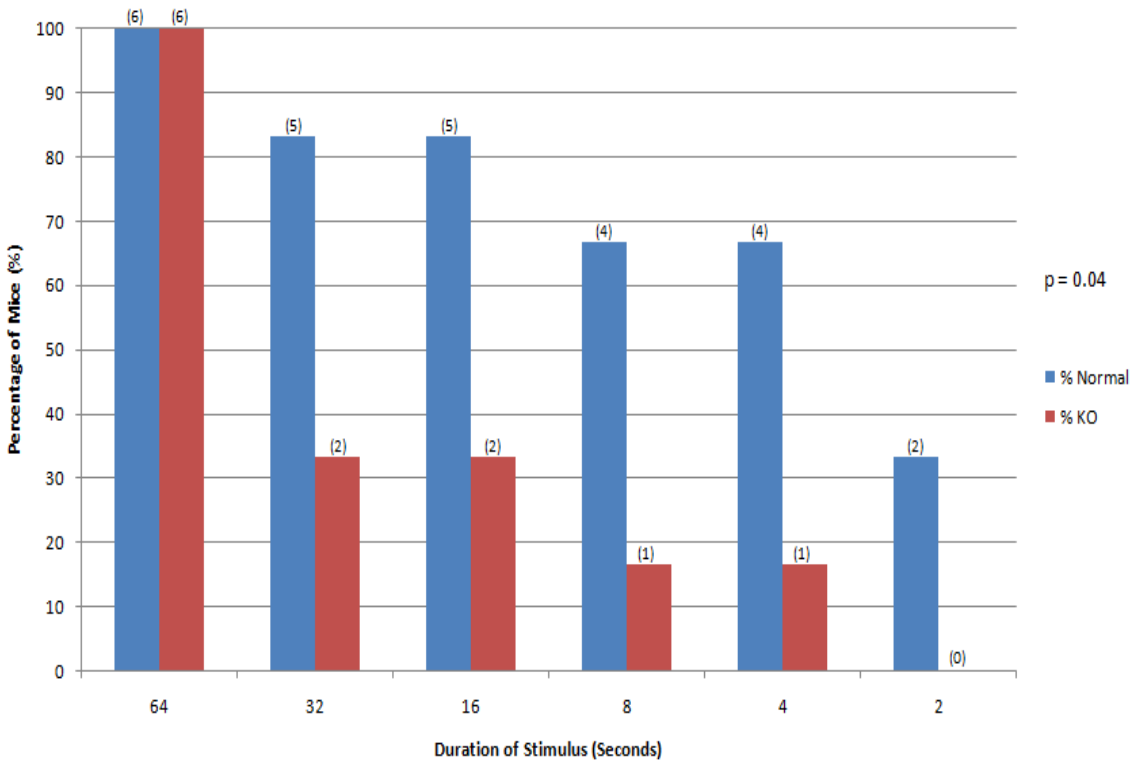

Figure 6: The number of mice who progressed to each stimulus duration on the 3CSRTT test. There was a significant (t = 2.83, df = 10, p = .04) difference in the progression to lower stimulus duration between KO mice and control mice.

As show in Fig. 7, there was a statistically significant difference between knockout and control mice in percent responses, percent omissions, and percent correct from total responses on the 32 sec stimulus duration 3CSRTT test. Control mice responded significantly greater than the knockout mice on the 32 sec stimulus duration (p=0.004). Knockout mice had a significantly greater percentage of omissions when compared to the control mice (p=0.004). On the 32 sec stimulus duration 3CSRTT test, control mice significantly had greater percentage of correct responses from total responses when compared to the knockout mice (p=0.02).

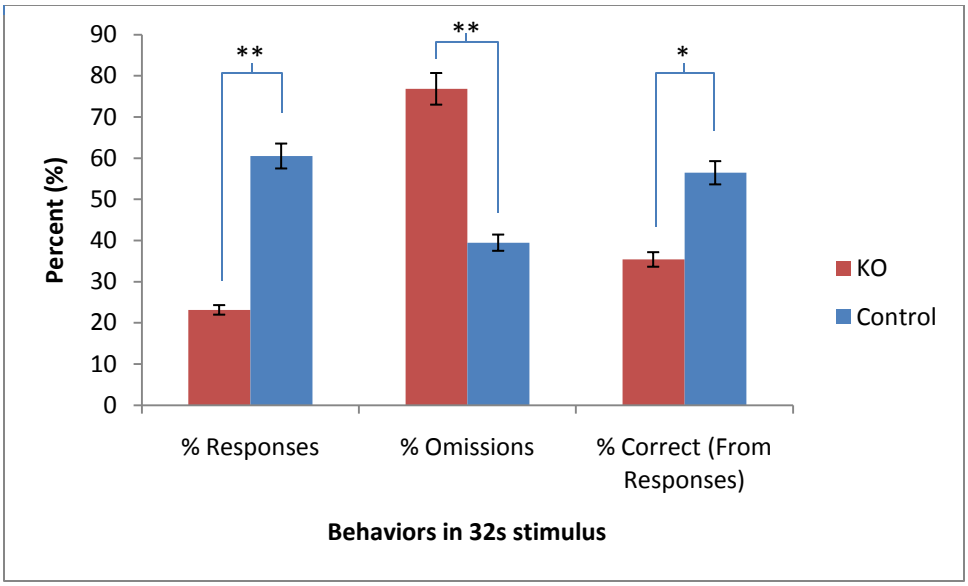

Figure 7: Percentage of overall behaviors for KO and controls on 32 sec stimulus duration on 3CSRTT test. Statistical significance was obtained for all categories with "" representing p<0.05 and "**" representing p<0.005 (p-values: % Responses=0.004, % Omissions=0.004, % Correct from Responses=0.02).*

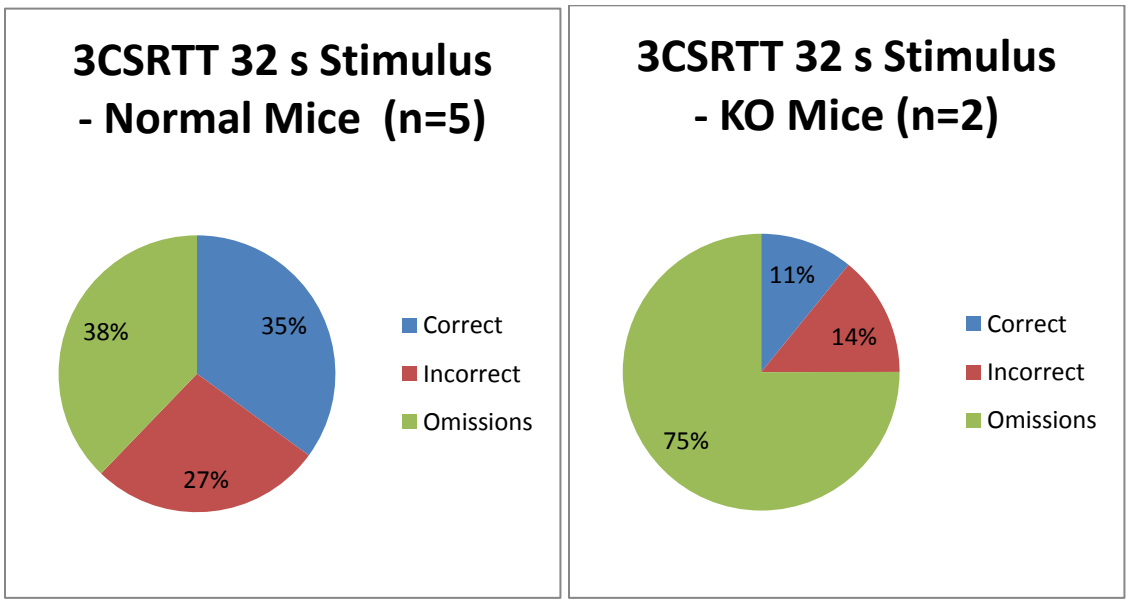

Figure 8a: Percentage of the different types of responses for various stimulus durations on the 3CSRTT test in knockout and control mice.

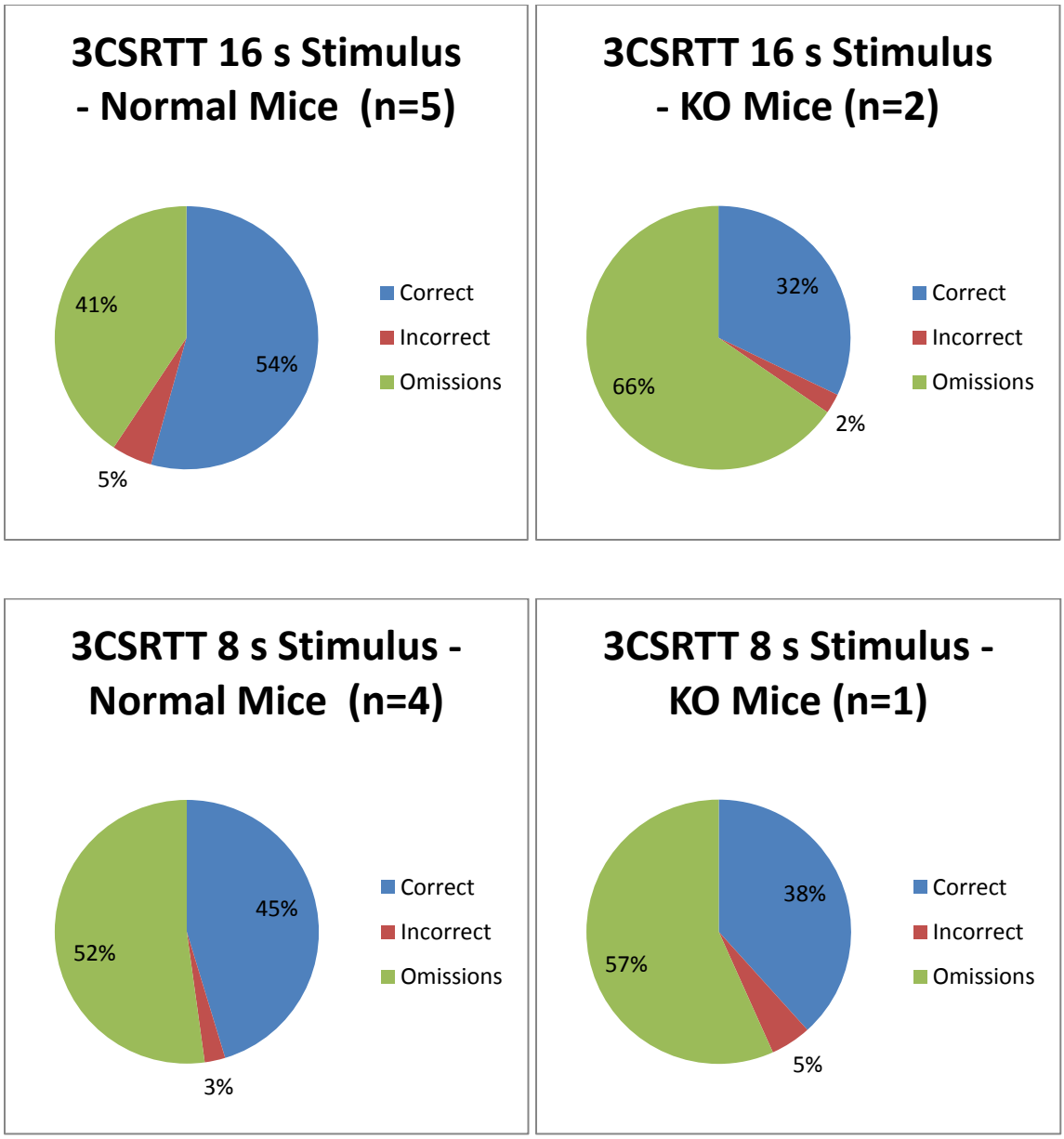

Figure 8b: Percentage of the different types of responses for various stimulus durations on the 3CSRTT test in knockout and control mice.

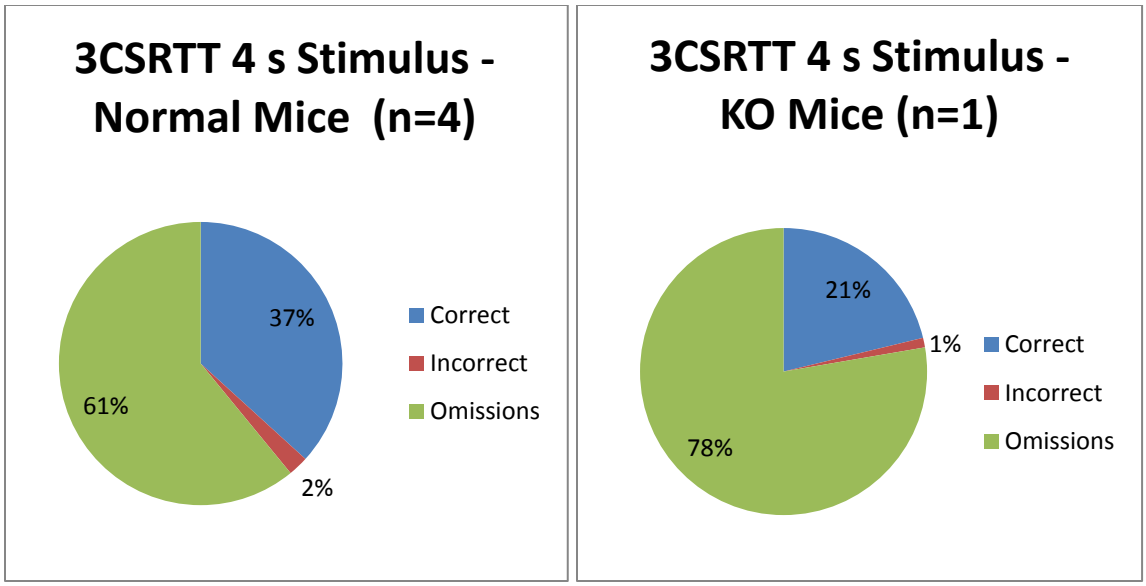

Figure 8c: Percentage of the different types of responses for various stimulus durations on the 3CSRTT test in knockout and control mice.

In the 32 sec stimulus duration, the control mice performed better on the 3CSRTT test in terms of the percentage of correct responses and errors of omission. Control mice responded correctly to 35% of the trials compared to knockouts at 11%. The knockouts were lower in the percentage of correct responses even though they had less responses overall. The knockouts had a higher percent of errors of omission at 75% compared to the controls at 38%. Knockouts were observed to continuously groom fur and nails, perhaps accounting for the high errors of omission when the 3CSRTT test is in progress.

Similar trends were observed at the 16 sec stimulus duration with lower errors of omission and higher correct responses for control mice. The control and knockout mice that progress to the 16 sec stimulus duration, display lower incorrect responses when compared to their performance on the 32 sec test. Comparing to the 32 sec test, errors of omission increased in the 16 sec stimulus duration test for both control and knockout mice.

For the 8 sec stimulus duration 3CSRTT test, the errors of omission continued to increase for the control mice when compared to the 32 and 16 sec stimulus duration 3CSRTT tests. However for the knockouts, the errors of omission decreased at 8 sec compared to the 32 sec and 16 sec stimulus durations. It is crucial to note there was only one knockout mouse that progressed to stimulus duration of 8 sec and less. This one knockout mouse still performed higher errors of omission compared to the average number of omissions observed by the control mice at 8 sec.

At 4 sec stimulus duration, the control mice continued to increase in the percentage of errors of omission when compared to their performance on the higher stimulus duration tests. The control mice had fewer errors of omission when compared to the one knockout mouse at 4 second stimulus duration. For control mice, the percentage of incorrect responses steadily decreased as the stimulus duration decreased. Overall, the control mice had a greater percentage of correct responses for each stimulus duration when compared to the knockout mice. In all stimulus durations, the control mice also had lower percentage of errors of omissions when compared to the knockout mice.

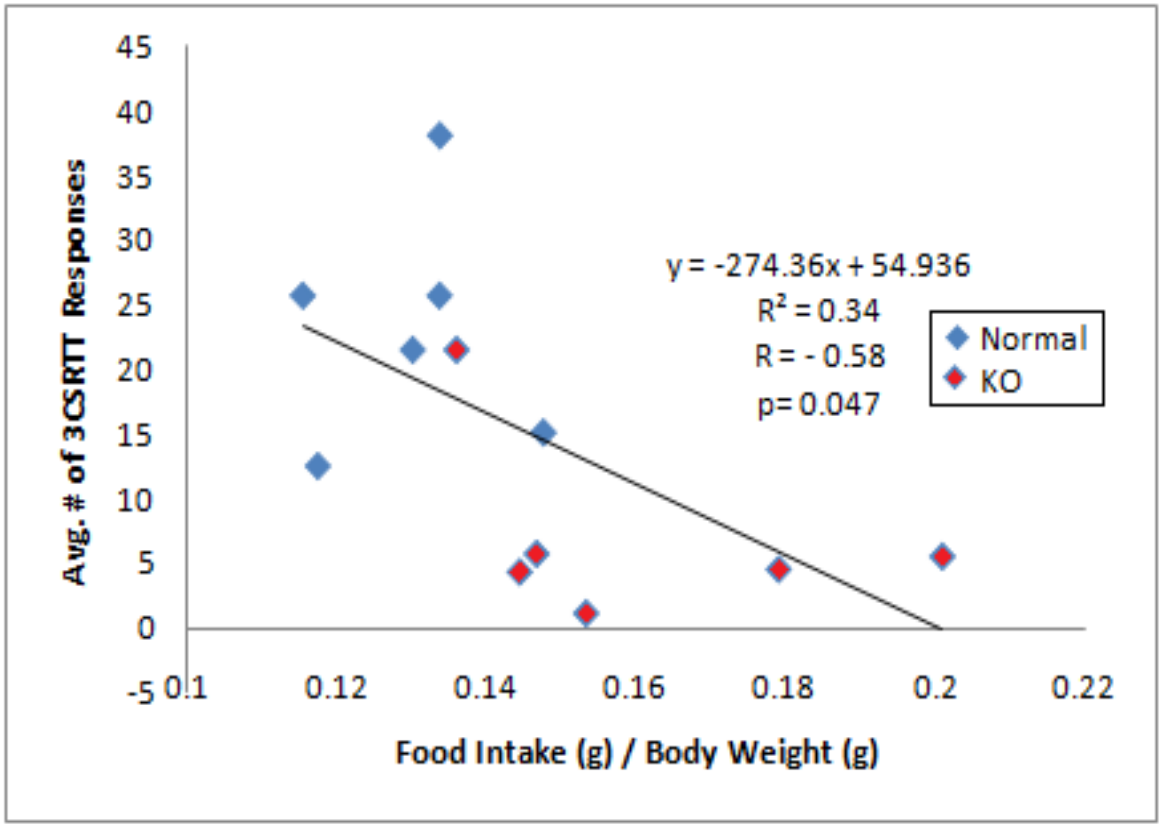

Figure 9: KO mice responded less on the 32 sec stimulus duration 3CSRTT test (r=-0.58, p=0.047).

Knockout mice that had a greater food intake per body weight ratio showed fewer responses in the 32 sec stimulus duration 3CSRTT test (Fig. 9). With a p-value of 0.047, food intake per body weight was inversely related to the average number of 3CSRTT responses on the 32 sec stimulus duration. Knockout mouse 3330 had a low food intake per body weight ratio and performed similar to control mice on the number of 3CSRTT responses.

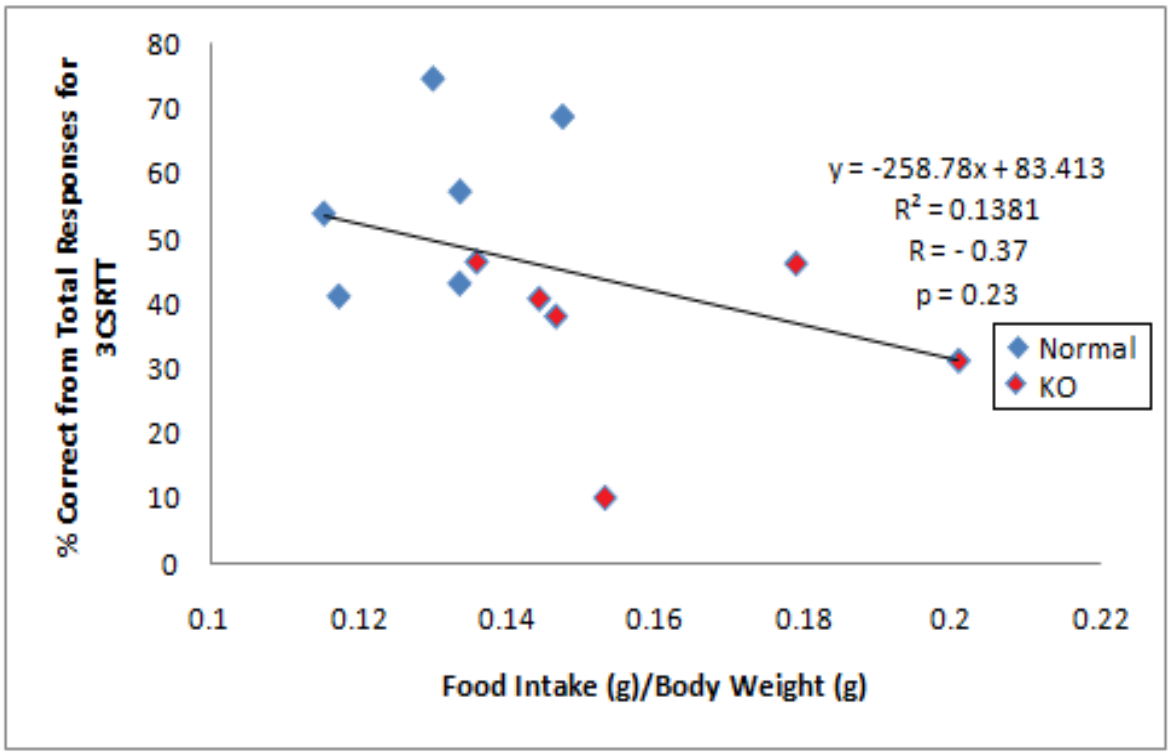

Figure 10: Percentage of correct responses from total responses observed on the 32 sec stimulus duration 3CSRTT test (r=-0.37, p=0.23).

Although there was a general trend that control mice had a higher percentage of correct responses from total responses when compared to knockout mice, there was no statistical significance with a p-value of 0.23 (Fig. 10). It is important to note, as displayed in Fig. 7, that the knockout mice responded significantly less than control mice on the 32 sec 3CSRTT test. Even with less total responses, knockout mice had a general tendency to nose poke the incorrect hole in the 3CSRTT test.

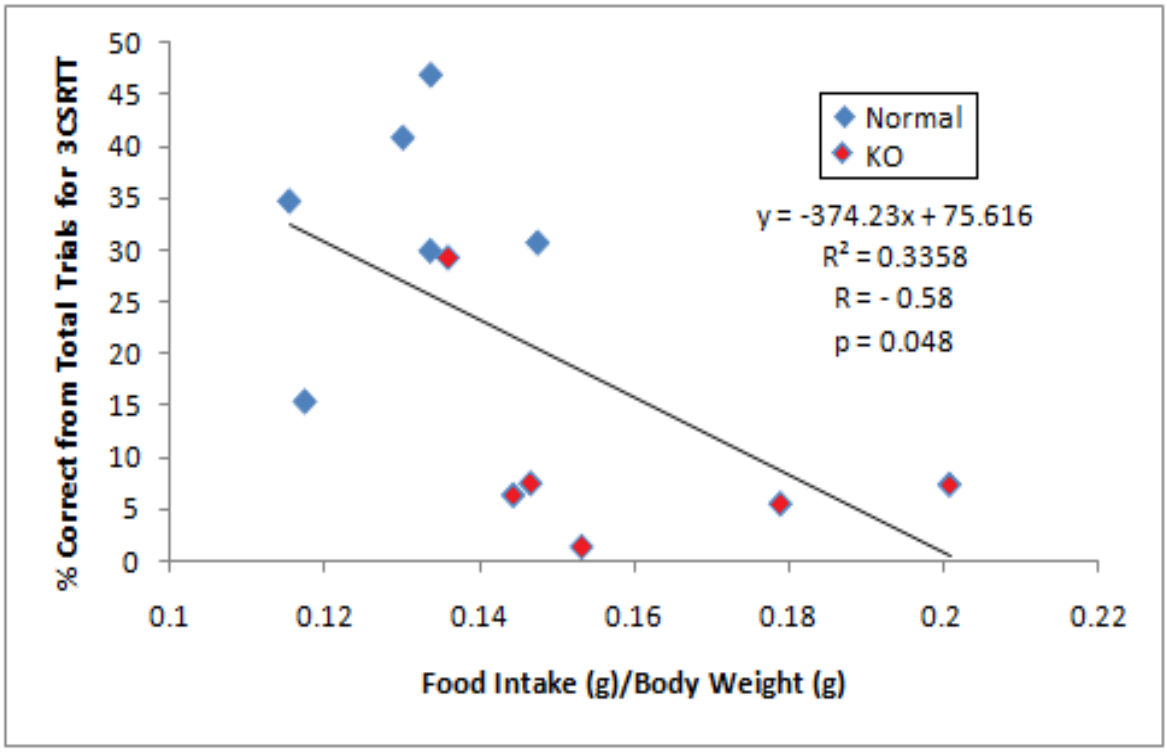

Figure 11: Percentage of correct responses from total trials on the 32 sec stimulus duration 3CSRTT test (r=-0.58, p=0.048).

As previously portrayed (Fig. 3), knockout mice statistically had a higher food intake per body weight ratio. As the food intake per body weight ratio increased, the statistically significant correlation showed less correct responses from the total trials conducted on the 32 sec stimulus duration 3CSRTT; this is shown in Fig. 11 above. Once again, knockout mouse 3330, having a similar food intake per body weight ratio as control mice, had approximately the same percent correct responses from total trials as control mice on the 32 sec stimulus duration of 3CSRTT.

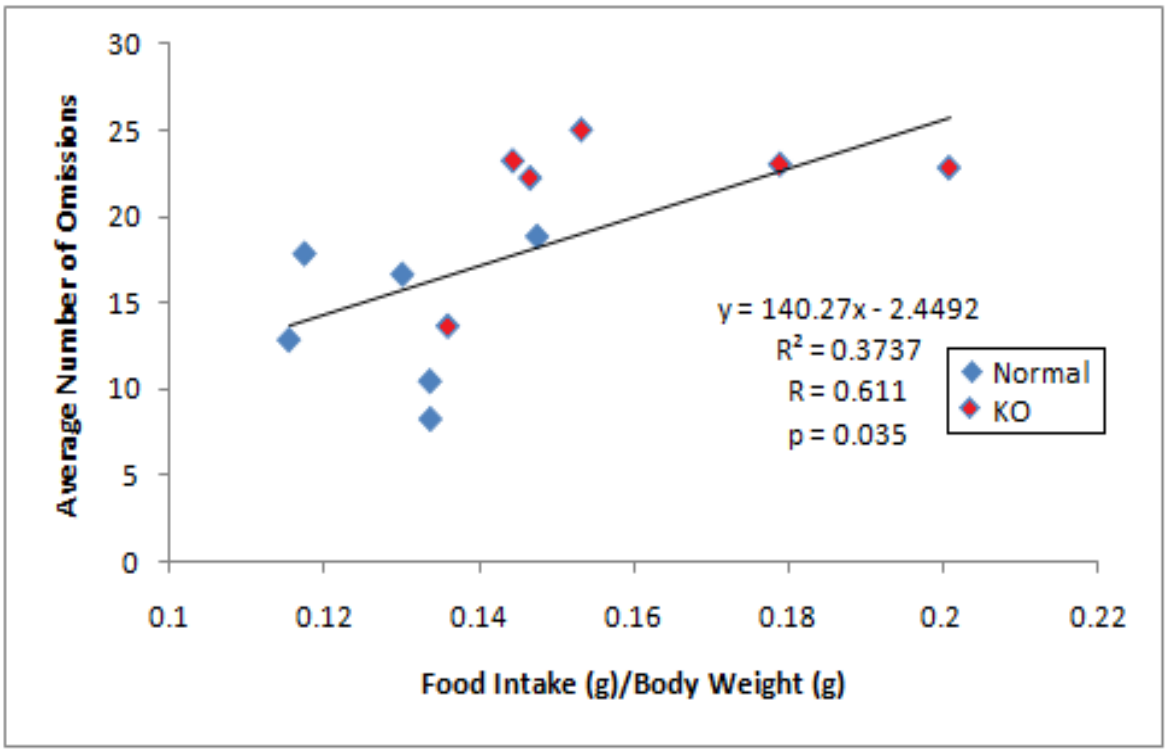

Figure 12: The number of omissions on the 32 sec 3CSRTT increased as the food intake per body weight ratio increased (r=-.61, p=0.035).

Knockout mice displayed higher percentages of errors of omission at all stimulus durations on the 3CSRTT test when compared to control mice (Fig. 8). In the 32 sec stimulus duration 3CSRTT test, a higher food intake per body weight was statistically related to higher numbers of omissions (Fig. 12, above).

DOPS Administration & 3CSRTT

Five knockout mice, who did not meet the criteria for continuation in the 3CSRTT test study, received subcutaneous DOPS injections. All DOPS injected mice were run on the 64 sec stimulus duration 3CSRTT test. Knockout mouse 3420, who failed to meet criterion for FR1, not surprisingly failed to nose poke during the 3CSRTT test following

DOPS administration. The data for mouse 3420 were therefore excluded based on the condition that the mouse never met the criterion to be run on any 3CSRTT test.

		DOPS Injection				Vehicle Injection			
Mouse	Run	Correct	Incorrect	Omissions	Responses	Correct	Incorrect	Omissions	Responses
3396(KO)	1	7	5	11	12	1	6	13	7
	2	4	3	13	7	2	2	13	4
3587(KO)	1	3	5	12	8	0	0	16	0
	2	2	3	13	5	1	1	14	2
3329(KO)	1	1	5	12	6	6	5	11	11
	2	2	3	13	5	7	7	11	14
3421(KO)	1	2	3	13	5	0	1	15	1
	2	0	1	15	1	0	1	15	1
Average		2.625	3.5	12.75	6.125	2.125	2.875	13.5	5

Table 3: Performance on the 64 sec stimulus duration 3CSRTT test after DOPS injection and vehicle injection. The rows numbered "1" represent performance 4-5 hrs after injection; the rows numbered "2" represent performance 6-7 hrs after injection..

In 3 of the 4 knockout mice tested, DOPS injection increased the number of responses and decreased the number of omissions when compared to vehicle injection for the period 4-5 hrs after injection.

DISCUSSION

The data indicate that the DBH -/- knockout mice, compared to DBH +/- controls, are notably impaired in performing the 3CSRTT test of attention. As shown in Fig. 6, more control mice progressed to lower stimulus durations ($p = 0.04$). In the 32 sec stimulation duration test shown in Fig. 7, knockout mice responded significantly less ($p=0.004$), had a greater percent omissions ($p=0.004$), and lower percent correct of responses ($p=0.02$) when compared to the control mice.

The core impairment of knockout mice on the 3CSRTT was the high number of omissions. At the 32 sec stimulus duration, knockout mice had a statistically significant *inverse* relationship of responding with food intake per body weight (Figure 12; $p = 0.035$). That is, even though the knockout mice ate more per unit body weight over 24 hours in their home cages, they responded less in the 3CSRTT test. In fact, knockout mice generally tended to show more percentage of errors of omission for all stimulus durations in the 3CSRTT test when compared with control mice. When knockout mice did respond, their responses showed a trend of higher percentage of incorrect responses compared to the controls. Control mice seemed to distinguish the stimulus better than knockout mice as evident by the continuous decrease in the percentage of incorrect responses as stimulus duration decreased.

The results of the DOPS injections, while only performed in a few knockout mice, strengthen the idea of the importance of norepinephrine in performing on the 3CSRTT task, and therefore in attention. In 3 out of 4 knockout mice tested, DOPS administration decreased the number of omissions, increased the number of correct responses, and increased the number of total responses in the knockout mice. It is important to note that

the subcutaneous DOPS injections in this research study should not alter the body's NE levels; instead it should have selectively increased NE levels in the brain. More sessions of 3CSRTT need to be conducted with DOPS injections to determine if, like the controls, their performance will continue to improve with training. There is not enough statistical power in the data at this point to make strong conclusions but the trends show that after DOPS administration there is a decrease in the number of omissions and an increase in the number of total responses in the knockout mice.

Knockout mice ate more per unit body weight in 24-hr tests, yet responded less on the 3CSRTT test, where responding delivered food. In fact, spontaneous food intake had a statistically significant inverse relationship with 3CSRTT responding ($p=0.047$). My interpretation of this relationship is that high spontaneous food intake is the mark of a "good" knockout mouse, and a "good" knockout will also perform poorly on the 3CSRTT. One knockout mouse (3330) performed similar to control mice in the 3CSRTT test at various stimulus durations. Knockout mouse 3330 had a 13.6 ratio of food intake per body weight, which was the lowest of any knockout mice and closest to the 12.7 average ratio observed in control mice. Despite being an exception to how most knockout mice performed on the 3CSRTT, knockout mouse 3330's ratio of food intake per body weight is still consistent with the trend observed in Figs. 11 and 12.

Alternative Explanations

One alternative explanation is that knockout mice were not interested in the food pellet even though they ate more food per body weight. As seen in the results section, knockout mice ate significantly more food per body weight in 24-hr measures raising the

concern that knockouts may nose poke more simply to eat more food. However, the 12-hr FR1 schedule and 15 minute FR1 schedule results indicate that there was no statistical relationship between FR1 nose poking and the ratio of food intake per body weight.

Another possible explanation is that the impaired performance of the knockout mice on the 3CSRTT test may be related to less locomotor activity. This concern was taken into consideration when modifying the program for 3CSRTT test. Norepinephrine knockout mice and control mice significantly differed in activity levels only in the light phase of the lighting cycle, being less active than controls (Swoap et al., 2003). In the dark phase, there was no significant difference between the two genotypes. Both knockout mice and control mice had significantly greater, and insignificantly different, activity in the dark phase (Swoap et al., 2003). This was one of the reasons why the 3CSRTT program was modified with house lights off and the mice were run during their dark cycle. In addition, knockout mice and control mice did not significantly differ in locomotor activity during the dark and light phases after caloric restriction (Swoap et al., 2003). Therefore it is probable that the impaired performance exhibited by knockout mice on the 3CSRTT test was not solely due to differences in locomotor activity.

During experimentation, there was concern that the knockout mice had visual deficits which impaired them from detecting the light stimulus. However by continuing to run the 3CSRTT tests, I discovered two knockouts that were able to meet the criteria to progress to 16 sec stimulus duration. It is very difficult for a mouse to meet the 3CSRTT test criteria to progress to lower stimulus duration, meaning that it is very unlikely that the two knockout mice that did progress did so by chance. Therefore the two knockouts that progressed to lower stimulus duration were able to distinguish the light stimulus.

Neuronal Alterations

Genetically altered mice that lack the gene that codes for DBH are unable to synthesize norepinephrine and are good candidates for examining the importance of norepinephrine in attention. Over the years, various studies and biological assays have led to the idea (Schank et al., 2006) that there is an up-regulation of dopamine receptors from the chronic lack of norepinephrine. This up-regulation of dopamine receptors makes the norepinephrine knockout mouse hypersensitive to cocaine and other psychostimulants (Schank et al., 2006). However, when norepinephrine is deficient for a short period of time, there is a decrease in dopamine release reducing the effects of cocaine, amphetamines, and other psychostimulants (Ventura et al., 2007). In DBH knockout mice exhibiting a chronic lack of norepinephrine, there is an up-regulation of dopamine receptors making the mouse more hypersensitive to dopamine (Schank et al., 2006).

DBH Knockout Mice & ADHD

Analogous to the 3CSRTT test, the continuous performance test (CPT) is used in humans to measure attention and impulsivity, two main cognitive deficits in ADHD patients (Barry et al., 2009). CPT is the most widely used neuropsychological test to differentiate patients with ADHD from those without the disorder (Kollins et al., 2008). Barry and colleagues (2009) found that when atomoxetine was used by ADHD patients, there was a significant reduction in errors of omission on the CPT. Also, significant associations have been made between a single nucleotide polymorphism (SNP) in the norepinephrine transporter gene and the variability in reaction time (measure for attentional lapses) measured by CPT (Kollins et al., 2008). Norepinephrine activity has

been hypothesized to be associated with a cognitive dysfunction characteristic of ADHD (Viggiano et al., 2004), which is consistent with our findings of impaired performance by norepinephrine knockout mice on the 3CSRTT test.

FUTURE DIRECTIONS

Successful completion of these studies, and publication of the results, will assist in establishing our credentials for neurobehavioral studies of ADHD with mice, particularly “knockout” mice. It will also offer the possibility of applications for extramural funding to further study the DBH-/- mice (e.g., do they have an attentional deficiency mimicking ADD/ADHD?), as well as studies of other mice with altered noradrenergic transmission (e.g., noradrenergic reuptake transporter knockouts). Other knockout mice which are deficient or lack norepinephrine can be run on the 3CSRTT test to further the investigation of norepinephrine in attention. Mice lacking α1b-adrenergic receptors (α1b-AR) fail to show increases in motor behavior with cocaine, amphetamine, and other psychostimulants (Drouin et al., 2002). Testing different adrenergic receptor knockout mice on attentional tasks may have clinical implications concerning which drugs are used to treat ADHD.

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