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Behavioral Phenotype of Fmr1 Knockout Mice During Active Phase in an Altered Light Dark Cycle

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Abstract:

Fragile X syndrome (FXS) is the most commonly inherited form of intellectual disability and is a disorder that is also highly associated with autism. FXS occurs as a result of an expanded CGG repeat sequence leading to transcriptional silencing. In an animal model of FXS in which *Fmr1* is knocked out (*Fmr1* KO), many physical, physiological, and behavioral characteristics of the human disease are recapitulated. Prior characterization of the mouse model was conducted during the day, the inactive phase of the circadian cycle. Circadian rhythms are an important contributor to behavior and may play a role in the study of disease phenotype. Moreover, changes in parameters of circadian rhythm are known to occur in FXS animal models. We conducted an investigation of key behavioral phenotypes in *Fmr1* KO mice during their active phase. We report that phase did not alter the *Fmr1* KO phenotype in open field activity, anxiety, and learning and memory. There was a slight effect of phase on social behavior as measured by time in chamber, but not by time spent sniffing. Our data strengthen the existing data characterizing the phenotype of *Fmr1* KO mice, indicating that it is independent of circadian phase.

Significance Statement:

This study seeks to characterize the behavioral phenotype of *Fmr1* KO mice during the active phase of the circadian rhythm. Given that for many behaviors the active phase is more physiologically relevant; our study is an important validation of *Fmr1* KO mice as a model for FXS. We find that classical behavioral phenotypes; such as hyperactivity, reduced anxiety, and learning and memory impairments; reported in the *Fmr1* KO mice are not influenced by circadian phase.

Introduction:

Fragile X syndrome (FXS) is the most commonly inherited form of intellectual disability, primarily affecting males with a prevalence of about 1 in 4,000 boys (Turner et al., 1996). Additionally, between 15-60% of patients with FXS are diagnosed as on the autism spectrum (Hagerman et al., 1986; Bailey et al., 1998; Hagerman et al., 2010). Patients with FXS account for about 5-8% of the total cases of autism (Muhle et al., 2004; Schaefer and Mendelsohn, 2008). Behavioral symptoms present in patients with FXS include: intellectual disability, anxiety, hyperactivity, social anxiety, and repetitive behaviors.

FXS is caused by an expanded CGG repeat sequence in the 5'UTR of the *FMR1* gene which leads to transcriptional silencing and subsequent loss of the gene product fragile X mental retardation protein (FMRP) (Verheij et al., 1993). This has been modeled in the mouse by deletion of the *Fmr1* gene (*Fmr1* KO). These mice recapitulate many of the clinical features, including physical, physiological, and behavioral, found in FXS patients (Kazdoba et al., 2014).

One important limitation of the phenotyping of FXS mice, particularly with regard to behavior, is that, to our knowledge, all studies have conducted behavior testing during the day, the inactive phase for mice. Given the circadian control of many physiological factors including: body temperature, corticosterone levels, hormones, gene expression, glucose metabolism, immune function, and sleep (Chung et al., 2011; Bass, 2012; Scheiermann et al., 2013), it is reasonable to expect that circadian phase might also be a strong contributor to behavior. Indeed, circadian phase has been shown to influence certain behaviors in rodents (including activity, anxiety, and learning) (Griebel et al., 1993; Jones and King, 2001; Bertoglio and Carobrez, 2002; Andrade et al., 2003; Hossain et al., 2004; Valentinuzzi et al., 2004), but may not affect others such as social behavior (Hossain et al., 2004; Yang et al., 2008). Circadian rhythm abnormalities are seen in both the *Drosophila* FXS model and *Fmr1* KO mice (Dockendorff et al., 2002; Zhang et al., 2008). We considered the possibility that the circadian cycle might differentially affect *Fmr1* KO mice and confound our understanding of the behavioral phenotype.

In the present study, we sought to determine if the behavioral abnormalities reported in *Fmr1* KO mice are also evident during the active circadian phase. We housed animals in an altered light/dark environment for at least one month prior to testing, and we performed open field, elevated plus maze (EPM), passive avoidance, and social behavior tests during the latter half of the active (dark) phase. We found that, in the active phase, *Fmr1* KO mice have the same phenotype as reported in the inactive phase in open field activity, anxiety, and learning and memory. There was a slight effect of phase on social behavior, but this was only reflected in time in chamber and not time spent sniffing. These results are an important verification of the mouse model of FXS, showing that they share many features associated with clinical FXS.

Materials and Methods:

Animals

These studies were conducted on male *Fmr1* KO and control mice (on a C57BL/6J background), which were generated through heterozygous female and control male breeding pairs maintained in-house. The original B6.129P2-Fmr1^{tm1Cgr}/J mice were

obtained from Jackson labs (Stock 003025). We have maintained the colony in house for six years, periodically backcrossing back into C57BL/6J mice (Stock 000664). Pups were weaned between 21 and 23 days of age. Genotyping of mouse tail DNA was performed by means of PCR amplification. All mice were group housed in a standard housing environment with up to five mice per cage in a climate-controlled central facility. Food (NIH-31 rodent chow, LabDiet, St Louis, MO, USA) and filtered tap water were available to mice *ad libitum*. From birth to one month of age, animals were maintained in a standard 12:12 hr light:dark environment (lights on at 6:00AM). At one month of age, and throughout behavioral testing, animals were shifted to a 12:12 hr light:dark environment (lights on at 1:00PM). Sixty mice were studied between the ages of 60 and 90 days. All procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and an animal study protocol approved by the National Institute of Mental Health Animal Care and Use Committee.

Behavior Testing

Behavior testing was performed on mice beginning at 60 days of age. Mice were allowed one week between tests. Testing was performed between 8:00 AM and 1:00 PM, during the active phase. Testing order was as follows: open-field, social behavior, elevated plus maze (EPM), and passive avoidance. Open-field and social behavior were conducted in the dark under red light conditions. Due to the nature of the tests, EPM and passive avoidance were performed in lighted conditions. Testing procedures are described below.

Open Field

Open field testing was used to determine levels of general activity, as well as anxiety. Activity was measured for 30 min (in five min epochs) by means of photobeam detection (Coulbourn Instruments, Whitehall, PA). Total distance traveled and the ratio of center to total distance traveled were analyzed.

Social Behavior

Mice were tested for social behavior by means of an automated three-chamber apparatus. Briefly, the test was performed in three stages, each lasting 5 min. 1.) Habituation: while the doors were open, mice were placed in the center chamber and allowed to freely explore. 2.) Sociability: the test mouse was isolated to the center chamber (Chamber 2) while a gender/age-matched stranger was placed inside a social enclosure (Noldus, Leesburg, VA) in either Chamber 1 or Chamber 3. The other chamber contained an empty social enclosure. The doors were opened and the test mouse was allowed to freely explore. The times spent in each chamber were recorded. 3.) Social novelty: immediately following the second phase, test mice were isolated back to the center chamber. In the previously empty social enclosure, a novel gender/age-

matched mouse was placed. Doors were opened and the test mouse was allowed to freely explore. Measures were taken as in Phase 2. Video-recording of the testing allowed for subsequent recording of sniffing time (determined by close proximity (<4cm) from the enclosure with the head directed toward the enclosure). For the social behavior testing conducted in the dark, video recording was performed by means of a UV-detecting camera and additional UV light (PhantomLite, East Greenville, PA).

Social behavior testing was performed in a second group of animals during the inactive phase. These animals were maintained throughout their life in the standard 12:12 hr light:dark environment (lights on at 6:00AM). Testing was performed between 1:00-3:00PM, also in the light. This group of animals did not receive any other testing.

Elevated Plus Maze

Mice were tested for general anxiety by means of the EPM. Mice were placed in the center of the apparatus facing an open arm. The times spent in the open arms, closed arms, and the center, were recorded for 5 min. The mouse was considered to be in a particular arm once the head and forepaws had crossed into an area. Testing for the EPM was conducted in the light so that the animal could perceive differences between open and closed arms. Data are presented as the % time spent in the open arms (open arm time/(open arm time + closed arm time)).

Passive Avoidance

Mice were tested for fear-based learning and memory impairments by means of the passive avoidance system (Coulbourn Instruments). The passive avoidance apparatus was composed of a lighted chamber and a dark chamber, separated by an automated door. The floor of the apparatus was capable of delivering an electric shock to the subject. The test was composed of three sessions over three consecutive days (24 hours apart). 1.) Habituation: the mouse was placed in the lighted chamber with the door to the dark chamber closed. After 30s, the door opened and the mouse was given 10 min to enter the dark chamber. Once the mouse entered the dark chamber, the door closed and the animal was removed. 2.) Training: the mouse was placed in the lighted chamber with the door to the dark chamber closed. After 30s, the door opened to the dark chamber. Once the mouse entered the dark chamber, the door closed and a 0.3 mA electric shock 1 s duration was delivered. After 15s, the mouse was removed from the apparatus and allowed 120s of rest before repeating the training session. 3.) Testing: the mouse was placed in the lighted chamber. After 30s, the door opened to the dark chamber. The latency to enter the dark chamber was recorded up to 570s. Given the necessity of a light chamber, passive avoidance training and testing were conducted in the light.

Statistical Analysis

For passive avoidance and EPM, statistical significance was determined by means of a Student's t-test, comparing control and *Fmr1* KO mice. For the other behavior tests, repeated measures ANOVA were used with genotype as the between subjects factor and within subjects factors as follows: epoch (open-field) and chamber (social behavior). Effects with $p \leq 0.05$ were considered to be statistically significant (*), though values $0.05 < p \leq 0.10$ are also reported here and noted on figures with a "~." Data are reported as means ± standard errors of the means.

Results:

Fmr1 KO mice are hyperactive in the open field during the active phase.

We measured distance traveled in 5 min epochs across 30 min of open field testing (Table 1, Figure 1). We found a statistically significant main effect of genotype indicating that, overall *Fmr1* KO mice were hyperactive compared to control mice. We also found a statistically significant effect of epoch indicating that both control and *Fmr1* KO mice displayed a burst of initial activity in response to the novel environment, and that both groups showed habituation to the environment as testing progressed (Figure 1).

Fmr1 KO mice display reduced levels of general anxiety during the active phase.

In the open field test, we determined the ratio of distance traveled in the center to total distance traveled as an index of anxiety-like behavior. We found a statistically significant main effect of genotype indicating that *Fmr1* KO mice moved more in the center of the field suggesting reduced general anxiety levels (Table 1, Figure 2A).

As an additional measure of anxiety levels in *Fmr1* KO mice, we determined behavior in the EPM. *Fmr1* KO mice had a significantly increased percent time in the open arms compared to control mice ($p < 0.001$) (Figure 2B) also suggesting reduced general anxiety levels.

Fmr1 KO mice have deficits in fear learning during the active phase.

In the passive avoidance test, we determined the latency to enter the dark chamber during and after training. During the initial training session mean latencies were similar for both genotypes, whereas during the second training session mean latency for *Fmr1* KO mice was 40% lower than that of controls. This difference was not statistically significant. The latency to enter the dark compartment during the testing session was

significantly lower in the *Fmr1* KO mice compared to controls (p=0.01), suggesting impaired learning and memory (Figure 3).

Social behavior is unaffected by circadian phase.

Results of initial studies of social behavior conducted in the active phase indicated some differences in *Fmr1* KO phenotype compared to our previous reports (Liu and Smith, 2009; Liu et al., 2011; Qin et al., 2015b; Qin et al., 2015a). We used a slightly altered protocol so we were uncertain as to whether the differences indicated an effect of circadian phase or were due to the altered procedures. Our previous studies were all conducted with the investigator in the room observing the mouse behavior. In the present studies, we recorded mouse behavior by means of a video camera, and the investigator left the room during testing. To understand these effects, we added testing in the inactive phase (in a separate group of animals maintained on a standard light: dark cycle (lights on at 6:00 AM)) to determine if this was a result of circadian phase or the change in testing procedures. We conducted social behavior testing in either the inactive or active phases.

In the test for sociability, in which we measured times in the chamber with a stranger mouse or in the chamber with a novel object, both *Fmr1* KO and control mice during either the active and inactive phases showed a preference for the stranger mouse (Table 1, Figure 4A). The only statistically significant effect was a main effect of chamber, indicating that regardless of phase or genotype, mice displayed a preference for the stranger mouse compared with the object. We also analyzed time spent sniffing either the stranger mouse or the object. For this measure also, the only statistically significant effect was a main effect of chamber, indicating no differences in sociability due to genotype or phase (Table 1, Figure 4B).

In the social novelty phase of the task, in which the mouse is tested for a preference for either a novel stranger mouse of the now familiar mouse, the Phase X Genotype X Chamber interaction for time in chamber approached statistical significance (p=0.075)(Table 1). *Post-hoc* pair-wise analyses indicate that, in the active phase, *Fmr1* KO mice spent more time in the chamber with the novel mouse than the familiar mouse (p=0.074), and control mice spent more time in the chamber with the familiar mouse than did *Fmr1* KO mice (p=0.024) (Figure 5A). We also analyzed time spent sniffing the novel and familiar mice. The only statistically significant effect was that of chamber, indicating that regardless of circadian phase or genotype, there was a preference for sniffing the novel mouse (Table 1, Figure 5B).

Discussion:

We found that in the active phase of the circadian cycle, *Fmr1* KO mice are hyperactive, and demonstrate reduced anxiety and impaired learning and memory.

These data are consistent with the behavioral phenotype reported in the inactive phase (Mineur et al., 2002; Qin et al., 2002; Spencer et al., 2005; Ding et al., 2014).

It is a bit surprising that, given the importance of circadian rhythm in physiological functions, it did not have a strong effect on these behaviors. It remains to be seen what, if any, behaviors would be sensitive to circadian phase. It has been suggested that testing during the active circadian phase may increase test sensitivity (Hossain et al., 2004). Perhaps the phenotypes observed in *Fmr1* KO mice are robust enough to be present even in the mouse's inactive phase. It is possible that conducting a more extensive behavioral battery of tests would reveal more subtle genotype differences only observable during one circadian phase or the other.

One limitation of our study is the need to use illumination during some of the testing, specifically for EPM and passive avoidance tests. There is evidence that the EPM may be influenced by high illumination (Bertoglio and Carobrez, 2002) resulting in decreased exploration of the open arm. However, our EPM data are consistent with the open field data, which were obtained in the dark. Overall results for both EPM and open field tests indicate that the *Fmr1* KO mice displayed decreased anxiety compared with controls, as previously reported in the inactive phase. Another potential limitation of our study is the fact that we shifted the light/dark cycle when mice were one month of age. While the 30 day period in the new environment would have allowed enough time for the mice to adjust to the new cycle, the switch was undoubtedly stressful for the mice. Since the behavioral phenotypes were similar in the active phase as previously reported in the inactive phase, it seems unlikely that this stress altered behavior.

For the passive avoidance data, latencies to enter the dark chamber during the first training session did not differ between *Fmr1* KO and control animals indicating that the baseline exploratory difference were not different. During the second training session, there was a 40% difference in mean latency; Fmr1 KO mice had a reduced latency to enter the dark. However, these data were highly variable and did not reach statistical significance. After 24 hours, during the test phase, latency to enter the dark chamber in *Fmr1* KO mice was statistically significantly lower than that of control mice indicating impaired learning and memory. Both *Fmr1* KO and control animals vocalized and jumped in response to the foot shock. Studies of *Fmr1* KO mice on a C57BL/6 background indicate normal acute nociceptive responses but reduced nociceptive sensitization (Price et al., 2007). Additionally, acute response to a foot shock did not differ between control and *Fmr1* KO mice (on a C57BL/6J x FVB/NJ mixed background) (Nielsen et al., 2009). It is unlikely that performance on this test of learning and memory was a reflection of a genotype difference in pain sensitivity.

We did not detect any differences between genotypes in social behavior, except a slight *increase* in preference for social novelty in the active phase for *Fmr1* KO mice.

This result suggests that, in the *Fmr1* KO mice, phase may affect social behavior, but the effect was seen only for the measure of time in chamber and not for time sniffing. In this present study, we did not find any effects of phase or genotype on sniffing time (considered to be the more sensitive measure of social behavior). Because the two measures do not show a consistent effect of phase on response to social novelty in *Fmr1* KO mice, we view this as a less robust effect.

Previous studies in which behavior was measured in the inactive phase have reported social behavior deficits in *Fmr1* KO mice, particularly in response to social novelty (Liu and Smith, 2009; Qin et al., 2015b; Sorensen et al., 2015). With our altered protocol, we did not observe genotype differences in social behavior even in the inactive phase. We interpret this lack of a genotype effect as due to the absence of the experimenter in the testing room, suggesting that even small changes to the behavioral testing procedure (across or within labs) can alter the observed phenotype. We conclude that the lack of a social behavior phenotype in *Fmr1* KO mice is not due to circadian phase.

Our results, apart from social behavior deficits, confirm the behavioral phenotype of *Fmr1* KO mice and indicate that they are not a function of circadian phase. These studies validate these mice as reliable models for FXS in which mechanisms of disease pathogenesis and novel therapies may be tested.

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Figure Legends:

Figure 1: *Fmr1* KO mice display hyperactivity in the open field during the active phase. Both control and *Fmr1* KO mice display habituation to the novel environment across the 30 min testing period (represented by a significant main effect of epoch). However, at all epochs, *Fmr1* KO mice are hyperactive with respect to controls. This is represented by a statistically significant main effect of genotype.

Figure 2: *Fmr1* KO mice display reduced levels of anxiety in the active phase. **(A)** By measuring the distance traveled in the center to the total distance traveled, testing in the open field environment revealed that *Fmr1* KO mice had increased relative distance traveled in the center, indicating reduced anxiety compared to controls. This is represented by a statistically significant main effect of genotype. **(B)** Testing in the elevated plus maze showed that *Fmr1* KO mice spent a significantly greater percent time in the open arms compared to controls ($p<0.001$).

Figure 3: *Fmr1* KO mice display learning and memory impairments in the active phase. Passive avoidance testing showed that *Fmr1* KO mice had a significantly reduced latency to enter the dark compared to controls ($p=0.01$) suggesting impaired learning and memory.

Figure 4: Sociability is unaffected by phase and does not differ by genotype. (**A**) Both control and *Fmr1* KO mice show sociability based on a preference for time spent in the chamber with a stranger mouse compared with an object. This did not differ by genotype or phase. (**B**) Both control and *Fmr1* KO mice show sociability based on time spent sniffing a stranger mouse compared with an object. This did not differ by genotype or phase.

Figure 5: Social novelty may be slightly affected by phase and genotype. (**A**) Time spent in the chamber shows that there was a near significant interaction between Phase X Genotype X Chamber. A post-hoc pair-wise analysis showed a significant difference between time in the chamber with the familiar mouse in the active phase between control and *Fmr1* KO mice. There was also a near significant preference in time in chamber with the novel mouse compared with the familiar mouse in the *Fmr1* KO mice during the active phase only. (**B**) Both control and *Fmr1* KO mice showed preference for social novelty based on time spent sniffing the novel mouse compared to the familiar mouse. This did not differ by genotype or phase.

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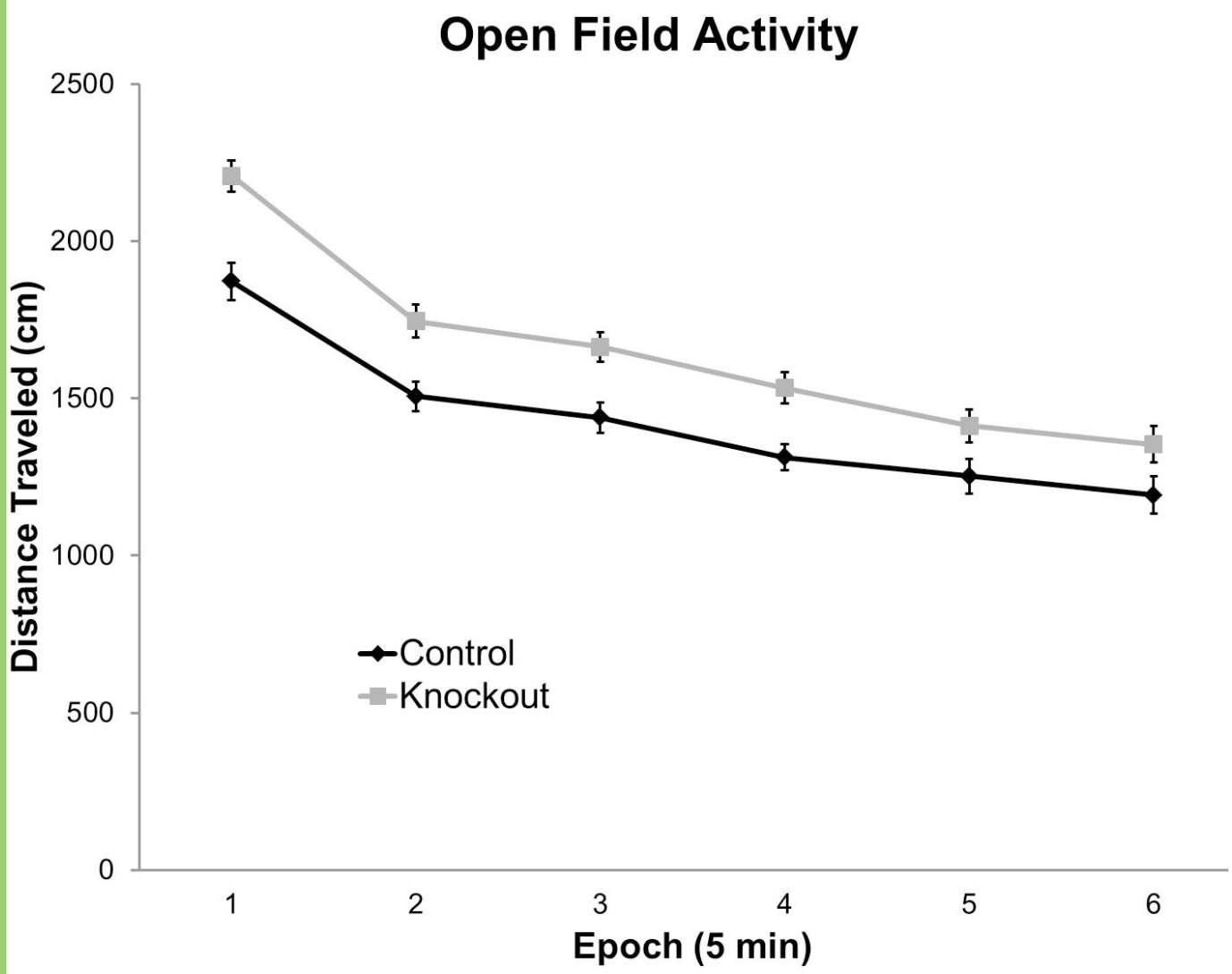
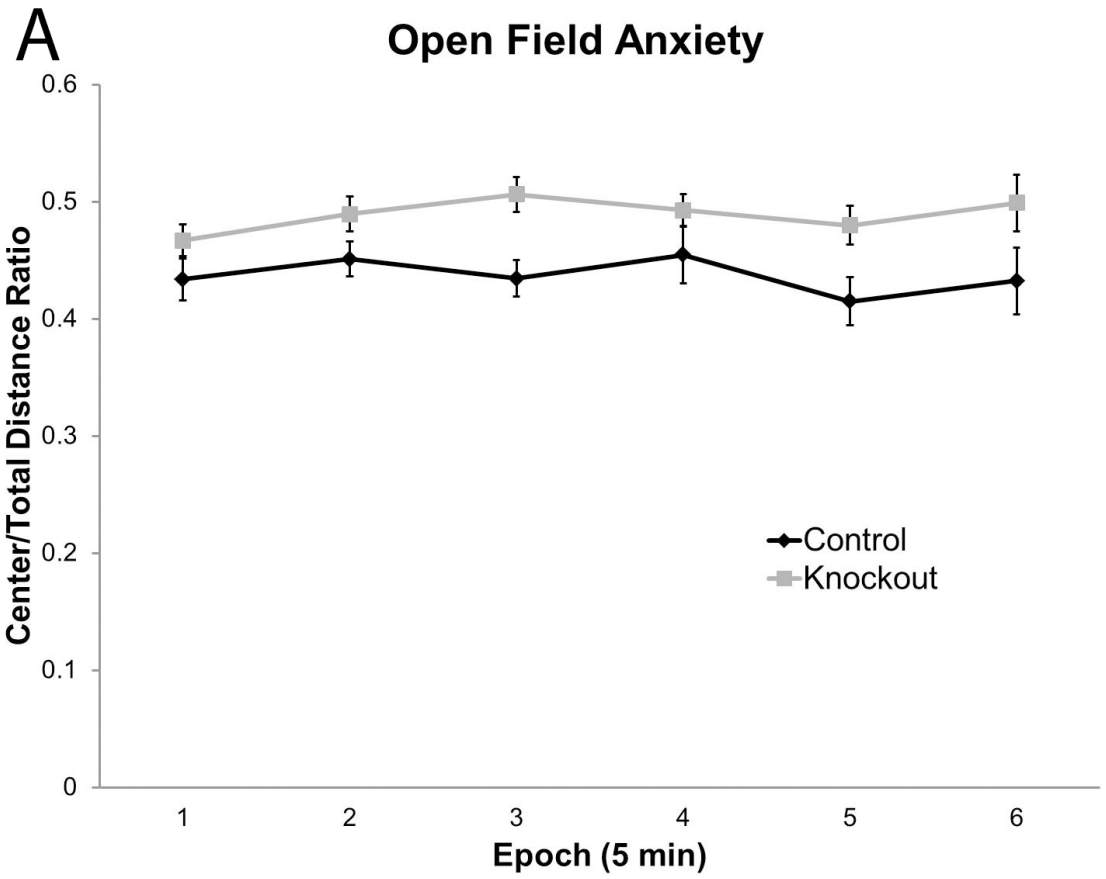
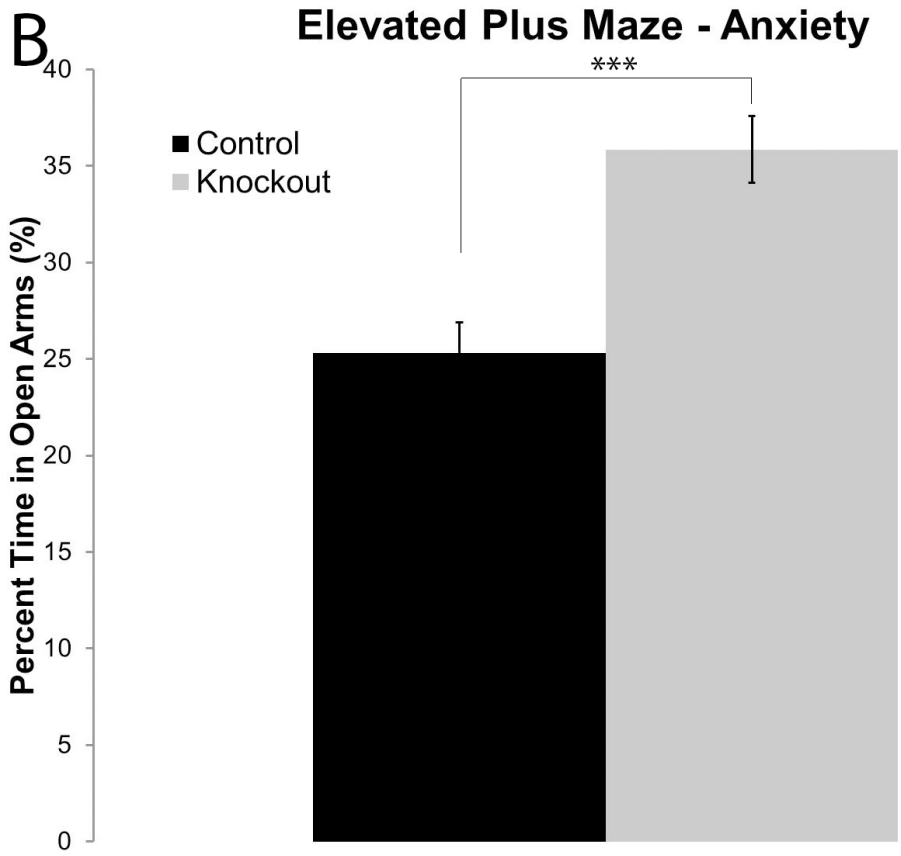
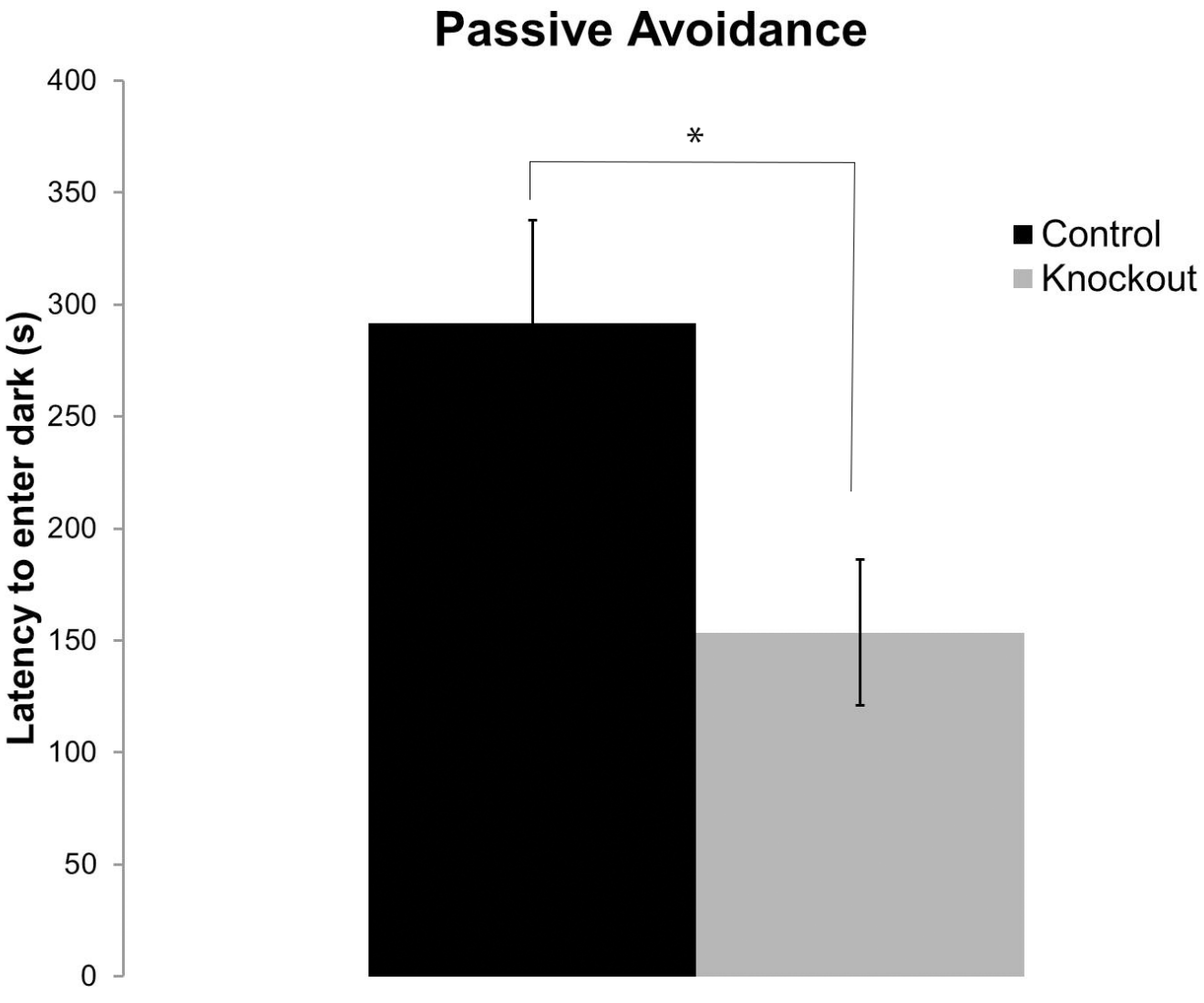
TABLE 1. Repeated Measures ANOVA Results			
BEHAVIOR	EFFECT	$F_{(df, error)}$ VALUE	P-VALUE
OPEN FIELD			
Total distance moved	Genotype x Epoch	$F_{(5,258)}$= 1.417	0.221
	Genotype	$F_{(1,54)}$= 13.943	<0.001*
	Epoch	$F_{(5,258)}$= 123.247	<0.001*
Center/Total Ratio	Genotype x Epoch	$F_{(5,228)}$= 0.594	0.675
	Genotype	$F_{(1,54)}$= 9.620	0.003*
	Epoch	$F_{(5,228)}$= 1.164	0.328
Social Behavior			
Sociability			
Chamber time	Phase x Genotype x Chamber	$F_{(1,99)}$= 0.080	0.778
	Genotype x Chamber	$F_{(1,99)}$= 0.040	0.842
	Phase x Chamber	$F_{(1,99)}$= 0.102	0.750
	Phase x Genotype	$F_{(1,99)}$= 0.047	0.829
	Phase	$F_{(1,99)}$= 0.497	0.483
	Genotype	$F_{(1,99)}$= 1.016	0.316
	Chamber	$F_{(1,99)}$= 32.244	<0.001*
Sniffing Time	Phase x Genotype x Chamber	$F_{(1,98)}$= 0.442	0.508
	Genotype x Chamber	$F_{(1,98)}$= 0.823	0.367
	Phase x Chamber	$F_{(1,98)}$= 0.073	0.787
	Phase x Genotype	$F_{(1,98)}$= 0.414	0.522
	Phase	$F_{(1,98)}$= 0.154	0.695
	Genotype	$F_{(1,98)}$= 0.057	0.812
	Chamber	$F_{(1,98)}$= 116.145	<0.001*
Social novelty			
Chamber time	Phase x Genotype x Chamber	$F_{(1,98)}$= 3.249	0.075~
	Genotype x Chamber	$F_{(1,98)}$= 0.189	0.664
	Phase x Chamber	$F_{(1,98)}$= 0.294	0.589
	Phase x Genotype	$F_{(1,98)}$= 1.461	0.230
	Phase	$F_{(1,98)}$= 0.133	0.716
	Genotype	$F_{(1,98)}$= 3.638	0.059~
	Chamber	$F_{(1,98)}$= 0.122	0.728
Sniffing time	Phase x Genotype x Chamber	$F_{(1,98)}$= 0.350	0.556
	Genotype x Chamber	$F_{(1,98)}$= 0.054	0.816
	Phase x Chamber	$F_{(1,98)}$= 0.042	0.837
	Phase x Genotype	$F_{(1,98)}$= 0.057	0.811
	Phase	$F_{(1,98)}$= 0.417	0.520
	Genotype	$F_{(1,98)}$= 2.688	0.104
	Chamber	$F_{(1,98)}$= 10.576	0.002*

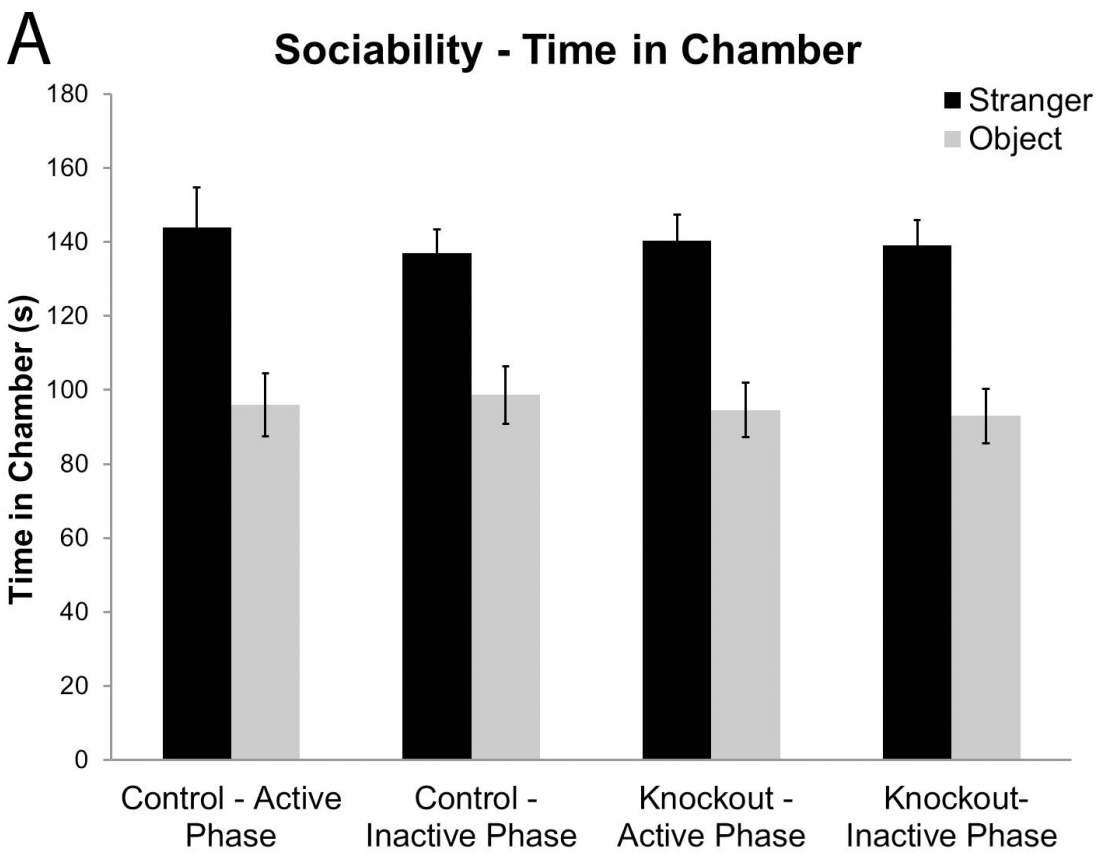
422

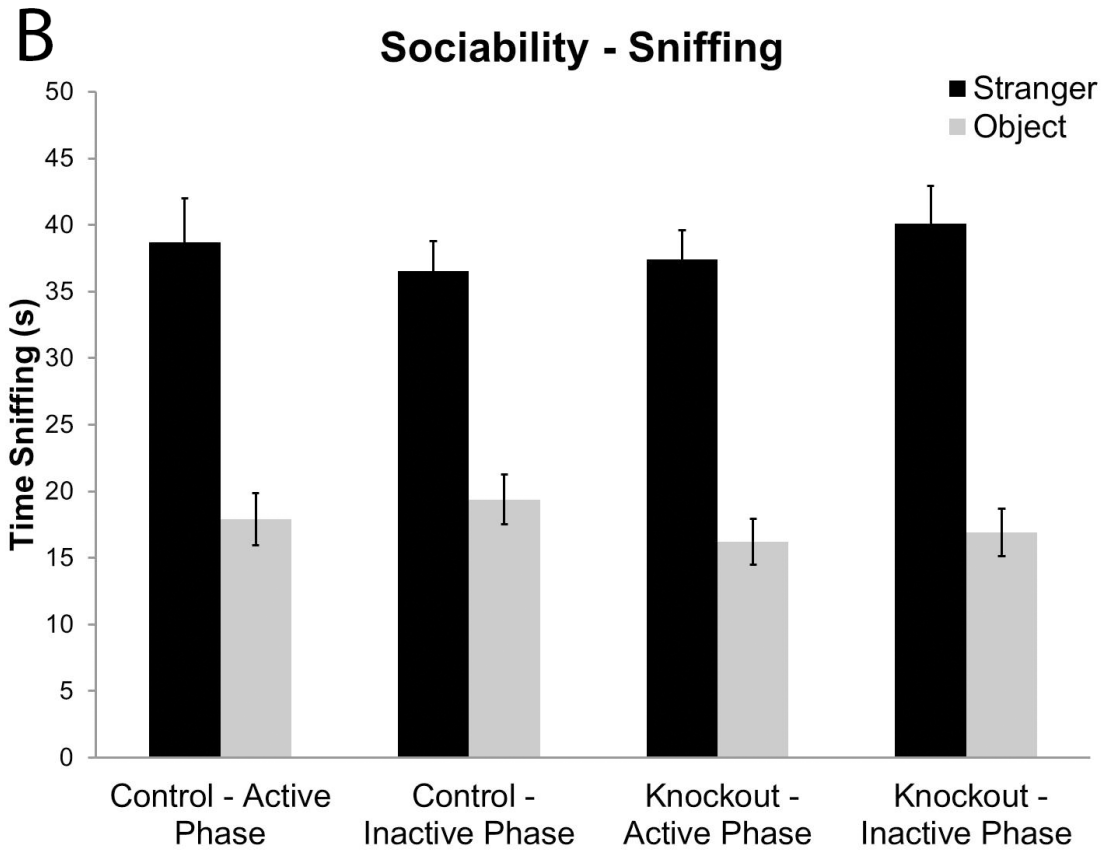




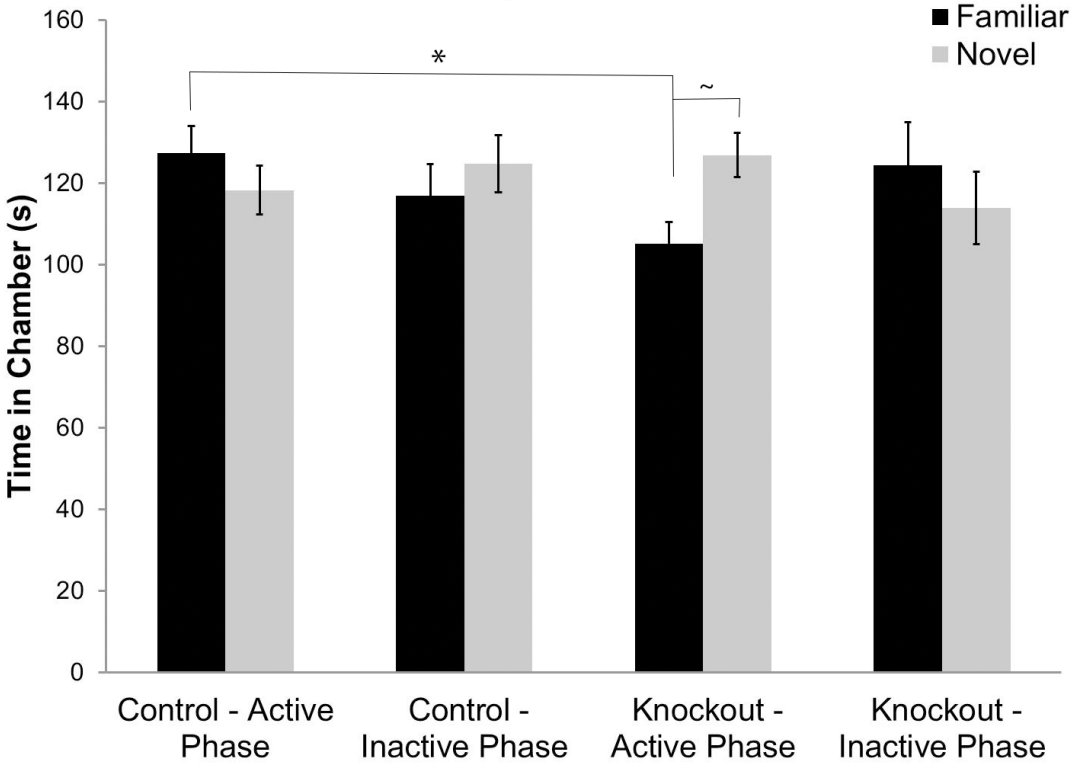








A



B