

# 5-HT<sub>1A</sub> Receptor Null Mutant Mice Responding Under a Differential-Reinforcement-of-Low-Rate 72-Second Schedule of Reinforcement

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**Abstract:** Over the last two decades, our ever-increasing ability to manipulate the mouse genome has resulted in a variety of genetically defined mouse models of depression and other psychiatric and neurological disorders. However, it is still the case that some relevant rodent models for depression and antidepressant action have been validated experimentally in rats only and not in mice. An important example of such models is the operant model of antidepressant action known as differential-reinforcement-of-low-rates 72-second (DRL 72-s). A specific set of drug-induced changes on the performance of rats responding under a DRL 72-s schedule of reinforcement has been shown to be a highly reliable predictor of antidepressant activity in human depressive disorders. The aim of this study is to validate the use of the DRL 72-s schedule in mice by both genetic and pharmacological means. We have analyzed the actions of the specific serotonin reuptake inhibitor (SSRI) fluoxetine and the tricyclic agent desipramine (DMI) on wild-type and 5-hydroxytryptamine 1A receptor-null mutant (5-HT<sub>1A</sub>R KO) mice. In agreement with the literature on rats, we found that fluoxetine produced an acute antidepressant-like effect in 5-HT<sub>1A</sub>R KO mice but not in wild-type (Wt) mice. Additionally, an antidepressant-like effect was observed when DMI was administered to both 5-HT<sub>1A</sub>R KO and Wt mice. In conclusion: through the use of both genetic and pharmacological strategies, this study validates the extension of a protocol involving the DRL 72-s operant schedule of reinforcement as a behavioral model for the action of antidepressants in mice.

## INTRODUCTION

Dysfunction of the serotonin (5-hydroxytryptamine, 5-HT) system has been shown to be a key factor in major depression, and serotonergic neurotransmission has been strongly implicated in the regulation of mood and anxiety states [1-3]. Consistent with the 5-HT dysfunction theory, there is an increased probability of depressive episodes *via* tryptophan depletion [4]. Also, a functional polymorphism in the promoter region of the 5-HT transporter, which decrease plasma membrane levels of this protein, is associated with increased incidence of depression [5]. This has also been supported clinically by the effectiveness of selective serotonin reuptake inhibitors (SSRI)[6].

Interestingly, there is a typical 3-6 week delay in the behavioral onset of antidepressant treatment effectiveness. The cellular and molecular nature of the time-dependent neuroadaptations underlying this phenomenon are incompletely understood, but appear to involve, among other things, long-term desensitization of 5HT<sub>1A</sub> receptors, a somatodendritic autoreceptor that regulates the firing of neurons in the dorsal raphe nucleus (DRN) [3]. SSRI treatment acutely reduces the

firing rate of DRN neurons, but over continued treatment, DRN firing gradually returns to normal [3]. Consistent with these electrophysiological data, acute SSRI treatment increases extracellular levels of serotonin in the DRN [7-9], which is enhanced after chronic administration of SSRI's [3, 10].

Polymorphisms associated with greater availability of 5HT<sub>1A</sub> receptors increase susceptibility to major depressive episodes, and are a predictor of treatment resistance to SSRI's [11]. Rat models of depression reveal treatment responses to behaviorally inert doses of SSRI's when these are supplemented with 5HT<sub>1A</sub> receptor antagonists [12, 13]. Analogous to preclinical investigations in rodents, clinical studies in humans suggest that the behavioral onset of antidepressant treatment can be accelerated with co-administration of 5HT<sub>1A</sub> receptor antagonists [3, 14, 15]. However, others have reported conflicting findings [7, 16, 17].

Over the last two decades a wide variety of potential animal models of depression and other psychiatric and neurological disorders have become available. Such models offer us a unique chance to probe into different theories that have been proposed to define the molecular etiology and pathogenesis of depression. However, some important behavioral rodent models of depression and antidepressant action, which have played a pivotal role in the identification of many antidepressant agents currently in use, only have been

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experimentally validated in rats, not mice. One such model of antidepressant action is the DRL 72-s operant schedule of reinforcement. This important behavioral model, first introduced by the Lewis Seiden's group in the 1980's, has been shown to reliably predict antidepressant drug efficacy in human beings [18]. Interestingly, studies involving the DRL 72-s schedule have shown that rats that display low hypothermic responses to the 5-HT<sub>1A</sub> receptor agonist ( $\pm$ )8-hydroxy-di-propylamino tetralin (8-OH-DPAT) respond to fluoxetine in a similar manner as rats pretreated with WAY-100535 [12, 19]. Under the DRL 72-s schedule, antidepressant agents typically produce a set of three characteristic effects on rats: 1) increased reinforcement rates; 2) decreased response rates; and 3) a shift to the right in interresponse times (IRT's) [18, 20, 21]. The validation of the DRL 72-s schedule in the mouse could provide an invaluable tool for a better understanding of depression and mechanisms underlying antidepressant action.

Here, we report for the first time that mice can perform under the DRL 72-s schedule. To show that mouse DRL 72-s performance is pharmacologically consistent with that in rats, we used 5HT<sub>1A</sub> receptor knockout (5-HT<sub>1A</sub>R KO) mice and evaluated their sensitivity to the antidepressant compounds fluoxetine and DMI. In agreement with the literature on rats, we found that fluoxetine produced an acute antidepressant-like effect on 5-HT<sub>1A</sub>R KO mice but not on wild-type (Wt) mice. Additionally, an antidepressant-like effect was observed when DMI was administered to both 5-HT<sub>1A</sub>R KO and Wt mice.

## MATERIALS AND METHODOLOGY

**Animals.** The generation of 5HT<sub>1A</sub>R KO mice has been previously described by Heisler *et al.* [1]. To minimize genetic variation, 5HT<sub>1A</sub>R KO mice were backcrossed 12 generations onto the C57BL/6J background. After backcrossing, mice for this study were produced by crossing heterozygous mice for the null mutation to obtain Wt and 5-HT<sub>1A</sub>R KO mice and were housed at the University of Denver. A total of 24 male mice were used: 12 homozygous for the null mutation, 5HT<sub>1A</sub>R KO mice and 12 wild-type (Wt, ie, non-mutant mouse littermates). Prior to the start of behavioral training, the mice were housed 4 to 5 per cage with *ad libitum* access to food and water under a 12 hour light/dark cycle. A week before the start of training, the mice were singly housed and weighed once a day (Monday through Friday) to determine free fed baseline weight. Mice were then food deprived the Friday before chamber acclimation, and their weight was maintained at 80-85% of their free fed weight throughout the testing by post-session feeding with NOYES precision pellets (PJAI-0500, Research Diets Brunswick, NJ). (Typically, the removal of food for the weekend mice produced weight drops of 15 to 20 % of free-fed weight.) During testing, mice had *ad libitum* access to water in their home cages. Feeding was performed at 3:00 P.M. during the week, and the weekend amount was given on Friday. Handling and care of mice were consistent with federal guidelines and all experimental methods have received the approval of the University of Denver's Animal Care and Use Committee.

**Animal Genotyping.** Genotyping was performed by polymerase chain reaction (PCR). Retro-orbital collecting technique was used to obtain blood samples and DNA was ex-

tracted with the Extract-N-Amp Blood PCR kit (Sigma St. Louis, MO). PCR mix was added with primers to the DNA extract according to kit's instructions. Two Wt primers were used; 1a forward: 5'-CTGCTCATGTGGTCCTCTATG-3'; 1a reverse: 5'-TAGGAGGTAGCTCCTGATTCGC-3'; to yield a band of approximately 250 b.p. when the Wt gene was present. Two mutant primers were used; Neo D: 5'-CACCTTGCTCCTGCCGAGAAA-3'; Neo H: 5'-AGAAGGCGATAGAAGGCGATG-3'; which produced a band of approximately 500 b.p. when the mutant gene was present. Primer mix was cycled using the following "step down" protocol: a) 94°C for 3 minutes b) 94°C for 20 seconds, 64°C for 30 seconds (-0.5°C per cycle), 72°C for 35 seconds (step 'b' is repeated 12 times) c) 94°C for 20 seconds, 58°C for 30 seconds, 72°C for 35 seconds (step 'c' is repeated 25 times) d) 72°C for 2 minutes e) 4°C until samples were removed. Products were separated by gel electrophoresis in a 2% agarose gel.

**Behavioral Training.** Mice started training at 10-12 weeks of age, with the training schedule lasting 14-18 weeks. Mouse operant conditioning chambers (Modular test chamber, ENV-307A, Med Associates, St. Albans, VT) were controlled by a PC running the Med Associates MED-PC research control and data acquisition system (Med Associates) as previously described by Wenger *et al.* [22]. Chambers were equipped with two photocell assemblies (Mouse nose-poke, ENV-313M, Med Associates) on the front wall on either side of the dipper (Liquid Dipper, ENV-302M, Med Associates) and one photocell assembly in the middle of the back wall. The interruption of a photocell beam in the nose-poke assembly (either the right or left of the dipper nose-poke assembly was assigned randomly to each mouse) defined a response and produced an audible click (feedback) upon the response. Responses on the two remaining nose-poke assemblies were not counted and had no programmed consequence in the experiments described in the present study. The mice were randomly assigned a chamber and photocell (either left or right). Photocell, chamber, and time of day remained constant throughout the training sessions.

Training sessions were run once a day Monday through Friday. On the Monday following the start of food deprivation, mice were auto-shaped by being placed in the operant chambers for 20 minutes a day; during which time they were presented with a non-response contingent dipper of evaporated milk (0.01 ml) every minute for 15 seconds (all reinforcers in this study were a dipper of milk for 15 seconds). By the end of four consecutive auto-shaping sessions, i.e., on the Thursday of the first week of training, we started the next training phase. (At this stage, all mice in this study were capable of finding the non-response-contingent dipper of evaporated milk.) On the following Friday, mice were trained to respond under a fixed-ratio 1 (FR1) schedule of milk presentation (each response on the assigned nose-poke assembly produced 15 seconds access to the dipper of milk). Each session under the FR1 schedule was terminated after 20 minutes or after 20 presentations of the dipper of milk, whichever occurred first. After one week, all mice reached criterion performance (80% of total possible reinforcements per session) and the schedule of reinforcement was changed to a DRL 4.5-s schedule. On weeks three and four, all mice also reached criterion performance (80% of total possible reinforcements per session) and were trained to respond un-

der DRL 9-s and DRL 18-s schedules, respectively. On week five, the schedule of reinforcement was changed to a DRL 36-s schedule. Finally, at the beginning of week seven, the schedule of reinforcement was changed to the DRL 72-s schedule and this schedule was left in place for all subsequent weeks. Each DRL schedule session was terminated after 60 minutes or 60 dipper presentations, whichever occurred first. After eight weeks of training under the DRL72-s schedule, the behavior stabilized within less than 10% variation in total responses from day-to-day and no observable trends, and the assessments of the dose dependence to different pharmacological agents were initiated.

**Drug Administration.** Stock solutions of fluoxetine HCl (Tocris, Ellisville, MO), WAY-100635 (Sigma, St. Louis, MO), and DMI (Sigma) were prepared and stored in a -80°C freezer until day of administration. The vehicle for all the drugs was distilled water and all injections were administered intraperitoneally (i.p.). Doses were expressed as the salt or in mg/kg and injections of drug or vehicle administered at a volume of 6.25 ml/kg. All injections of drugs were administered 15 minutes before testing.

The schedule of drug injection was based on aforementioned studies by Lewis Seiden's research team [12, 19]. However, we made two major modifications to the original protocols. (1) We avoided doing any drug injections on Mondays, because, immediately after weekends, the animals were coming from a two-day interval in which training had been interrupted; in addition, on Mondays, the animals also had been subjected to non-standard food restriction, which sometimes resulted in a slightly less stable behavior than those recorded on the following four weekdays. (2) Injections were performed on Tuesdays and Fridays, i.e., twice a week, with two days of no drug injection, to minimize the potential development of tolerance or cumulative (chronic) effects due to the application of antidepressants.

In experiments involving WAY-100635, a 5-HT<sub>1A</sub> receptor antagonist, plus fluoxetine both compounds were prepared in the same solution. Injections were performed on Tuesdays and Fridays. The baseline was derived from data compiled on three days in which mice had been injected with vehicle only prior to the start of experiments involving fluoxetine and fluoxetine plus 0.03 mg/kg WAY-100635. For experiments involving DMI, data derived from experiments performed on the four days of vehicle injection preceding the beginning of the DMI injections were used to construct the baseline. The dose-dependence for fluoxetine and fluoxetine plus 0.03 mg/kg WAY-100635 were assessed over four weeks, on Tuesdays (fluoxetine 2.5, 5, 10, and 20 mg/kg) and Fridays (Way-100635 0.03 mg/kg with fluoxetine 0, 2.5, 5, and 10 mg/kg). For experiments involving DMI, this drug was administered over two weeks, on Tuesdays and Fridays (1.25, 2.5, 5, and 10 mg/kg). The order of dosing was in ascending order for all mice.

In summary, the schedule of drug injection was as follows: 1) to derive a first baseline level, data were averaged from experiments involving vehicle-only injections (these experiments occurred on a Tuesday, Friday, and Tuesday); 2) experiments to assess the behavioral response to the administration of fluoxetine and fluoxetine plus 0.03 mg/kg WAY-100635 followed (Friday-0.03 mg/kg WAY-100635, Tuesday- 2.5 mg/kg fluoxetine, Friday-0.03 mg/kg WAY-

100635 and 2.5 mg/kg fluoxetine, Tuesday- 5 mg/kg fluoxetine, Friday-0.03 mg/kg WAY-100635 and 5 mg/kg fluoxetine, Tuesday- 10 mg/kg fluoxetine, Friday-0.03 mg/kg WAY-100635 and 10 mg/kg fluoxetine, and Tuesday- 20 mg/kg fluoxetine); 3) after this first round of experiments, a second baseline level was determined, by averaging data collected from four days of experiments involving vehicle-only injections (Tuesday, Friday, Tuesday, and Friday); 4) This was followed by experiments involving the administration of increasing doses of DMI (Tuesday-1.25 mg/kg, Friday-2.5 mg/kg, Tuesday- 5 mg/kg, and Friday-10 mg/kg).

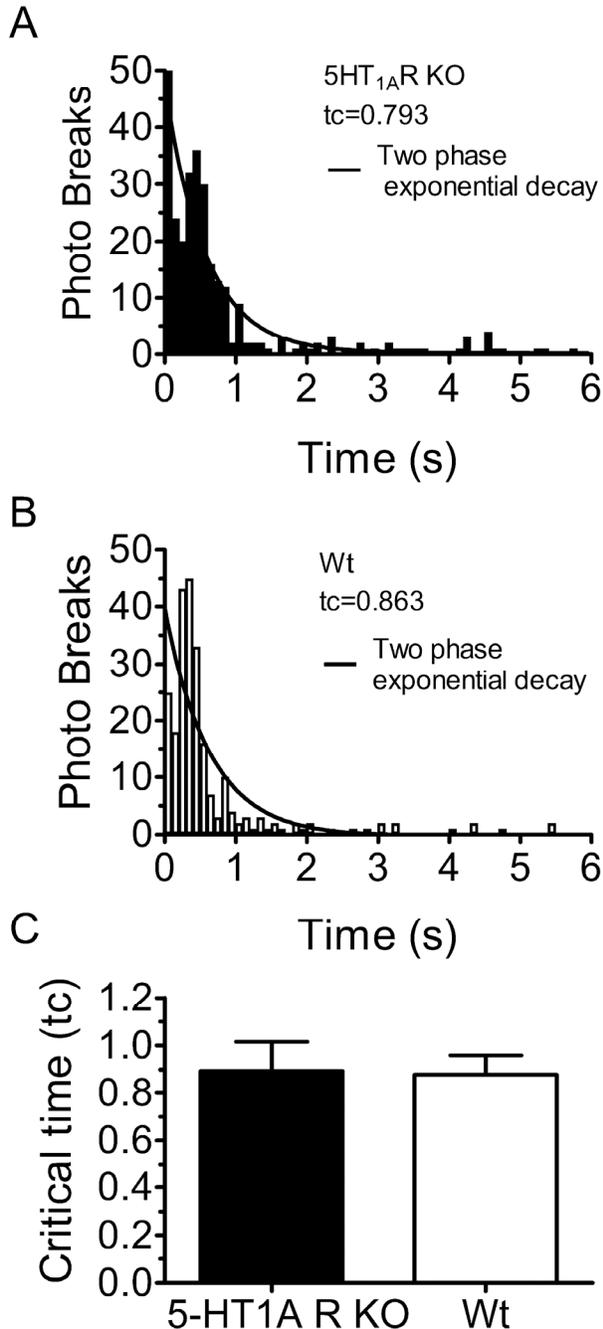
**Data Analysis.** Data are expressed as mean and standard error of the mean (SEM). A corresponding negative exponential (CNE) was fitted to the IRT distributions (Prism version 4, Graph-Pad Software, San Diego, CA). This curve represents the theoretical IRT distribution that would be produced if the mouse randomly produced the same number of responses. To determine the effects of drug treatment on the IRT distribution, peak deviation analysis [20, 21] was used to determine peak location (PkL) (median location of IRT distribution above the CNE), peak area (PkA) (area of distribution of IRT's above CNE), and burst ratio (BR). A 6-s bin width was used to construct the IRT histograms used in peak deviation analysis. Because no significant differences were found for BR, in order to save publication space, data for this measure were not displayed in figures. (Notice, however, that results of statistical analyses for BR are presented in the text.) As described in Richards and Seiden [21], a minimum of 25 responses was required for peak analysis. Our data analysis only departed from the methods introduced by Dr. Seiden's group in one significant way: because the data acquisition rate was set at 10 ms, this had the potential to produce an artificially high number of very fast responses due to the artifact of multiple responses being recorded for a single nosepoke. It is not difficult to envision that, at this high temporal resolution, phenomena such as head bobbing, tremor, or stereotypical movements could all produce artifactual responses.

To minimize potential data analysis errors generated by such phenomena, we used the following strategy: 1) the histogram for the fast IRT (<6 sec) distribution was fitted by a second order exponential decay (Prism version 4); 2) to determine the burst terminator criterion, we used an algorithm developed by electrophysiologists in the early nineties to define burst terminator times in an unbiased fashion [23, 24]. This algorithm consists of mathematically determining the criterion time ( $t_c$ ) by numerically solving the equation below:

$$A_1 \int_0^{t_c} \exp(-tc \cdot t/\tau_1) dt = A_2 \int_{t_c}^{\infty} \exp(-tc \cdot t/\tau_2) dt$$

In this equation, the amplitudes of the exponential components are represented by  $A_1$  and  $A_2$ . Decay rates are represented by  $\tau_1$  and  $\tau_2$ . These two exponential components correspond to the best-fit IRT negative exponential probability density functions. The  $t_c$  determined by this equation characterize the burst terminator time at which the probability of miscounting short IRT's as belonging to the population of long events (described by the first negative exponential component) is equal to the probability of miscounting long IRT's (second exponential component) as short IRT's. Based on this analysis (Fig. 1), any IRT shorter than one second

were considered artifactual (or at least not belonging to the same type of events as IRT's longer than one second in duration) and, therefore, were removed from the analysis. In other words, the equation above aims at finding the time point ( $t_c$ ) in which the probability of type I errors (or false positives, i.e., the inclusion of artifactual IRTs) and type II errors (false negatives, i.e., the exclusion of IRTs due to actual responses made by the animal) in the data analysis are balanced.



**Fig. (1).** Representative fast IRT (<6 sec) responses for (A) an individual 5-HT<sub>1A</sub>R KO and (B) an individual Wt mouse. Non-linear regression was used to fit second order exponential decay functions to determine the critical time,  $t_c$ , which was used to remove artifactual responses from the IRT analysis. (C) Averaged mean  $t_c$ 's for Wt and 5-HT<sub>1A</sub>R KO mice. For A and B, data was binned in 0.1s increments for the first 6 seconds.

Baseline DRL 72-s data (vehicle injections) were analyzed by two-tailed unpaired  $t$  test with Welch's correction (Prism version 4). DRL 72-s dose response data for fluoxetine, fluoxetine plus WAY-100635, and DMI were analyzed by one-way repeated measures analysis of variance (RM ANOVA) (Statistica 7.0, Stat-soft, Tulsa, OK) or two-way RM ANOVA with genotype and dose as the factors. Bonferroni post hoc tests were performed for all significant RM ANOVA. Statistical significance is expressed as \*, \*\*, \*\*\* (one-way RM ANOVA) for 5-HT<sub>1A</sub>R KO and †, ††, ††† for Wt, or ‡, ‡‡, ‡‡‡ for (two-way RM ANOVA, to assess potential interactions between genotype and drug) for  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ , respectively.

## RESULTS

**Baseline Analysis.** After eight weeks of training on the DRL 72-s schedule, the baseline behaviors of Wt and 5-HT<sub>1A</sub>R KO mice were compared. As described in Methods, histograms for the fast IRT's (<6 sec) of both Wt and 5-HT<sub>1A</sub>R KO mice were generated and fitted with a second order exponential decay function to determine the burst terminator criterion,  $t_c$  (Fig. 1A, B). This analysis was performed to exclude potentially artifactual multiple nose poke counting of single responses and yielded very similar  $t_c$ 's for Wt and 5-HT<sub>1A</sub>R KO mice (Wt:  $0.88 \pm 0.08$ ,  $N=12$ ; 5HT<sub>1A</sub>R KO:  $0.89 \pm 0.12$ ,  $N=12$ ;  $t=0.10$ ,  $df=38$ ,  $p=0.92$ ) (Fig. 1C). Also,  $\tau_1$ ,  $\tau_2$ , and  $A_2$  were not significantly different between the two mouse genotypes ( $F_{1,110} = 1.22$ ,  $p=0.27$ ;  $F_{1,2942} = 0.022$ ,  $p=0.88$ ; and  $F_{1,110} = 0.011$ ,  $p=0.92$ , respectively). However,  $A_1$  was significantly different between Wt and 5-HT<sub>1A</sub>R KO mice ( $F_{1,110} = 3.97$ ,  $p=0.049$ ). Based on these results, only IRT's longer than one second in duration were used to determine PkA, PkL, and BR for both genotypes in this entire study.

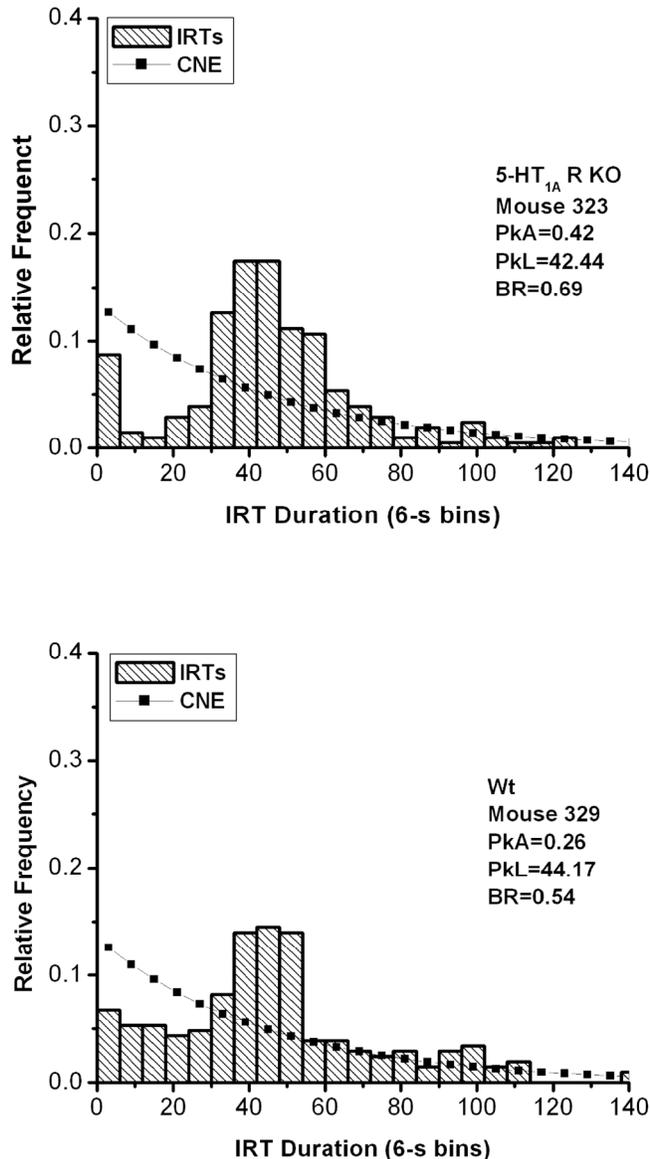
Data compiled from three days of experiments in which the animals received vehicle injections prior to drug administration (Tuesday-Friday-Tuesday) were used to generate baseline IRT distribution histograms (Fig. 2). These histograms were used to determine PkA, PkL, and BR baselines [20, 21]. Baseline behaviors for the two genotypes were assessed in terms of number of responses, reinforcers, PkA, PkL, and BR (Table 1). Analysis of these data showed no significant difference between genotype before or after drug administration.

**Fluoxetine Effects.** Consistent with the expected effects for an antidepressant agent, for the 5-HT<sub>1A</sub>R KO mice, fluoxetine produced a significant decrease in response rate ( $F_{4,44} = 8.88$ ,  $p=0.00002$ ), and a significant increase in reinforcers ( $F_{4,44} = 4.67$ ,  $p=0.0031$ ). It is important to note that these effects occurred at different doses. IRT distribution analyses showed significant shifts in PkA, PkL, and BR ( $F_{4,44} = 3.37$ ,  $p=0.019$ ;  $F_{4,44} = 3.45$ ,  $p=0.017$ ; and  $F_{4,44} = 2.96$ ,  $p=0.032$ , respectively; data not depicted graphically). In addition, these antidepressant-like effects of fluoxetine on 5-HT<sub>1A</sub>R KO mice were clearly dose-dependent (Fig. 3). In contrast, Wt mice showed significant changes from baseline response rates with increasing doses of fluoxetine ( $F_{4,44} = 2.75$ ,  $p=0.040$ ), but no significant change in reinforcers ( $F_{4,44} = 1.09$ ,  $p=0.37$ ). Additionally, IRT analysis showed no significant shift in PkL ( $F_{4,44} = 1.79$ ,  $p=0.15$ ), whereas PkA and BR (data not shown) displayed significant shifts from baseline values ( $F_{4,44} = 4.08$ ,  $p=0.0067$ ; and  $F_{4,44} = 2.98$ ,

$p=0.029$ , respectively). In summary, acute injections of fluoxetine had an antidepressant-like effect on 5-HT<sub>1A</sub>R KO, but not on Wt mice (Fig. 3).

Finally, two-way RM ANOVA analysis showed a significant interaction for genotype and dose of fluoxetine on reinforcers when comparing Wt and 5-HT<sub>1A</sub>R KO mice (Fig. 3A) ( $F_{4,88}=3.63$ ,  $p=0.0087$ ). Although similar analysis was performed on all other measures, no other instance of significant genotype and dose interaction was found (data not shown).

**Effects of Fluoxetine Plus WAY 100365.** For 5-HT<sub>1A</sub>R KO mice, injections of fluoxetine combined with WAY 100365 did not decrease response rate ( $F_{4,44}=2.56$ ,  $p=0.052$ ),



**Fig. (2).** Representative baseline IRT histograms for a 5-HT<sub>1A</sub>R KO mouse and a Wt mouse. Analysis of baseline parameters showed no significant differences between the two mouse genotypes. CNE, corresponding negative exponential.

or increase reinforcers ( $F_{4,44}=2.06$ ,  $p=0.10$ ). Analysis of IRT distribution, however, showed significant shifts for PkA and PkL ( $F_{4,44}=3.47$ ,  $p=0.015$ ) and ( $F_{4,44}=5.21$ ,  $p=0.0016$ ), re-

spectively. In contrast, BR was not shifted ( $F_{4,44}=0.81$ ,  $p=0.53$ ; data not graphically shown). In summary, the combination of 0.03 mg/kg of WAY 100365 plus fluoxetine, did not produce a complete antidepressant-like effect in 5-HT<sub>1A</sub>R KO mice (Fig. 4). Interestingly, for Wt mice, fluoxetine plus WAY 100365 significantly decreased response rate ( $F_{4,44}=4.52$ ,  $p=0.0038$ ), but did not increase reinforcers ( $F_{4,44}=2.56$ ,  $p=0.052$ ). Analysis of IRT distribution showed significant shifts for PkA, PkL ( $F_{4,44}=3.43$ ,  $p=0.016$ ), and ( $F_{4,44}=5.46$ ,  $p=0.0012$ ), respectively. Again, BR was not shifted ( $F_{4,44}=0.69$ ,  $p=0.60$ ; data not depicted graphically). Therefore, fluoxetine, when injected in conjunction with WAY 100365, also did not produce a complete set of antidepressant-like effects on the Wt mice (Fig. 4).

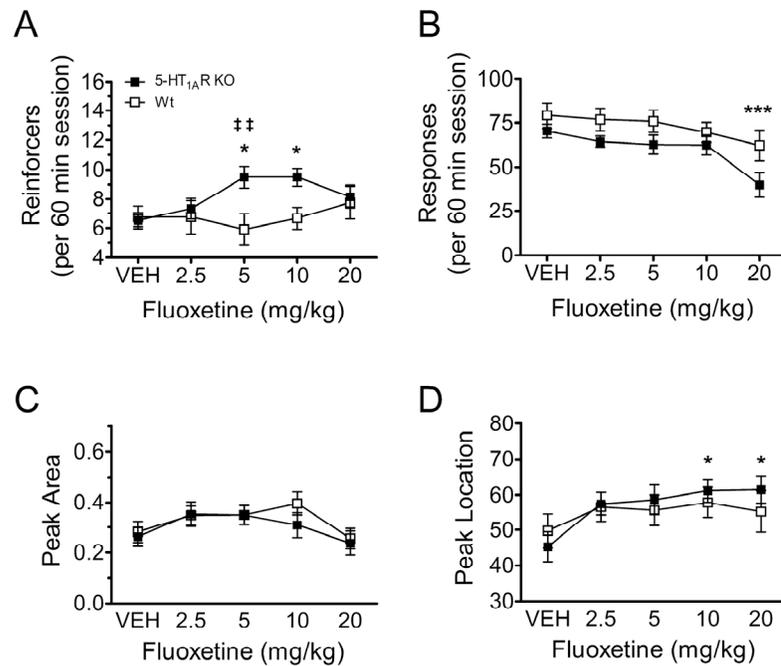
**Table 1. Comparison of Baseline Parameters Between Wt and 5-HT<sub>1A</sub>R KO Mice**

Baseline	Wt n=12	5HT <sub>1A</sub> R KO n=12	P Value
<i>Before fluoxetine</i>			
Reinforcers	6.73 ± 0.79	6.50 ± 0.39	0.79
Responses	79.11 ± 7.21	70.33 ± 3.92	0.30
PkA	0.28 ± 0.04	0.26 ± 0.04	0.75
PkL	49.72 ± 4.82	45.24 ± 4.19	0.49
BR	1.40 ± 0.27	1.95 ± 0.26	0.20
<i>After fluoxetine</i>			
Reinforcers	6.23 ± 0.76	6.22 ± 0.63	0.99
Responses	73.39 ± 5.34	68.39 ± 5.16	0.56
PkA	0.31 ± 0.03	0.32 ± 0.05	0.86
PkL	51.77 ± 3.77	50.80 ± 3.75	0.86
BR	1.21 ± 0.18	1.78 ± 0.45	0.26

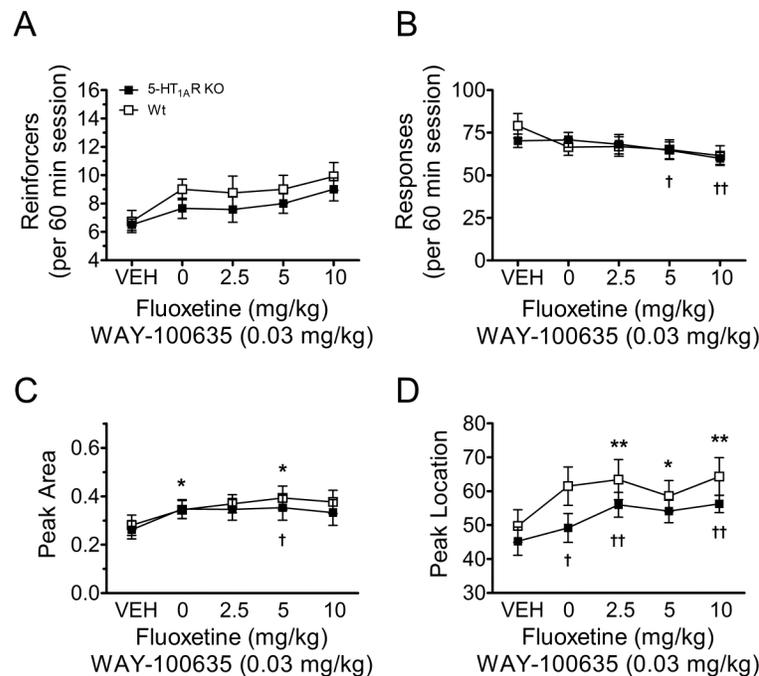
**Desipramine Effects.** For the 5-HT<sub>1A</sub>R KO mice, DMI decreased response rates ( $F_{4,44}=13.00$ ,  $p<0.0001$ ) and increased reinforcers ( $F_{4,44}=9.56$ ,  $p<0.0001$ ). The IRT distribution analysis showed a significant shift in PkA and PkL ( $F_{4,44}=6.68$ ,  $p=0.00027$ ) and ( $F_{4,44}=5.23$ ,  $p=0.0016$ ), respectively. However, there was no shift for BR ( $F_{4,44}=0.62$ ,  $p=0.65$ ; data not graphically shown). Therefore, DMI showed an antidepressant-like effect on the 5-HT<sub>1A</sub>R KO mice, which followed a dose dependent pattern (Fig. 5). Similar to 5-HT<sub>1A</sub>R KO mice, for Wt mice, DMI decreased response rates ( $F_{4,44}=10.69$ ,  $p<0.001$ ) and increased reinforcement rate ( $F_{4,44}=3.74$ ,  $p=0.01$ ). Again, IRT distribution analysis of revealed significant shifts in PkA and PkL ( $F_{4,44}=7.60$ ,  $p<0.0001$ ) and ( $F_{4,44}=5.66$ ,  $p<0.00091$ ), respectively. Finally, no shift for BR was observed ( $F_{4,44}=1.40$ ,  $p=0.25$ ; data not graphically shown). Therefore, similar to what was observed in 5-HT<sub>1A</sub>R KO mice, DMI also produced a dose-dependent antidepressant-like effect on the Wt mice (Fig. 5).

## DISCUSSION

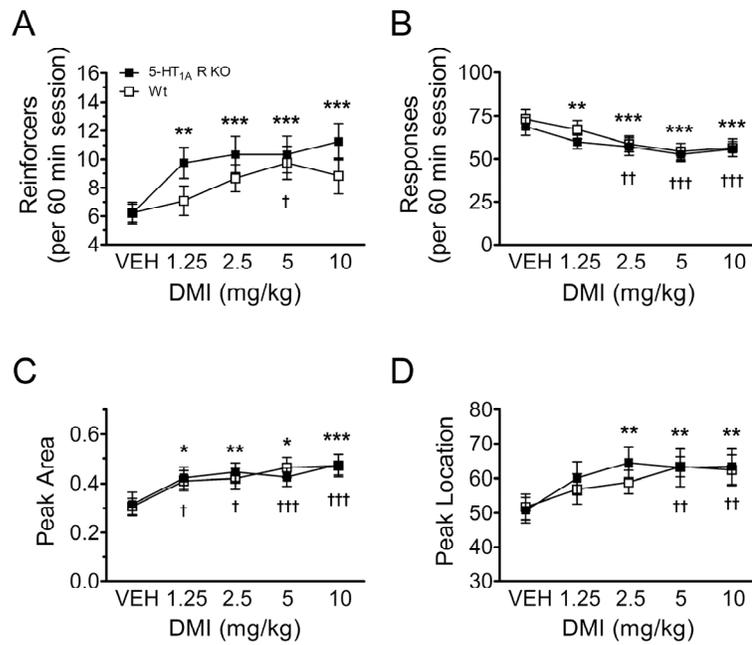
The aim of this study has been to validate the use of the DRL 72-s schedule in mice. To this end, we have analyzed the actions of the SSRI fluoxetine, alone and in combination



**Fig. (3).** Dose dependence for responses to fluoxetine. (A) Reinforcement rate. We found a significant increase in reinforcers for 5-HT<sub>1A</sub>R KO mice at 5 mg/kg ( $p=0.012$ ) and 10 mg/kg ( $p=0.012$ ) of fluoxetine. We also found a significant interaction between genotype and dose at 5 mg/kg ( $p<0.001$ ) of this drug. (B) Response rate. Significant decreases in this parameter were only observed at 20 mg/kg of fluoxetine in 5-HT<sub>1A</sub>R KO mice ( $p=0.000018$ ). Although the RM-ANOVA was significant for Wt the *post hoc* test did not show significance for any dose. (C) PkA. For both Wt and 5-HT<sub>1A</sub>R KO mice the *post hoc* test did not show significant for any drug dose. (D) PkL. Significant deviations to the right in PkL were observed in 5-HT<sub>1A</sub>R KO mice at 10 mg/kg ( $p=0.024$ ), 20 mg/kg ( $p=0.047$ ) of fluoxetine. 5-HT<sub>1A</sub>R KO  $n=12$ . Wt  $n=12$ . Two 5-HT<sub>1A</sub>R KO mice did not generate enough responses (i.e., <25 responses) and, therefore, were not used for PkA and PkL analysis of data from experiments involving the injection of 20 mg/kg of fluoxetine (5-HT<sub>1A</sub>R KO  $n=10$ ).



**Fig. (4).** Dose dependence for responses to fluoxetine and WAY-100635. (Note that, in all experiments here, WAY-100635 was injected at a dose of 0.03 mg/kg). (A) Reinforcement rate. No significant dose-dependence was found for this parameter. (B) Response rate. Significant dose-dependence was observed for Wt mice at 5 mg/kg ( $p=0.027$ ), and 10 mg/kg ( $p=0.0026$ ), whereas no significant dose dependence was observed for 5-HT<sub>1A</sub>R KO mice. (C) PkA. We observed significant dose dependence for this parameter in 5-HT<sub>1A</sub>R KO mice at the following doses of fluoxetine 0 mg/kg ( $p=0.047$ ), and 5 mg/kg ( $p=0.028$ ). Wt mice injected with 5 mg/kg ( $p=0.017$ ) of fluoxetine showed a significant dose dependence. (D) PkL. 5-HT<sub>1A</sub>R KO mice showed dose dependence at 2.5 mg/kg ( $p=0.0075$ ), 5 mg/kg ( $p=0.046$ ), and 10 mg/kg ( $p=0.0059$ ), whereas Wt mice displayed dose dependence at 0 mg/kg ( $p=0.019$ ), 2.5 mg/kg ( $p=0.0039$ ), and 10 mg/kg ( $p=0.0018$ ) of fluoxetine. 5-HT<sub>1A</sub>R KO  $n=12$ . Wt  $n=12$ .



**Fig. (5).** Dose dependence for responses to DMI. **(A)** Reinforcement rate. 5-HT<sub>1A</sub>R KO mice showed significant dose dependence at 1.25 mg/kg ( $p=0.0028$ ), 2.5 mg/kg ( $p=0.00035$ ), 5 mg/kg ( $p=0.00035$ ), and 10 mg/kg ( $p=0.000012$ ). Significant dose dependence was observed for Wt mice injected with 5 mg/kg ( $p=0.015$ ) of DMI. **(B)** Response rate. 5-HT<sub>1A</sub>R KO mice displayed dose dependence at 1.25 mg/kg ( $p=0.0062$ ), 2.5 mg/kg ( $p=0.00017$ ), 5 mg/kg ( $p<0.001$ ), and 10 mg/kg ( $p=0.000025$ ). We observed significant dose dependence for Wt mice at 2.5 mg/kg ( $p=0.0012$ ), 5 mg/kg ( $p=0.000018$ ), and 10 mg/kg ( $p=0.00014$ ) of DMI **(C)** PkA. Regarding this parameter, we detected significant dose dependence for 5-HT<sub>1A</sub>R KO mice at 1.25 mg/kg ( $p=0.024$ ), 2.5 mg/kg ( $p=0.0030$ ), 5 mg/kg ( $p=0.018$ ), and 10 mg/kg ( $p=0.00016$ ). Wt mice injected with DMI displayed dose dependence at 1.25 mg/kg ( $p=0.033$ ), 2.5 mg/kg ( $p=0.015$ ), 5 mg/kg ( $p=0.00031$ ), and 10 mg/kg ( $p=0.00014$ ). **(D)** PkL. 5-HT<sub>1A</sub>R KO mice showed dose dependent responses to DMI at 2.5 mg/kg ( $p=0.0027$ ), 5 mg/kg ( $p=0.0094$ ), and 10 mg/kg ( $p=0.0071$ ). Significant dose dependence was observed in Wt mice injected with the following doses of DMI 5 mg/kg ( $p=0.0013$ ), and 10 mg/kg ( $p=0.0037$ ). 5-HT<sub>1A</sub>R KO  $n=12$ . Wt  $n=12$ .

with the 5-HT<sub>1A</sub>R antagonist WAY-100635, and the tricyclic antidepressant DMI. Although, in the process of validating behavioral models, it is customary to write a first report including data derived exclusively from wild-type animals before the introduction of results from genetically modified animals to the literature, given the advanced state of this field, we found it appropriate to complement our results by including data from experiments performed on 5-HT<sub>1A</sub>R KO mice, i.e., data generated from combined genetic and pharmacological manipulations. We found that both Wt and 5-HT<sub>1A</sub>R KO mice could reliably be tested on the DRL 72-s schedule. In addition, we found that DMI produced an antidepressant like effect on both Wt and 5-HT<sub>1A</sub>R KO mice similar to what had been seen in rats. Therefore, our data indicate that the DRL 72-s schedule seems to be as reliable of a model in mice as it is in rats to determine the potential of new antidepressant compounds.

Baseline analysis of the behavior of Wt and 5-HT<sub>1A</sub>R KO mice under the DRL 72-s did not demonstrate significant differences in responses, reinforcers, PkA, PkL, or BR. These results are consistent with published data on the performance of 5-HT<sub>1A</sub>R KO mice responding under a DRL 36-s schedule of reinforcement [25]. After analyzing second order exponential decays for individual mice, we determined that most IRT's shorter than one second were likely the result of a disproportionately large number of very short IRT's being recorded due to multiple responses resulting from effectively a single nosepoke and a high sampling rate (e.g., 10

ms). As mentioned in the Methods section, at high sampling rates, phenomena such as head bobbing, tremor, or stereotypical movements could all produce artifactual short IRT's. Therefore, we recommend that, in future experiments involving DRL 72-s, similar to the procedure we adopted in the present work, any IRT shorter than one second in duration should be excluded from the data analysis. However, mouse responses approaching 3 responses/sec under FR30 schedules of milk presentation have been previously reported [26]. In these experiments, Wenger used electromechanical equipment where each beam interruption produced a square-wave pulse of 100 ms duration, independent of the duration of the photocell beam interruption. Therefore, at present, our recommendation is only applicable to mice responding under DRL 72-s and when responses are detected with high sampling rates. Still, due to the widespread use of photocell-based nosepoke detectors for mice, which may lack the typical debouncing mechanisms embedded in many electromechanical switches, similar IRT distribution analysis may reveal disproportionately large numbers of very fast IRT's in experimental data derived from mice responding to schedules of reinforcement other than the DRL 72-s.

Fluoxetine had an anti-depressant-like effect on 5-HT<sub>1A</sub>R KO mice but not on the WT mice. We found that acute injections of fluoxetine produced dose-dependent increases in reinforcers, decreases in the number of response, and a shift to the right in PkL in 5-HT<sub>1A</sub>R KO mice. Overall, BR was not affected significantly by acute injections of fluoxetine,

except for the highest dose used in this study (20 mg/kg). This response pattern is consistent with anti-depressant effects that have been reported for rats in the DRL 72-s schedule [18]. Fluoxetine also produced a shift in the PkA of both Wt and 5-HT<sub>1A</sub>R KO mice. In contrast, we have not observed antidepressant-like effects on Wt mice, which is consistent with findings by Balcells-Olivero *et al.* [19] and Cousins *et al.* [27] in rats that have high sensitivity to the hypothermic effects of the 5-HT<sub>1A</sub>R agonist 8-OH-DPAT.

Concomitant acute administration of fluoxetine and WAY-100635 to Wt mice produced reduction in response rate and a shift to the right in PkL. These results are consistent with the findings in the DRL 72-s study by Cousins and Seiden [12], in which these authors used Holtzman Sprague-Dawley rats. In the same study, however, Cousins and Seiden observed no significant changes on the DRL 72-s parameters following the acute administration of WAY-100635 alone. In contrast to these findings, we observed that administration of WAY-100635 alone to Wt mice produced a similar effect as the combination of fluoxetine and WAY-100635. It is worth noting that the same research group has shown that Holtzman Sprague-Dawley rats have a low level of hypothermic response to the 5-HT<sub>1A</sub>R agonist 8-OH-DPAT. Therefore, these animals may not be as sensitive to the effects of WAY-100635 as the related substrain of Sprague-Dawley rats known as Harlan Sprague-Dawley [19]. Based on these results, we had hypothesized that 5-HT<sub>1A</sub>R KO mice, which show no detectable hypothermic response to 8-OH-DPAT [1], should respond to WAY-100635 in a similar fashion as Holtzman Sprague-Dawley rats. Accordingly, we observed that acute injections of WAY-100635 in 5-HT<sub>1A</sub>R KO mice produced no significant effect on reinforcers earned, number of responses, or PkL. (We did observe a small, but significant, increase in PkA.) When WAY-100635 was administered with fluoxetine to 5-HT<sub>1A</sub>R KO mice, however, the observed effect deviated from our simple predictions based on 5-HT<sub>1A</sub>R sensitivity. Although we have observed no significant change in PkL compared to injections of fluoxetine alone, the increase in reinforcement rate produced by fluoxetine was lost, and all the fluoxetine doses (including the 0 dose) produced shifts in PkA. Therefore, our original prediction proved to be too simplistic, which may be due to the fact that WAY-100635 also has high affinity for D<sub>2A</sub>, D<sub>3</sub>, and  $\alpha_1$  receptors [27]. Such non-serotonergic binding of WAY-100635 could potentially explain its effect on mice lacking 5-HT<sub>1A</sub>R during experiments involving the DRL 72-s schedule.

These observations have important consequences to the interpretation of previously published data. For example [19, 28] had linked differences in performance in the DRL 72-s schedule between two substrains of Sprague-Dawley rats to different hypothermic responses to the 5-HT<sub>1A</sub>R agonist 8-OH-DPAT. But, again, the 5-HT<sub>1A</sub>R KO line used in this study show no hypothermic response to 8-OH-DPAT [1]. This suggests that another component of the neural circuitry involved in pharmacologically-induced hypothermia in rodents, other than 5-HT<sub>1A</sub>Rs might be disrupted in Holtzman Sprague-Dawley rats. Recent studies in our lab have shown a reduction in the hypothermic response to 8-OH-DPAT in *Girk2* null mutant mice [29]. Therefore, a polymorphism or mutation in *Girk2* or a related gene in Holtzman rats could explain the depressed, but not completely absent, hypothermic

response to 8-OH-DPAT in this substrain of Sprague-Dawley rats.

DMI produced anti-depressant effects on both Wt and 5-HT<sub>1A</sub>R KO mice. We observed reductions in response rates, increases in reinforcers, and shifts to the right in the PkL in mice of both genotypes. These findings were particularly robust for 5-HT<sub>1A</sub>R KO mice, in which all doses, but 1.25 mg/kg of DMI, produced significant effects. For Wt mice, DMI at 5 mg/kg produced an increase in reinforcers, a decrease in responses, and a shift to the right in PkL. DMI doses of 5 and 10 mg/kg, however, produced a significant shift in PkL and a decrease in the number of responses in Wt. Again, these observations are consistent with previous studies in rats [19, 28], which have shown that DMI had similar antidepressant-like effects, regardless of the 8-OH-DPAT sensitivity of the Sprague-Dawley substrains being investigated.

With the growing number of mouse models for depression it is important to have reliable behavioral models for screening anti-depressants in mice. The DRL 72-s has been a reliable model for rats for two decades. It has been used to screen tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, and norepinephrine reuptake inhibitors [18]. Some investigators, have suggested that the implementation of DRL 72-s in mice might be particularly problematic. This is primarily due to the erroneous, but unfortunately widespread belief that "mice are generally not very apt subjects for operant conditioning experiments" [30]. In the present study, however, we demonstrated: 1) that mice are capable of performing on DRL 72-s experiments on par with rats; and 2) that the application of this operant schedule has the potential to provide new interpretations to previously published data from the rat literature as well as expanding our understanding of the molecular mechanisms underlying the action of existent and future antidepressants.

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