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Learning and Memory Abnormalities in Mice Deficient in the RNA-Binding Protein KSRP

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by

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ABSTRACT

Post-transcriptional regulation plays a critical role in the function and development of the nervous system; especially in the control of specific subsets of mRNAs localized to dendrites and axons. One such regulatory mechanism is the stabilization or disruption of mRNA through association of RNA binding proteins (RBPs) such as the KH-type splicing regulatory protein (KSRP). We have previously shown that loss of KSRP results in dysregulation of GAP-43 and increased growth of hippocampal neurons *in vitro.* Therefore, $KSRP^{-/-}$ and $KSRP^{+/-}$ animals were subjected to a battery of behavioral tests including Novel Object Recognition and Trace Fear Conditioning to assess hippocampal function as well as the Attentional Set Shifting Task to determine executive control capabilities. $KSRP^{-/-}$ animals have significantly greater recognition of the novel object, but are impaired in trace conditioning compared to age and sex matched WT controls. They also exhibit deficits in set-shifting of species-specific stimulus domains within the ASST task. $KSRP^{-/-}$ mice also display novelty induced hyperactivity that is present in several tests including novel open field, zero maze, and novel object recognition. We conclude that loss of KSRP leads to abnormalities in both hippocampal dependent and independent learning and memory as well as hyperactivity.

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CHAPTER 1
INTRODUCTION

1.1 Post-Transcriptional Regulation

Post-transcriptional regulation (PTR) plays a critical role in the function and development of the nervous system. Independent from transcription and translation, PTR is especially important in control of specific sets of mRNAs localized to dendrites and axons (Eberwine et al, 2001). Furthermore, the stability of these mRNAs is also critically important for the regulation of gene expression as changes in the decay of mRNA rates are rapid and precise. One such regulatory mechanism is *cis*-acting mRNA sequences interacting with trans-acting factors like RNA binding proteins (RBPs) and miRNA (Bolognani and Perrone-Bizzozero, 2008). Binding of RBPs to mRNA can stabilize the mRNA by protecting it from nucleases and may promote translation by targeting to ribosomes (Szostak and Gebauer, 2012). Conversely, binding of RBPs can promote the decay of mRNA by targeting to the exosome.

1.2 KH-Type Splicing Regulatory Protein

The KH-type splicing regulatory protein (KSRP) is an RBP associated with the decay of AU-rich element (ARE)-containing mRNAs by targeting them to the exosome for degradation (Gherzi et al, 2004; Chen et al, 2001). KSRP was originally identified as a DNA Far Upstream Element (FUSE) binding protein 2 (FBP2) which enhanced splicing of the neuron-specific c-src N1 exon (Davis-Smyth et al, 1996; Min et al, 1997). The KSRP orthologue in rats is MARTA1 and is described as transporting MAP-2 to neuronal processes (Rehbein et al, 2002). Similarly, the chicken homologue nuclear zipcode binding protein 2 (ZBP2) is involved in cytoplasmic β-actin mRNA targeting (Gu et al,

2002). Recently, KSRP has been characterized to destabilize growth associated protein GAP-43 mRNA (Bird et al, 2013).

1.3 Growth Associated Protein GAP-43

The growth associated protein GAP-43 is a neuronal-specific phosphoprotein critical in normal axonal growth and pathfinding, therefore, its proper and precise regulation is essential for correct neuronal function. GAP-43 is localized to the growth cones of developing neurons (Fallini et al, 2016; Strittmatter et al, 1995). Complete knock out of GAP-43 is lethal within the first 3 weeks of life, while knock down of GAP-43 results in decreased hippocampal dependent learning and memory (Rekart, Meiri, and Routtenberg, 2005). Overexpression (OE) of GAP-43, however, results in aberrant axonal sprouting and increased learning and memory in a hippocampal dependent win-shift task (Benowitz and Routtenberg, 1997; Routtenberg et al, 2000). We have previously shown that loss of KSRP results in dysregulation of GAP-43 and increased growth of hippocampal neurons *in vitro* (Bird et al, 2013).Therefore, $KSRP^{-/-}$ mice may have a similar increased hippocampal dependent learning as shown by the GAP-43 OE mice.

1.4 HuD

GAP-43 is stabilized by the neuronally enriched RBP HuD and leads to increased outgrowth of axons (Beckel-Mitchener et al, 2002). It has been previously shown that transgenic mice overexpressing HuD have decreased freezing to tone in a contextual fear behavioral paradigm which relies on proper hippocampal function (Bolognani et al, 2007). Since HuD and KSRP compete for binding sites (Bird et al, 2013) we hypothesized that KSRP knockout mice may have similar behavioral abnormalities.

CHAPTER 2
METHODOLOGY

2.1 Animals

All animal studies were conducted in accordance with guidelines for animal use and care established by the University of New Mexico Health Science Center Institutional Animal Care and Use Committee.

KSRP$^{-/-}$ animals were generated by Cre-Lox recombination as described in Lin et al., 2011. Animals were maintained on a reverse 12-hour dark/12-hour light cycle (lights on at 20:00 hours) in grouped-housed cages. Water and standard chow was available ad libitum in all cages unless otherwise specified. Behavioral testing was conducted on male and female KSRP$^{-/-}$, KSRP$^{+/-}$, and age and sex matched wild type (WT) controls. KSRP has also been recently identified to interact with the circadian rhythm by targeting PER2 (Chou et al, 2015), therefore, all behavioral measurements were conducted during the dark period between 09:00 and 17:00 in behavioral rooms lit with red lighting.

Cohort 1 consisted of 7 M WT, 7 F WT, 10 M KSRP$^{-/-}$, and 8 F KSRP$^{-/-}$ that began the preliminary behavioral screen between the ages of 9-18 weeks. Followed by the zero maze at the ages of 9-19 weeks, then the open field at the age of 10-21 weeks, then novel object recognition at the age of 11-21 weeks, lastly they were assessed on ASST at ages 17-27 weeks.

Cohort 2 consisted of 8 M WT, 6 F WT, 5 M KSRP$^{+/-}$, 5 F KSRP$^{+/-}$, 2 M KSRP$^{-/-}$, and 5 F KSRP$^{-/-}$ that began the preliminary behavioral screen between the ages of 10-21 weeks, followed by the marble burying task and nestlet shredding at the ages of 9-23 weeks, then the zero maze at the ages of 10-24 weeks, then the open field at the age of 11-24 weeks, then novel object recognition at the age of 12-24 weeks, then they were

assessed on ASST at ages 18-29 weeks (3 additional F KSRP$^{-/-}$ were assessed only on ASST at the ages of 12-14 weeks), lastly they were assessed on trace fear conditioning at the age 23-33 weeks. An additional 5 M KSRP$^{-/-}$ were assessed only on trace fear conditioning at the age of 10 weeks.

2.2 Preliminary Behavioral Screen

Mice were assessed using a subset of tests derived from the Irwin screen as previously described (Zhao et al, 2006; Crawley, 1998; Irwin, 1968) for physical health, sensory, motor, and neurological function. General physical health and empty cage behaviors were observed by placing the mouse in a corner of a clear box (45 x 45 x 22 cm) and recording exploratory behavior for 10 min.

2.3 Elevated Zero-Maze Test

The elevated zero-maze test was conducted on a white circular platform (5 cm runway, 60 cm diameter and 50 cm from the floor) consisting of 2 opposing open quadrants with a 0.5 cm raised lip to prevent mice from falling off the maze and 2 opposing closed quadrants with 15 cm high walls. The room was illuminated with red fluorescent lights and two single white lights on either side of the open arms providing an average of 90 lux on the open arms and 45 lux in the closed arms. Mice were allowed to freely explore the arena for 5 min. Locomotor activity and time spent in the open vs closed arms was measured using Ethovision videotracking system (Noldus Information Technology, Leesburg, VA, USA).

2.4 Novel Open Field Test

The novel open field test was conducted in a square arena (40 x 40 x 35 cm) constructed from white Plexiglas. The room was illuminated with red fluorescent lights

and 2 white spot lights (center 80 lux, corner 35 lux). Each mouse was placed in the NW corner and allowed to freely explore for 30 min per day for 5 consecutive days to establish a baseline of anxiety and locomotor activity. Distance traveled, velocity, and duration in the (20 x 20 cm) center was measured using Ethovision videotracking system (Noldus Information Technology, Leesburg, VA, USA).

2.5 Novel Object Recognition

Testing took place inside a Plexiglas arena (45 x 45 x 20 cm) with white walls and floor. Familiar objects were three identical dark glass vials and the novel object was a plastic bottle of similar size and weight. Mice were transported to the testing room and after 30 min elapsed were placed in the empty arena and allowed to freely explore for 5 min to become familiar with the context. 24 hours later the mice were subjected to a single test trial consisting of a sample phase, a rest period, and a test phase. The sample phase consisted of placing 2 of the familiar glass objects into opposite corners of the arena (NW and SE), placing the mouse facing the wall opposite the objects (SW), and allowing them to freely explore for 5 min. Next mice were placed in a holding cage for a 5 min rest period while the arena was wiped with 70% isopropanol and the objects replaced with the third non-scent marked familiar object (NW corner) and the novel object (NE corner). Lastly the mouse was placed back in the arena (SW corner) for the 5 min test phase. Distance Traveled, velocity, frequency of visits to each object and duration of time spent with each object was measured using Ethovision videotracking system (Noldus Information Technology, Leesburg, VA, USA). Animals with a total exploration time of less than 3 s for either object during the testing session were excluded from analysis.

During the sample phase the location preference was calculated to rule out the influence of the location of the object on exploratory behavior using the following formula: Location preference (LP) = ($T_{standard1}$/[$T_{standard1}$+$T_{standard2}$] *100)

During the testing phase each animal's Recognition Index was calculated using the standard formula: Recognition Index (RI) = (T_{novel}/[T_{novel}+$T_{standard}$] * 100)

2.6 Measures of OCD-like behavior

Marble Burying was conducted as previously described (Angoa-Perez et al, 2013; Zhao et al, 2006) in a (45 x 22 x 20 cm) rat cage with 5 cm of corn cob bedding in the bottom. 20 Marbles were placed in 5 rows equally spaced on top of the media. Number of marbles at least 2/3 buried after two 30 min sessions 1 week apart were scored by 2 investigators, 1 of which was blinded to the genotypes.

Nestlet shredding was conducted in the home cage of mice housed in groups of 2-3. Each nestlet was weighed prior to use. After a 4 hour exposure the nestlet was removed and allowed to dry overnight before taking a second weight. Percent shredded was calculated as 100-((weight 1/ weight 2) * 100). (Angoa-Perez et al, 2013).

2.7 Attentional Set Shifting Task (ASST)

Testing was conducted in an acrylic apparatus (30 x 18 x 12 cm) divided into a start box and 2 choice chambers. Each choice chamber contained a ceramic digging bowl (4.5 x 2.5 cm) placed on a platform (11 x 5 cm). Access to digging bowls was limited by a removable divider. Scented medium was made by mixing 150g of cob bedding with 20 crushed 14 mg dustless precision pellets (#F0568, BioServ, Frenchtown, NJ) and 3 g of commercially available powdered spices: nutmeg, ginger, garlic, coriander, thyme, and cinnamon (Kroger Co, Cincinnati, Ohio 45202). Approach platforms were manufactured

in house from commercially available materials: sandpaper, wood, neoprene, metal wire, tile, and a plastic fiber sponge.

ASST was conducted as previously described (Young et al., 2010, Marquardt et al., 2014, Thompson et al., 2015). Briefly, day 1 consisted of acclimation to the testing chamber and training to dig in unscented cob medium for food reward. Initially, pellets were available on the floor and in the bottom of empty digging bowls. Mice were allowed to explore the apparatus until all pellets were consumed. For all proceeding trials the pellets were restricted to digging bowls beginning with 3 pellets per side. Digging medium was incrementally increased while successively burying one additional pellet each trial. When the digging bowls were full of medium and all pellets buried, the pellets were restricted to a single bowl, randomly assigned per trial. Pellet number was then decreased with each proceeding trial, with the final 9 trials containing only 1 pellet. A total of 50 pellets were consumed on day 1. Trials were timed from divider lifting until all pellets were consumed. Mice who ceased digging for pellets were returned to their home cage for a 10 min rest period before resuming at last attempted trial.

Day 2 training introduced the mice to each odor and platform combination that they may encounter during day 3 testing (Table 1). A single pellet was placed approximately ¾ of the way below the digging medium surface in one bowl, randomly assigned between trials. Placement of a pellet was mimicked in the empty bowl to "sham bait" and prevent mice learning experimenter related cues. 24 pellets were received.

Day 3 mice were tested in succession with no inter-session-breaks on 7 discrimination tasks (Table 1). In the simple discrimination (SD) mice were initially trained to discriminate 2 exemplars in either the odor or platform dimension

(counterbalanced across genotypes and gender n=34). Upon reaching criterion mice were moved to compound discrimination (CD) in which the second, non-reward dimension, was added. Mice were required to respond in accordance to the first discrimination learned. The first 4 trials of the SD and CD stages mice were allowed to dig in the incorrect bowl without consequence, although an error was recorded. In all other trials the opposite chamber was blocked upon an error to prevent reward collection. In successful trials, the mouse was allowed to collect the pellet before continuing to the next trial. If the mouse did not dig in either bowl by 2 min the trial was recorded as 'no choice' and the mouse repeated the trial until a choice was made. Criterion was set to 6 consecutive correct responses.

Upon completion of the CD the rewarded exemplar in the initially rewarded dimension was reversed to form a compound discrimination reversal (CDR). Following the CDR, a novel set of exemplars in each dimension was introduced and mice were rewarded for responding to one exemplar in the initially learned dimension (intra-dimensional shift (IDS)). Next, the intra-dimensional reversal (IDR) reversed the correct stimuli within the same dimension. Following IDR, a second novel set of exemplars in both dimensions were introduced in the extra-dimensional shift (EDS) where the rewarded exemplar was in the previously irrelevant dimension. Finally, the correct exemplar within the newly learned dimension was reversed to form an extra-dimensional reversal (EDR).

Trials to criterion and errors were recorded for each stage. Trial latencies to respond were measured from the time the barrier was raised until digging was initiated. A dig was defined as the moment when the mouse's nose or paw broke the surface of the

cob-digging medium. Mice were discontinued if they required 60 trials on any 1 problem or 150 trials total.

WT animals in Cohort 1 did not form an attentional set due to high performance on the EDS. Upon examination of the stages the first 3 trials have the same odor/platform combinations on the CD and IDS stages but not on the EDS stage so for Cohort 2 the odor/platform combination of the first 3 trials on the EDS were paired resulting in a slight increase in difficulty which produced a WT attentional set. Therefore, data displayed is only Cohort 2.

Problem Stage	Dimensions		Exemplars	
	Relevant	Irrelevant	S+	S-
Simple Discrimination (SD)	Odor	n/a	O1	O2
Compound Discrimination (CD)	Odor	Platform	O1/P1	O2/P2
			O1/P2	O2/P1
Compound Discrimination Reversal (CDR)	Odor	Platform	O2/P2	O1/P1
			O2/P1	O1/P2
Intradimensional Shift (IDS)	Odor	Platform	O3/P4	O4/P4
			O3/P3	O4/P3
Intradimensional Shift Reversal (IDR)	Odor	Platform	O4/P4	O3/P4
			O4/P3	O3/P3
Extradimensional Shift (EDS)	Platform	Odor	P5/O5	P6/O5
			P5/O6	P6/O6
Extradimensional Reversal (EDR)	Platform	Odor	P6/O5	P5/O5
			P6/O6	P5/O6

Table 1 Example problem stages and stimulus combinations for the attentional set shifting task. Starting dimension was counterbalanced across sex and genotype

2.8 Trace Fear Conditioning

Studies were conducted between 0900 and 1200 hrs. under dim red illumination, as previously described (Brady et al, 2012). Briefly, animals were placed into a Coulburn Instruments (Whitehall, PA) Habitest System for 90 secs of habituation, followed by 7 trials each consisting of the Condition Stimulus (CS, 10 secs, 80 dB 6 Hz clicker), a 30 second trace, the Unconditioned Stimulus (US, 1 sec, 0.8 mA scrambled foot Shock), and a 180 sec inter-trial interval; the subject was removed from the chamber 60 secs following the delivery of the last US.

Twenty four hours later, freezing to the CS in a novel context (a standard, clean mouse cage with minimal bedding) was assessed. The CS was delivered at 180 secs while the animal's behavior was videotaped. The amount of time spent freezing during the 10 sec CS and during the 30 sec trace was scored by 2 investigators, one of which was blinded to the genotype.

2.9 Statistical Analysis

Repeated Measures (RM) ANOVA was used for open field percent center duration, distance and velocity days 1-5, distances and velocity day 1 mins 0-5 through 20-25. Tukey post hoc tests were utilized for open field distance and velocity days 1-5, distances and velocity day 1 mins 0-5 through 20-25. 2-way ANOVA was used to determine if there was a main effect of sex and genotype for the percent open arm duration, distance, and velocity in the zero maze. Upon elimination of a main effect of sex in the zero maze parameters sexes were combined. RM ANOVA was used to determine overall effects of problem stages in ASST, to compare starting dimension effects of specific stages 1-way ANOVA was used. To compare each genotype between

stages (CDR-CD, IDR-IDS, and EDR-IDS) t-tests were employed. 2-way ANOVA was used to determine if there was a main effect of sex and genotype for all parameters measured in novel object recognition (NOR) upon elimination of a main effect of sex the sexes were combined and 1-way ANOVA was used thereafter. For the locomotor effects in NOR RM ANOVA was used followed by Tukey post hoc tests. Similarly, RM ANOVA was used to identify time differences between Day 1 and Day 2 of marble burying, followed by 1-way ANOVA of each day to discover where the Genotype differences lay. Lastly, RM ANOVA was utilized for the Tone Training, Trace Training, as well as the Tone-Trace Test followed by individual 1-way ANOVAs for each time point in Trace Fear Conditioning

CHAPTER 3
RESULTS

3.1 General sensory motor assessment of KSRP$^{-/-}$ and KSRP$^{-/+}$

3.1a Preliminary behavioral screen

A preliminary behavioral screen was adapted from a subset of tests derived from the Irwin screen as previously described (Zhao et al, 2006; Crawley, 1998; Irwin, 1968) for physical health, appearance, sensory utility, motor coordination, locomotor activity and neurological function. As shown in Table 2, KSRP$^{-/-}$ and KSRP$^{-/+}$ exhibit normal mouse features including weight, whiskers, eyes, eyelids, teeth, tail, and fur. They also show no differences in normal mouse behaviors including gait, time spent grooming, latency to first groom, and rearing, but KSRP$^{-/-}$ animals display increased circling (ANOVA: $F(2,57)=4.887$, $P<0.01$) and were the only group to display wild running, which may indicate hyperactivity in these mice.

	Males			Females		
	WT	HT	KO	WT	HT	KO
Physical Health						
Whiskers	0	0	0	0	0	0
Bald Patches	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0
Exophthalmos	0	0	0	0	0	0
Palpebral closure	0	0	0	0	0	0
Straub tail	0	0	0	0	0	0
Kinked Tail	0	0	0	0	0	0
Head bobbing	0	0	0	0	0	0
Waltzing	0	0	0	0	0	0
Prancing	0	0	0	0	0	0
Retropulsion	0	0	0	0	0	0
Clear Box Behaviors						
Sniffing	100	100	100	100	100	100
Abnormal Gait	7	0	17	0	0	23
Circling	20	40	75*	23	20	46
Jumping	27	20	25	0	20	23
Freezing	27	20	33	23	40	46
Wild running	0	0	17*	0	0	15*
Defecation	67	40	75	69	20	15*
Urination	13	20	17	8	20	15
Rearing Count	95	103	100	87	80	103
Total Time Grooming (s)	15.9	18.8	17.8	21.2	14.8	20.9
Latency to first Groom (s)	185.8	186.4	164.1	134.4	115.4	113.2
Weight (g)	23.7	22.6	23.1	23.2	22.1	22.8

Table 2: Preliminary behavioral screen of KSRP$^{-/-}$ and KSRP$^{-/+}$ mice. Data are percentage of animals that displayed a response unless units are given in parenthesis. * indicates significance of $p<0.05$ from WT controls. N= 15 M WT, 13 F WT, 5 M HT, 5 F HT, 12 M KO and 13 F KO.

3.1b Anxiety-like behavior

Mice were assessed for anxiety-like behavior, locomotion, and exploratory tendencies in the novel open field test and on the elevated zero maze. There is no significant main effect of genotype or sex nor a genotype x sex interaction for center duration in the open field, therefore, sexes were combined (Figure 1A). However, there is a significant main effect of time (RM ANOVA $F(4,240)=3.987$, $P=0.0039$). Tukey post hoc test revealed this to be due to an elevation of center time on day 2 compared to days 3 and 4.

There is no significant main effect of genotype or sex, nor a genotype x sex interaction for open arm time on the zero maze, therefore sexes were combined (Figure 1B) KSRP$^{-/-}$ and KSRP$^{-/+}$ mice demonstrate no differences in percent open arm time on the zero maze (ANOVA: $F(2,59)=0.6047$ $P>0.05$). Taken together this indicates that overall there is no difference in anxiety-like behavior between genotypes.

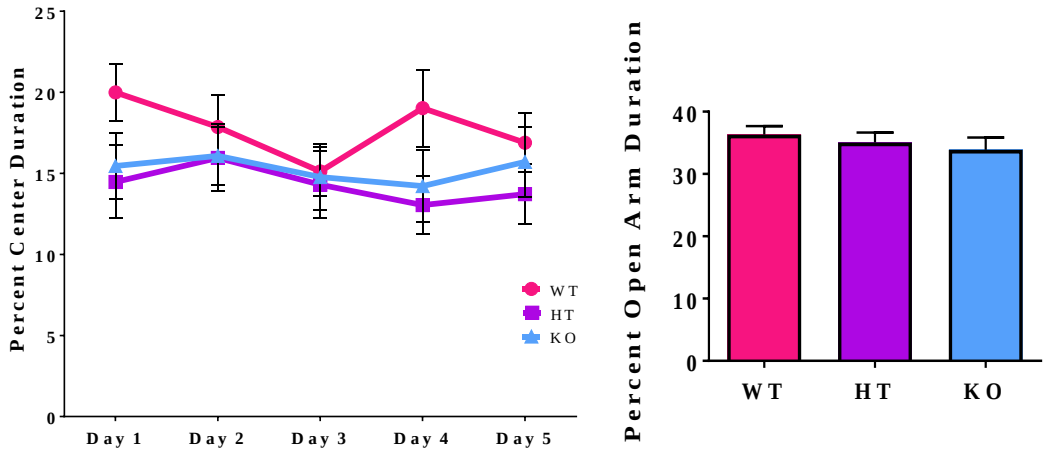

Figure 1: KSRP$^{-/-}$ and KSRP$^{-/+}$ mice display no differences in anxiety-like behaviors from WT mice. *A.* Percent center duration in the open field on days 1-5. *B.* Percent open arm duration on the zero maze. Mean ± SEM. N= 15 M WT, 13 F WT, 5 M HT, 5 F HT, 12 M KO and 13 F KO.

3.1c Locomotor activity

There is no significant main effect of sex nor a genotype x sex interaction for either measure of locomotor activity on the zero maze, therefore sexes were combined (Figure 2A and 2B). KSRP$^{-/-}$ and KSRP$^{-/+}$ mice have increased locomotor activity in the zero maze (ANOVA Distance traveled: $F(2,52)=4.606$ $p=0.0143$; Velocity: $F(2,53)=6.915$, $p=0.0022$).

In the Open Field there is no significant main effect of genotype or sex, nor a genotype x sex interaction for distance traveled (Figure 2C), however, there is a significant main effect of sex for velocity (Figure 2D; RM ANOVA $F(1,57)=4.250$, $P=0.0438$). Both Distance and velocity have a significant main effect of time (RM ANOVA Distance: $F(4,228)=54.016$, $P<0.0001$; Velocity $F(4,228)=23.690$, $P<0.0001$). Tukey post hoc analysis determined this is because day 1 is significantly elevated compared to all other days.

Further examination of day 1 revealed a significant main effect of time (RM ANOVA Distance: $F(4,228)=84.998$, $P<0.0001$; Velocity: $F(4,228)=69.649$, $P<0.0001$) and a time x genotype interaction (RM ANOVA Distance: $F=8,228)=3.263$) $P=0.0015$; Velocity: $F=2.898$, $P=0.0043$) but no significant main effect of genotype, sex, genotype x sex, time x sex, or time x genotype x sex for either measure of locomotion. Tukey post hoc test revealed that minute 0-5 is significantly different from all other time points for both distance traveled and velocity which is indicative of novelty induced locomotion. This difference is driven by the KSRP$^{-/+}$ mice for distance traveled (ANOVA $F(2,60)=0.2306$, $P=0.0449$), while for velocity KSRP$^{-/-}$ and KSRP$^{-/+}$ animals are both

significantly elevated compared to WT controls (ANOVA F(2, 60)=1.895, WT vs HT P=0.0064, WT vs KO P=0.0442).

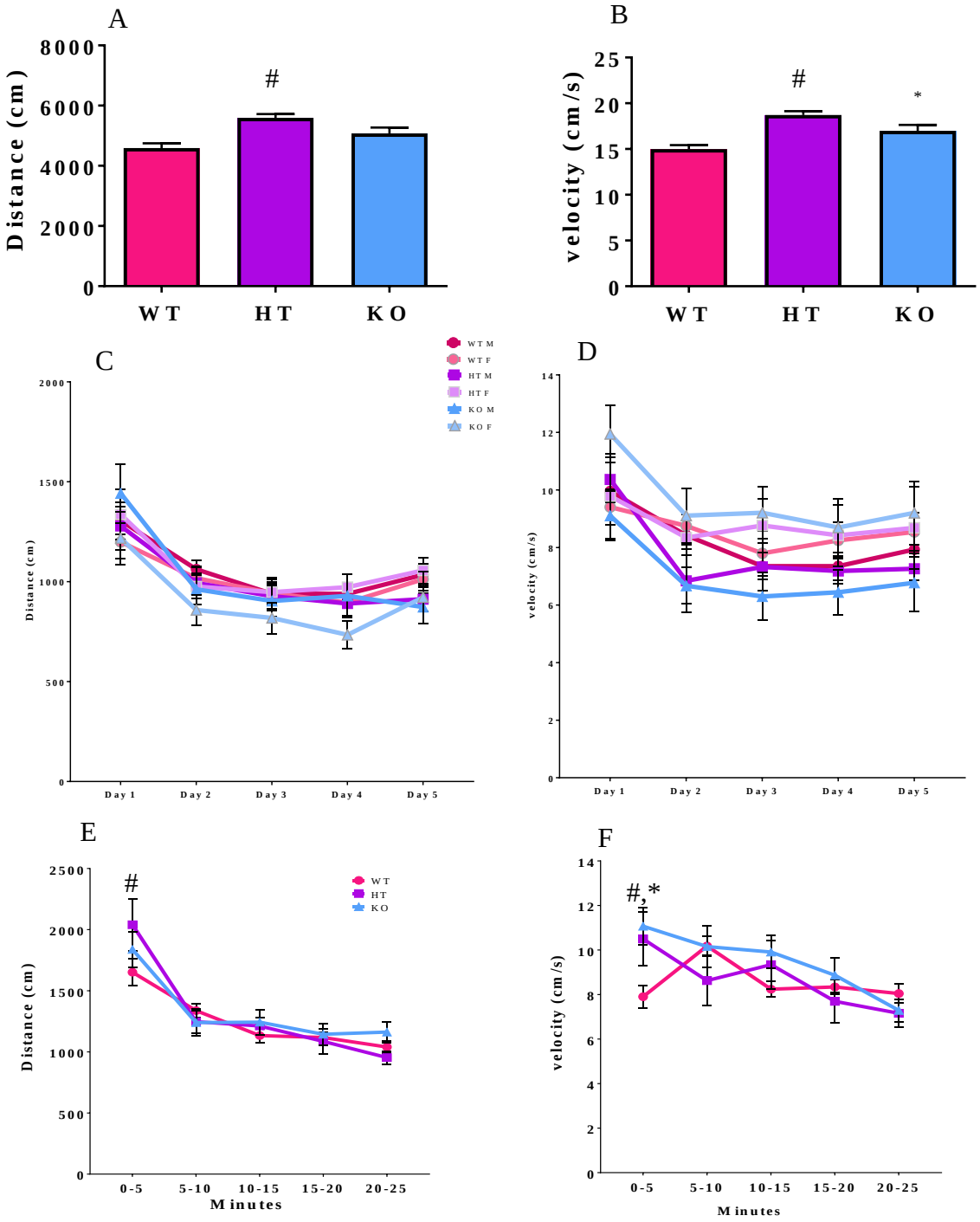

Figure 2: KSRP$^{-/-}$ and KSRP$^{-/+}$ mice locomotor activity. *A.* and *B.* increased locomotor activity during the 5 min zero maze task. *C.* and *D.* Locomotor activity over days 1-5 separated out by sex. *E.* and *F.* KSRP$^{-/-}$ and KSRP$^{-/+}$ mice have increased locomotor activity during the first 5 mins of Day 1. Values are mean ± SEM. N= 15 M WT, 13 F WT, 5 M HT, 5 F HT, 12 M KO and 13 F KO. # $p<0.05$ WT vs HT; * $p<0.05$ WT vs KO

3.3 Executive Control and the Attentional Set Shifting Task

The attentional set shifting task (ASST) assessed executive control by exposing the animals to a series of problem stages that include discrimination-reversal learning within one dimension, such as Odor, an intradimensional shift (IDS) with novel exemplars within the previously learned dimension (i.e. Odor to Odor), as well as an extradimensional shift (EDS) to the previously unrewarded dimension (i.e. Odor to Platform). It is well established that reversal learning is mediated by the orbitofrontal cortex (OFC) (Chudasama and Robbins, 2003; Rudebeck and Murray, 2011; Rudebeck et al., 2013) and attentional set shifting is mediated by regions of the ventromedial prefrontal cortex (vmPFC) (Floresco and Jentsch; 2011; Hamilton and Brigman, 2015; Birrell and Brown, 2000; Floresco et al., 2008).

There are no significant differences observed by sex, however, there was a significant main effect of starting dimension (Figure 3A) during the SD and CD stages that was eliminated by the CDR stage (ANOVA SD: $F(1,28)=12.998$, $P<0.01$; ANOVA CD: $F(1,28)=5.025$, $P>0.05$). It is commonly observed that tactical learning is more difficult to initially acquire than olfactory (Thompson et al, 2015).

Reversal learning is expectantly requires more trials to reach criterion than the preceding stage for WT mice (Figure 3B) (CDR-CD: $T=2.708$, $P<0.05$; IDR-ID: $T=2.393$, $P<0.05$), however, this was not true for $KSRP^{-/-}$ mice (CDR-CD: $T=5.482$, $P>0.05$; IDR-ID: $T=0.900$, $P>0.05$). Interestingly, $KSRP^{-/+}$ mice did have increased number of trials to criterion for the first reversal (CDR-CD: $T=5.482$, $P<0.01$), but this was not sustained on the second reversal (IDR-ID: $T=0.058$, $P>0.05$). Neither $KSRP^{-/-}$ or $KSRP^{-/+}$ mice established an attentional set as measured by EDS-IDS ($KSRP^{-/+}$:

T(9)=0.065, P>0.05; KSRP$^{-/-}$: T(9)=1.087, P>0.05), however, WT mice did form an attentional set (T(13)=3.787, P<0.01). Taken together this indicates that KSRP$^{-/-}$ and KSRP$^{-/+}$ are able to perform each discrimination but are cognitively inflexible.

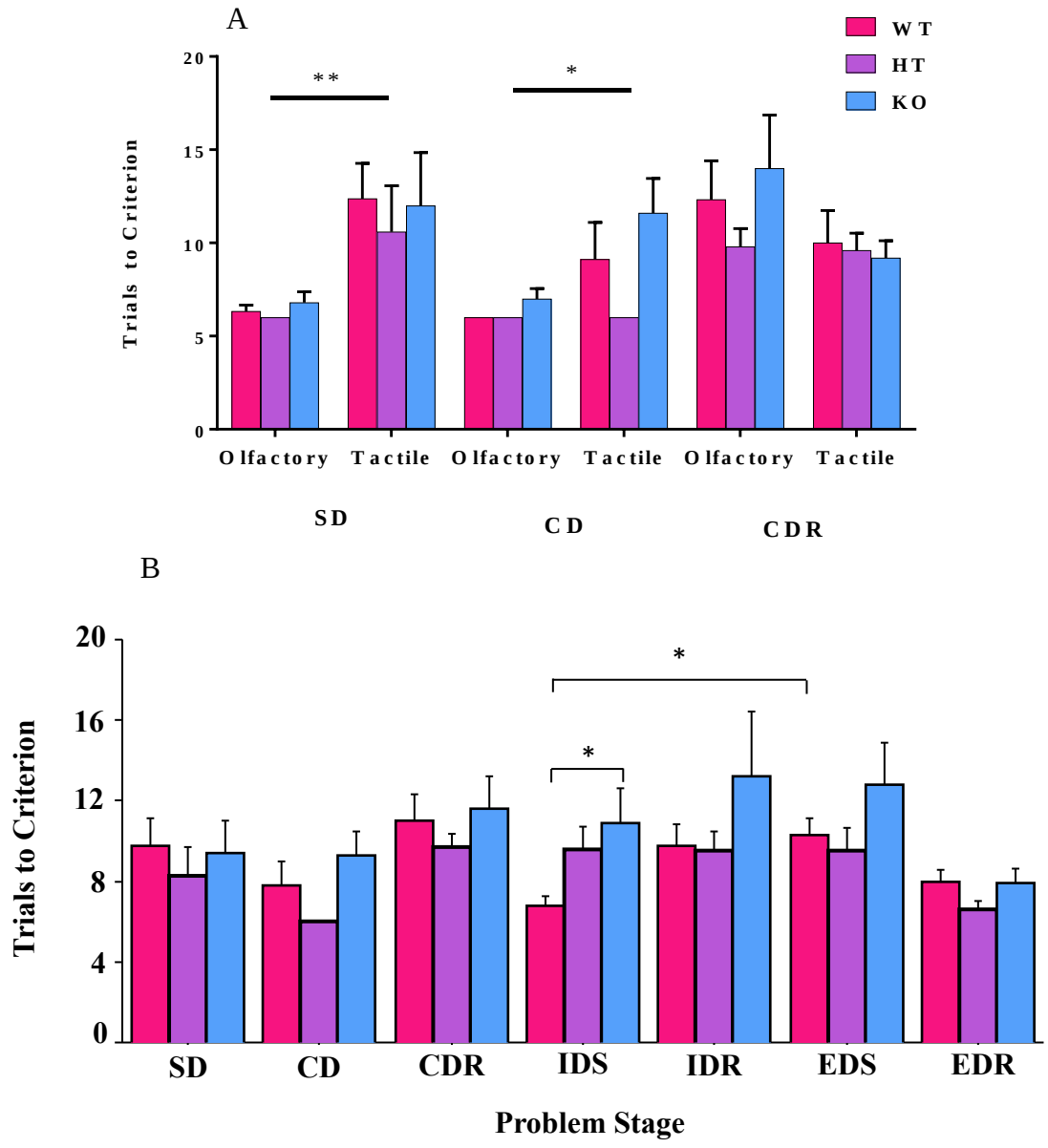

Figure 3: KSRP$^{-/-}$ and KSRP$^{-/+}$ mice display deficits in the attentional set shifting task. *A.* There was a significant main effect of starting stimulus dimension for the SD and CD stages that was eliminated by the CDR problem stage. *B.* All mice were able to perform discrimination (SD, CD, and IDS) and reversals (CDR, IDR, EDR), although the reversals were no more difficult for KO mice than the preceding stage (CDR-CD, and IDR-IDS). KO mice took significantly more trials to perform the IDS. WT mice formed an attentional set (EDS-IDS) however, KSRP$^{-/-}$ and KSRP$^{-/+}$ did not. SD=Simple Discrimination; CD=Compound Discrimination; CDR=Compound Reversal; IDS=Intradimensional Shift; IDR= Intradimensional Reversal; EDS= Extradimensional Shift; EDR= Extradimensional Reversal. N= 5-7/ genotype/sex. *p<0.05

3.4 Hippocampal Dependent Learning and Memory

3.4a Novel Object Recognition (NOR)

To address whether loss of KSRP affected recognition memory the novel object recognition task was employed which takes advantage of a rodent's spontaneous behavior of exploring novelty. We found no significant main effect of sex for any measure of novelty interaction and therefore combined sexes (Figure 4A-D). There is no significant difference in the time that mice spent exploring the two identical objects during the sample phase (Figure 4A); indicating the right/left location of the objects does not affect the curiosity or exploratory behavior of $KSRP^{-/-}$ and $KSRP^{-/+}$ mice. The Recognition Index (RI), calculated as the mean time spent at the novel object divided by the total time spent at each of the objects, was significantly elevated for $KSRP^{-/-}$ mice at 70% (Figure 4B) in comparison to the WT controls with an RI of 55% (ANOVA $F(2,51)=3.502$, $P=0.0373$). The $KSRP^{-/+}$ mice fell in between with and RI of 60%. The increased RI in $KSRP^{-/-}$ animals is a result of increased duration at the novel object (Figure 4C; ANOVA $F(2,51)=5.560$, $p=0.0065$) and increased frequency of visits to the novel object (Figure 4D; ANOVA $F(2,52)=10.15$ $p=0.0002$).

Examination of locomotor activity (Figure 4E and 4F) displays no significant main effect of genotype, sex, nor a genotype x sex interaction there is, however, both a time x genotype interaction (RM ANOVA Distance: $F(4,110)=6.461$, $P=0.0001$; Velocity $F(4,110)=6.977$, $P<0.0001$) as well as a time x sex interaction (RM ANOVA Distance: $F(2,110)=7.225$, $P=0.0011$; Velocity $F(2,110)=5.912$, $P=0.0036$). Interestingly, there is a significant main effect of time for both distance traveled and velocity (RM ANOVA Distance: $F(2,110)=25.579$, $P<0.0001$; Velocity: $F(2,110)=25.423$, $P<0.0001$), according

to Tukey Post Hoc analysis this is a result of the test time point. Further examination of the test time point revealed that the KSRP$^{-/-}$ male and female mice have significantly elevated distance traveled (Males: T(23)=5.753, P<0.0001; Females: T(26)=2.42, P=0.0228) and velocity (Males: T(23)=5.568, P<0.0001; Females: T(26)=2.326, P=0.0281) compared to WT males and females, respectively.

Male and female KSRP$^{-/+}$ and WT controls have decreased locomotor activity during the test period compared to the preceding 2 stages (WT male Distance Acclimation vs Test: T(12)=5.97, P<0.0001; WT female Distance Acclimation vs Test: T(14)=5.479, P<0.0001; WT male Velocity Acclimation vs Test: T(12)=5.517 P<0.0001; WT female Velocity Acclimation vs Test: T(14)=5.484 P<0.0001; WT male Distance Sample vs Test: T(12)=3.02, P=0.0107; WT female Distance Sample vs Test: T(14)=5.972, P<0.0001; WT male Velocity Sample vs Test: T(12)=3.056, P=0.01; WT female Velocity Sample vs Test: T(14)=5.972, P<0.0001; HT male Distance Acclimation vs Test: T(4)=5.634, P=0.0049; HT female Distance Acclimation vs Test: T(4)=5.227, P=0.0064; HT male Velocity Acclimation vs Test: T(4)=5.05, P=0.0072; HT female Velocity Acclimation vs Test: T(4)=5.331, P=0.006; HT male Distance Sample vs Test: T(4)=2.288, P=0.084; HT female Distance Sample vs Test: T(4)=5.995, P=0.0039; HT female Velocity Sample vs Test: T(4)=6.405, P=0.0031), except the male HT velocity for the sample phase (HT male Velocity Sample vs Test: T(4)=2.192, P=0.0935)

In contrast, KSRP$^{-/-}$ male mice do not display differences in their ambulation between stages, however, KSRP$^{-/-}$ female mice do have decreased velocity during the test phase compared to the previous 2 stages (Acclimation vs Test: T(12)=2.712, P=0.0189; Sample vs Test: T(12)=2.585, P=0.0239). This result led us to hypothesize that the

KSRP$^{-/-}$ mouse increased RI and heightened locomotor activity when the novel object is present could be due to obsessive-compulsive-like behavior.

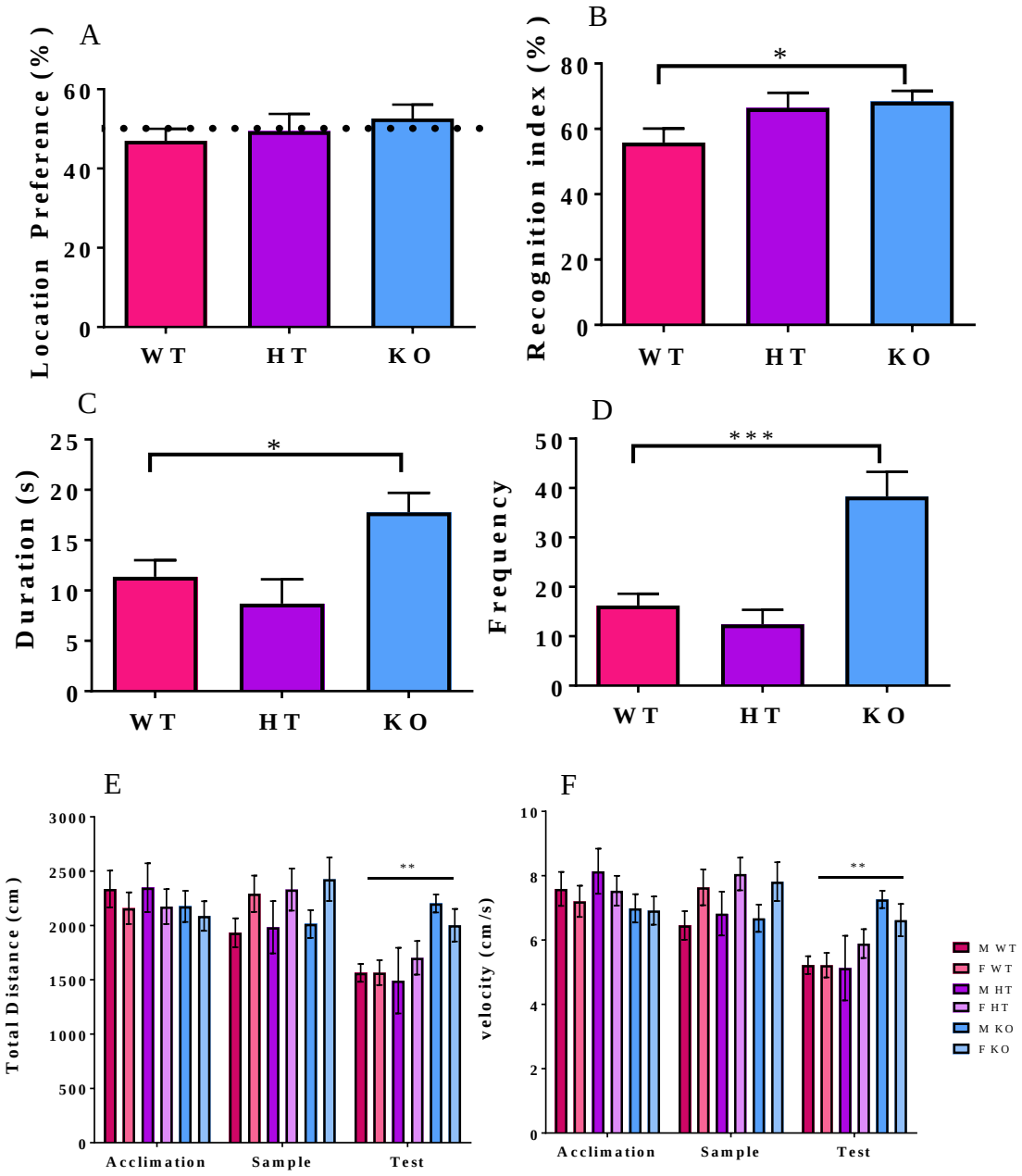

Figure 4: KSRP$^{-/-}$ mice have increased novel object recognition. *A.* none of the mice exhibited location preference of sample objects, dotted line represents 50%. *B.* KSRP$^{-/-}$ mice have an increased recognition index during the test phase of NOR. *C.* KSRP$^{-/-}$ mice spend more time within 1cm of the novel object. *D.* KSRP$^{-/-}$ mice also have increased visits to the novel object. Locomotor activity was increased in KSRP$^{-/-}$ mice during the test phase for both total distance traveled *E.* and velocity *F.* Data displayed mean ± SEM, N= 15 M WT, 13 F WT, 5 M HT, 5 F HT, 12 M KO and 13 F KO. *p<0.05

Therefore, we conducted the marble burying task which utilizes a rodent's innate compulsion to bury objects. There is no significant main effect of genotype, sex, or a genotype x sex interaction, therefore sexes were combined (Figure 5A). There is, however, a significant main effect of time (RM ANOVA $F(1,21)=15.620$, $P=0.0007$). Individual ANOVA for each time point revealed that $KSRP^{-/-}$ mice had decreased marble burying (MB) on day 2 (ANOVA $F(2,49)=4.575$ $P=0.0151$) compared to WT controls, while $KSRP^{-/+}$ mice fell in between. Both $KSRP^{-/-}$ and $KSRP^{-/+}$ mice display decreased disturbance of the media (Figure 5B) compared to WT controls. While the $KSRP^{-/-}$ and $KSRP^{-/+}$ mice do not engage in burying marbles to the same degree as their WT counterparts they do interact with the arena by digging in the medium as well as by nose poking the marbles, however, they spend significantly more time grooming, rearing, and generally exploring the arena than WT mice. This led us to use a second test of OCD-like behavior—Nestlet shredding.

The $KSRP^{-/-}$ male and female mice display decreased Nestlet shredding (Figure 5C), while the $KSRP^{-/+}$ males display increased shredding and the $KSRP^{-/+}$ females display decreased shredding compared to WT controls. Taken together these results indicate that the elevation of novel object recognition by $KSRP^{-/-}$ mice is not due to increased obsessive-compulsive-like behavior.

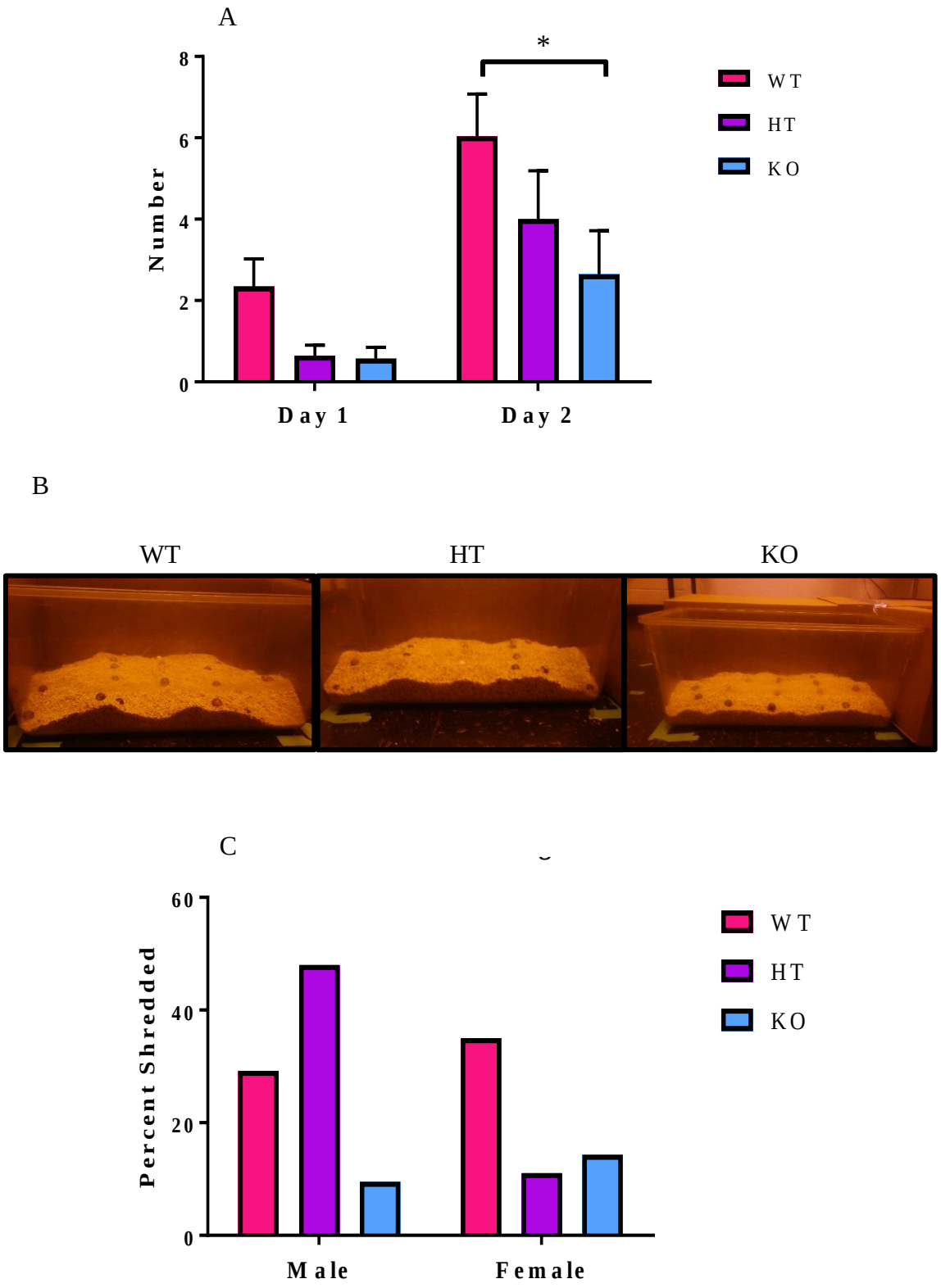

Figure 5: KSRP$^{-/-}$ mice display decreased OCD-like behavior. *A.* number of marbles buried after 2 exposures 1 week apart. *B.* representative photos of the disturbed media after the second 30 min session. *C.* Percent Nestlet shredded after a 4 hour exposure in the home cage of each genotype/sex. Data is mean ±SEM, N= 8 M WT, 6 F WT, 3 M HT, 3 F HT, 2 M KO, and 5 F KO. *p<0.05

3.4b Trace Conditioning

Trace conditioning was employed to test the KSRP$^{-/-}$ and KSRP$^{-/+}$ mice ability to associate the conditioned stimulus (CS, Tone) to the unconditioned stimulus (US, shock) with a 30 second trace interval between. This association requires a functional hippocampus ((McEchron et al, 1998). Initial learning of the task was assessed by secs freezing during training which includes 7 CS and US presentations. All mice progressively increase freezing to the CS (Figure 6A $F(6, 156) = 23.550$, $P<0.0001$) and during the Trace interval following each CS presentation (Figure 6B $F(6, 156) = 128.968$, $P<0.0001$).

During the CS presentation of training there is no main effect of genotype, sex, nor a sex x genotype interaction. There is a time x sex interaction ($F(6, 156)=3.221$, $P=0.0052$), but no time x genotype x sex interaction. The time x sex interaction is driven by decreased freezing of WT Females during Tone 3 ($T(9)=2.305$, $P=0.0466$) compared to their male counterparts.

During trace training there is a main effect of genotype ($F(2,26)=5.243$, $P=0.0122$) but no main effect of sex nor a sex x genotype interaction. There is a significant main effect of time ($F(6,156)=128.968$) $P<0.0001$). Tukey post hoc tests reveal that Trace 1, 2, and 3 are significantly different from all succeeding time points indicating that all mice have learned the Tone/Shock pairing by Trace 4.There is also a time x genotype interaction ($F(12, 156)=4.545$, $P<0.0001$), but no time x genotype x sex

interaction. Tukey post hoc test reveals that KSRP$^{-/-}$ animals are significantly different from KSRP$^{-/+}$ and WT mice.

To assess retention of trace fear conditioned responses KSRP$^{-/-}$ and KSRP$^{-/+}$ mice were evaluated for response to the CS 24 hours post training by measuring freezing behavior (Figure 6C). The CS was delivered in a novel context to examine the response independent of the original fear context. There is no significant main effect of genotype, sex, nor a sex x genotype interaction, nor a time x sex interaction, nor a time x genotype x sex interaction, therefore sexes were combined. There is a main effect of time ($F(1, 26)=13.470$, $P=0.0011$). Individual ANOVA for each time point reveals no significant main effect of sex for either the CS (Tone) or Trace, neither was there a main effect of genotype during the CS delivery, however KSRP$^{-/-}$ mice display decreased freezing during the trace interval compared to WT controls ($F(2,26)=4.138$, $P=0.0262$).

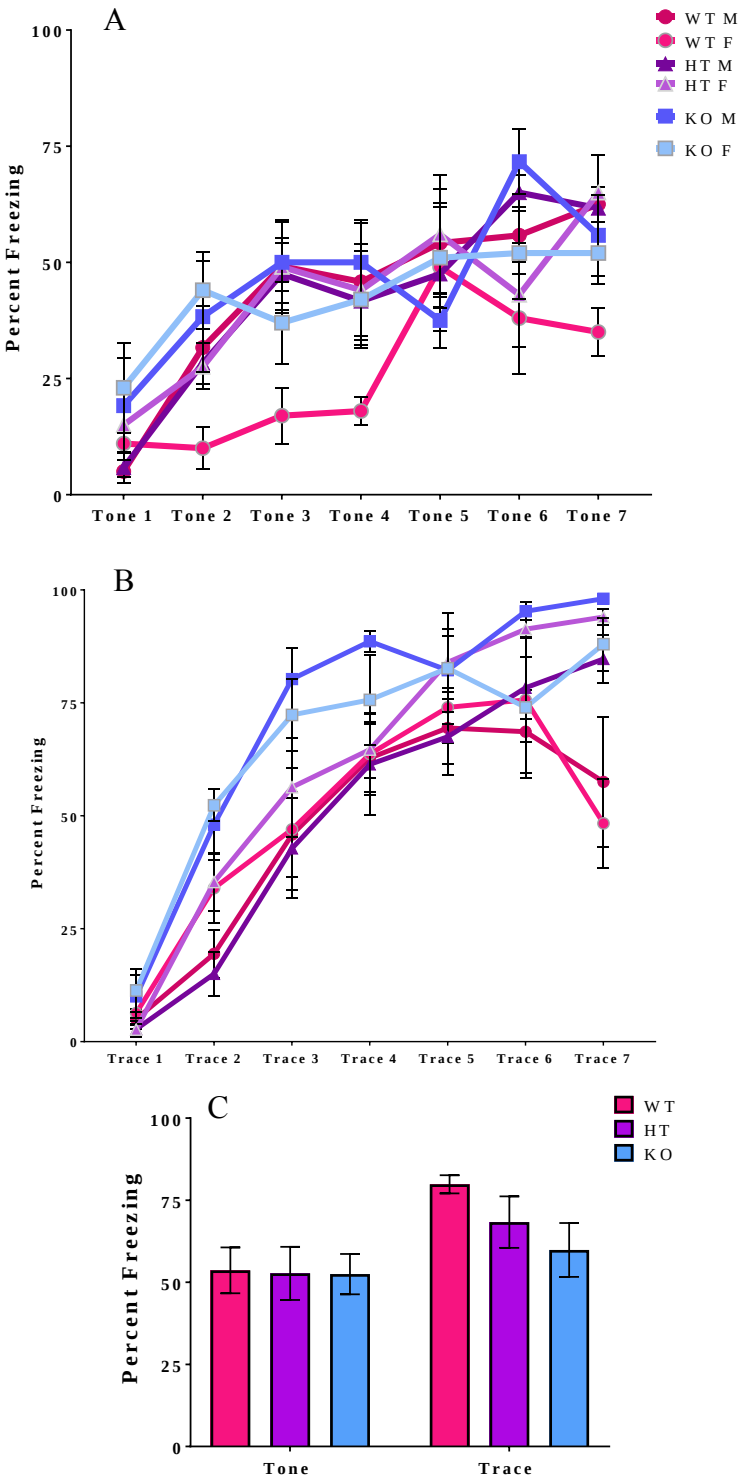

Figure 6: KSRP$^{-/-}$ mice initially learn the CS-US pairing, but display decreased freezing following CS presentation approximately 24 hours after training. All mice progressively freeze during each CS presentation *(A.)* and during the Trace interval *(B.)* KSRP$^{-/-}$ mice demonstrate increased freezing during the Trace interval following CS presentation. *C.* All mice freeze equaling during CS presentation approximately 24 hours after training, but KSRP$^{-/-}$ mice display decreased freezing during the Trace interval compared to WT controls. Data is mean ± SEM, n= 5-6/genotype/sex, *p=0.0149

CHAPTER 4
DISCUSSION

It is increasingly clear that post-transcriptional regulation of mRNA plays a dynamic role in the regulation of neuronal genes and subsequent changes in behavior (Bolognani et al, 2007; Perrone-Bizzozero and Bolognani, 2008). This includes the critical role of RNA binding proteins (RBPs) to increase or decrease the stability of mRNAs. Here we show that loss of the RBP KSRP leads to a unique set of behavioral changes including increased locomotor activity and alterations in hippocampal dependent and independent learning and memory.

It has been previously reported that loss of KSRP leads to aberrant axonal outgrowth *in vitro* due to dysregulation of GAP-43 (Bird et al, 2013). Correct axonal growth regulation is critical for proper development and maintenance of neuronal networks. While overexpression of GAP-43 on its own also leads to aberrant axonal sprouting (Fallini et al, 2016; Strittmatter et al, 1995) it has also been reported to increase learning and memory of a radial maze win-shift paradigm which relies on proper hippocampal function (Collier, Quirk, and Routtenberg, 1987; Routtenberg et al, 2000). This coincides with our observed increase in novel object recognition in $KSRP^{-/-}$ mice.

The novel object recognition (NOR) test utilizes the hippocampus to recognize objects in both humans and rodents (Lyon et al, 2012; Zhang et al, 2012). NOR is based on a rodent's spontaneous behavior to explore novelty and thus avoids the confounding effects of stress or reward motivation. Here we observe an increase in recognition of the novel object in our mice that corresponds to increased frequency of visits and duration of visits with the novel object as well as increased locomotion when the object is present.

This led us to explore the possibility of an OCD-like phenotype by utilizing 2 other tasks that rely on mouse spontaneous behavior. The first relied on the rodent's spontaneous compulsion to bury objects in which we observed a decrease in marble burying in our animals and an increase in other mouse activities. The second, utilized a rodent's compulsion to chew and shred material for nest building. This nestlet shredding task was conducted one time and without statistics no definite conclusion can be drawn, however, the KSRP$^{-/-}$ mice do display decreased shredding compared to WT controls, which is consistent with our MB data.

The hippocampus is also necessary for formation of the association between the CS and US in trace fear conditioning (McEchron et al, 1998). Phosphorylation of GAP-43 in the hippocampus by PKC is necessary for learning fear conditioning (Young et al, 2000). As seen here the KSRP$^{-/-}$ mice are able to initially learn the association between the CS and US during training, however, they display decreased freezing to the CS approximately 24 hours later. This indicates that the KSRP$^{-/-}$ mice have deficits in temporal processing of information due to the time separation between the training and testing (Meck, Church, and Matell, 2013; Ranganath and Hsieh, 2016). This may indicate a deficit in hippocampal dependent short-term vs long-term memory.

Proper executive control is critical for many cognitive functions including planning, attention, behavioral flexibility, and working memory. Lesion studies have demonstrated the role of the Prefrontal cortex (PFC) in reversal learning (Dias et al, 1996; Chudasama and Robbins, 2003) which requires the ability to shift response to a previously unrewarded stimuli within the previously rewarded dimension (i.e. odor to odor or platform to platform). While, KSRP$^{-/-}$ mice can perform reversals, these problems

are no more difficult than the preceding discrimination (CDR-CD and IDR-ID) indicating that these mice approach each problem as if it were novel.

The vmPFC plays a critical role in the shift between stimulus features such as odor to platform or platform to odor (Ragozzino et al., 1999; Bissonette et al, 2008; Floresco et al., 2008). Lesions of the vmPFC lead to deficits in the ED-ID shift (Birrell and Brown, 2000; Rudebeck and Murray, 2012). Here we demonstrate that global loss of KSRP results in impairments of attentional set formation of species-appropriate stimuli.

$KSRP^{-/-}$ animals have increased novelty induced locomotion during the zero maze (Figure 2A-B), open field (Figure 2C-D) and the test phase of NOR (Figure 4E-F). Locomotor hyperactivity is observed in mouse models of ADHD, a human condition that manifests by impulsivity, hyperactivity, and inattention (DSM-IV). It is well established that the dopamine system plays a critical role in locomotor activity. Dopamine transporter (DAT) knockout mice have elevated extracellular dopamine and increased activity in the open field (Gainetdinov, et al., 1999; Giros, et al., 1996). These mice also display deficits in attention as measured by a radial arm maze win-shift assay (Gainetdinov, et al., 1999). Stimulation of the dopamine receptor type 1 (DRD1), a putative target of KSRP, also increases locomotor activity (Pezze et al., 2015). Both pharmacological antagonism and stimulation of DRD1 blocked novelty seeking in rats (Besheer, Jensen, and Bevins, 1999; Pezze et al., 2015).

Here we observed a decrease in attention, an increase in locomotor activity, and an increase in recognition of a novel object with loss of KSRP suggesting that KSRP may play a role in fine tuning DRD1 levels and may be a model for ADHD.

ABBREVIATIONS LIST

PTR: Post-Transcriptional Regulation

RBPs: RNA Binding Proteins

KSRP: KH-Type Splicing Regulatory Protein

ARE: AU-Rich Element

FUSE: DNA Far Upstream Element

FBP2: Fuse Binding Protein 2

ZBP2: Nuclear Zipcode Binding Protein 2

GAP-43: Growth Associated Protein

OE: Overexpression

KO: Knockout

HT: Heterozygous

WT: Wild Type

NOR: Novel Object Recognition

ASST: Attentional Set Shifting Task

SD: Simple Discrimination

CD: Compound Discrimination

CDR: Compound Discrimination Reversal

IDS: Intra-Dimensional Shift

IDR: Intra-Dimensional Reversal

EDS: Extra-Dimensional Shift

EDR: Extra-Dimensional Reversal

OFC: Orbitofrontal Cortex

vmPFC: Ventromedial Prefrontal Cortex

MB: Marble Burying

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