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# Title: Analysis of Kalirin-7 knockout mice reveals different effects in female mice

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Running title page

**Kalirin-7 knockout mice: different effects in females**

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List of nonstandard abbreviations: PSD, post-synaptic density; E2, 17β-estradiol

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## ABSTRACT

Estradiol treatment of ovariectomized rodents is known to affect the morphology of dendritic spines and produce behavioral and cognitive effects. Kalirin-7 (Kal7), a PSD-localized Rho-guanine nucleotide exchange factor, is important for dendritic spine formation and stability. Male Kal7 knockout (Kal7$^{KO}$) mice exhibit a number of abnormal behavioral and biochemical phenotypes. Given that chronic estradiol (E2) replacement of ovariectomized rats enhanced Kal7 expression in the hippocampus and in primary hippocampal cultures, we assessed the behavioral and biochemical effects of chronic E2 treatment of ovariectomized female wildtype and Kal7$^{KO}$ mice. Both intact and ovariectomized Kal7$^{KO}$ female mice exhibited decreased anxiety-like behavior compared to the corresponding wildtype in the elevated zero maze, unaffected by E2 treatment. Chronic E2 decreased locomotor activity in the open field and enhanced performance in a passive avoidance fear conditioning task, which were both unaffected by genotype. Kal7$^{KO}$ female mice engaged in significantly more object exploration, both familiar and novel, than did wildtype females. E2 enhanced the acute locomotor response to cocaine, with no significant effect of genotype. Similar to Kal7$^{KO}$ males, Kal7$^{KO}$ females had decreased levels of NR2B in hippocampal post-synaptic density fractions, with no effect of E2 treatment. The differing behavioral effects of Kal7 ablation in female and male mice may offer insight into the molecular underpinnings of these differences.

## INTRODUCTION

Dendritic spine density fluctuates across the estrus cycle, with the greatest number of dendritic spines following proestrus, the stage with the highest levels of E2 (Woolley et al., 1990;Yankova et al., 2001). Ovariectomy results in decreased spine density in CA1 rat hippocampal pyramidal neurons, an important region for learning and memory (Gould et al., 1990), while administration of exogenous E2 increases spinogenesis in CA1 hippocampal pyramidal neurons of ovariectomized rats (Ma et al., 2011;Gould et al., 1990;Woolley et al., 1990;Yankova et al., 2001). In mice, CA1 hippocampal dendritic spines change shape in response to E2, but the total number of spines does not increase (Li et al., 2004).

The ability of estrogen to affect spine formation and shape is thought to contribute to sex-specific differences in the response to cocaine. One of the enduring effects of cocaine on the nervous system is changes in dendritic spine morphology (Robinson and Kolb, 1999;Robinson et al., 2001;Li et al., 2003). Female rats show a larger behavioral sensitization to cocaine during estrus (following high serum estradiol) than during diestrus (following lower estradiol) (Becker, 1999). Women and female rodents respond to cocaine more intensely than males, acquire drug-seeking or self-administration behavior more quickly, and develop addiction and addictive behaviors more readily (Festa et al., 2004;Segarra et al., 2010;Parylak et al., 2008;Kuhn et al., 2001).

Spine morphogenesis is controlled in large part by the actin cytoskeleton (Hering and Sheng, 2003;Togashi et al., 2002;Fukazawa et al., 2003;Ehlers, 2002;Star et al., 2003;Nimchinsky et al., 2002). Kal7, a Rho-guanine nucleotide exchange factor (Rho-GEF) localized to the post-synaptic density (PSD) of excitatory synapses, activates Rac, a key regulator of actin dynamics. Golgi staining of hippocampal CA1 pyramidal neurons from mature male mice shows decreased spine density in Kal7 knockout (Kal7$^{KO}$) mice (Ma et al., 2008). Kal7 plays an essential role in the behavioral response of male mice to cocaine; Kal7$^{KO}$ mice display increased cocaine locomotor sensitization and impaired cocaine-induced spinogenesis (Kiraly et al., 2010). Expression of Kal7 in rat hippocampal neurons in culture is increased by 17β-estradiol (Ma et al., 2011). Likewise, hippocampal lysates from ovariectomized rats implanted with a subcutaneous estradiol pellet show increased Kal7 levels relative to ovariectomized rats receiving vehicle pellets (Ma et al., 2011). These studies suggest that Kal7 could contribute to sex-specific differences in the response to cocaine.

Given the potential for an interaction between Kal7 and E2, the goal of these studies was to compare the effects of the absence of Kal7 in female mice to the changes documented in Kal7$^{KO}$ male mice. Male Kal7$^{KO}$ mice show a decrease in anxiety-like behavior and impaired acquisition of a passive avoidance task but perform normally in the open field and in tests of novel object recognition (Ma et al., 2008). Based on studies using ovariectomized rodents, E2 treatment affects each of these behaviors, although specific results vary with the type of rodent, the dose of E2, the delay between ovariectomy and E2 replacement, and whether the E2 treatment was acute or chronic (Lewis et al., 2008;Koss et al., 2004;Walf and Frye, 2009;Walf and Frye, 2010;Tomihara et al., 2009). We examined the effects of chronic E2-replacement on the behavior of ovariectomized wildtype (Wt) and Kal7$^{KO}$ mice.

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## MATERIALS AND METHODS

**Animals:** All experiments were performed using C57BL/6 Wt and Kal7$^{KO}$ mice (Ma et al., 2008). Wt and Kal7$^{KO}$ female littermates from Kal7$^{+/KO}$, Kal7$^{+/+}$, and Kal7$^{KO/KO}$ pairings were ovariectomized (OVX) between P60 and P100 for behavioral experiments and between P50 and P120 for biochemical experiments. The Kal7$^{+/KO}$ mice have been backcrossed into C57BL/6 from Jackson Laboratories for more than 20 generations, and no mice were more than two generations from Kal7$^{+/KO}$ x Kal7$^{+/KO}$ matings. Every effort to use littermates from Kal7$^{+/KO}$ matings for behavioral experiments was made, but some animals from homozygous breeders were necessary to achieve adequate power for the experiments (due to the number of groups needed and size of litters). No behavioral differences were noted between mice from heterozygous and homozygous breeders. Mice were group housed in the University of Connecticut Health Center animal facility on a 12-hour light/dark cycle (lights on 0700). Food and water were available *ad libitum*. Experiments were conducted in agreement with the University of Connecticut Health Center Institutional Animal Care and Use Committee guidelines.

**Ovariectomy:** Mice were anesthetized using isoflurane/$O_2$ inhalation (5% for induction, 2-3% for maintenance) (Li et al., 2011). Small incisions through skin and muscle tissue were made bilaterally to expose the ovaries. The ovary and fallopian tubes were excised and the tip of the uterus was cauterized to prevent bleeding. The incisions in the muscle tissue were sutured and wound clips were applied bilaterally to close the skin (Li et al., 2011). A small incision was then made at the nape of the neck and a 60-day continuous-release pellet (Innovative Research of America) containing either a placebo or 0.01 mg of estradiol (nominal release rate, 165 ng/day) was implanted roughly 1 cm from the incision site. The incision was closed using a suture, and the animals were allowed to recover for at least one week before being used in an experiment.

**Behavioral Analyses:** All behavior experiments were performed using our previously published methods (Kiraly et al., 2010;Ma et al., 2008). Mice were moved to the behavior room in their home cages at least one hour before training or testing. Starting at least two days prior to the first behavioral experiment, mice were handled for one minute once per day to acclimate them to the experimenter. In each experiment, mice were subjected to behavioral testing at a consistent time of day, either 0800-1300 or 1300-1800.

Elevated Zero Maze. A white plastic elevated zero maze (San Diego Instruments) was used. Under low light conditions, each animal was placed head-first into one of the walled quadrants. Over a single 5-min trial, a trained observer blinded to the genotype and treatment of the mice monitored the number of entries made into and the duration of time spent in the open quadrants. A mouse was considered to be in an open arm once all four paws passed the edge of the wall of a walled quadrant, and considered to be in a closed quadrant once all four paws left an open quadrant.

Dark-light transition. A conditioned place preference apparatus (San Diego Instruments) was modified to have a dark side (dark rubber mat, ~10 lux) and a light side (white floor and walls, ~950 lux) separated by a small closed door (~4x4 cm). Mice were acclimated for 5 min to the dark chamber before the door was opened, and the time for all 4 paws of the mouse to be in the light chamber was recorded as the time to emerge (Tsuda and Ogawa, 2012;Verleye et al., 2011).

Open Field. Horizontal spontaneous locomotor activity was evaluated using a PAS Open Field system (San Diego Instruments). Animals were placed into the center of the open field under low light conditions and were allowed to freely explore the chamber for one hour. Ambulatory activity was recorded as the number of sequential beam breaks in a 16x16 photobeam grid in each 5 minute time bin.

Passive Avoidance Fear Conditioning. A Gemini passive avoidance system (San Diego Instruments) was used. On the training day, the inter-chamber door was closed and mice were placed into a compartment with the lights off. After a 5 sec habituation, the chamber lights came on only in the compartment with the mouse and the inter-chamber door was simultaneously opened. The latency to cross to the dark compartment was recorded. Upon crossing, the inter-chamber door closed and the mouse received a 2 sec 0.3 mA scrambled footshock; 30 sec later, mice were returned to their home cages. Shock delivery was confirmed by a readout on the apparatus and an audible vocalization from each animal receiving the foot shock. The apparatus was thoroughly cleaned between each subject. Twenty-four hours later, mice were placed into the same initial chamber for 5 sec; the light turned on and the inter-chamber door opened. Time to cross to the dark compartment was recorded, but no footshock was delivered.

Novel Object Recognition. On the training day, mice were placed into a clean, empty rat cage and allowed to habituate for 5 min. After habituation, mice were briefly moved to a holding cage while two identical objects (LEGO$^R$ blocks or 50 ml tubes weighted down with salt) were placed into the cage. The mouse was then placed into the center of the cage and allowed to explore the objects for 5 min. Twenty-four hours later, the mice underwent the same protocol, except one object was replaced with a novel object (the one not used on the training day). Both the training session and testing session were videotaped and time spent exploring the objects was measured by a blinded scorer. Object exploration was defined as direct nasal contact with the object.

Cocaine Sensitization. Cocaine sensitization experiments were conducted in the same open field boxes used to assess locomotor activity. On each experimental day, locomotor activity was monitored for one hour immediately after an intraperitoneal (i.p.) injection. On days 1-3, mice received an i.p. injection of 200 µl of 0.9% saline (S1 to S3). Development of locomotor sensitization was assessed using a protocol developed by Pierce and colleagues (Pierce et al., 1996). On day 4 (C1), mice received a 10 mg/kg injection of cocaine (courtesy of National Institute on Drug Abuse, Bethesda, Maryland) dissolved in 0.9% saline. On days 5-9 (C2 to C6), mice received 20 mg/kg injections of cocaine. On day 10, mice received an injection of 10 mg/kg cocaine (C7). The ratio of locomotor activity on the two 10 mg/kg days (C1 and C7) was used as the primary measure of sensitization (Pierce et al., 1996). Following day 10, mice remained in their home cages without treatment for 9 days. On day 20, mice received an injection of 10 mg/kg cocaine (Challenge) and were placed in the open field for one hour.

**Biochemical analyses:** Post-synaptic density fractions (PSDs) were prepared from hippocampal and cortical samples from drug-naïve mice according to previously published methods (Ma et al., 2008). Briefly, tissue was pooled from 3-4 mice that had been implanted with a subcutaneous placebo or E2-containing pellet and homogenized in isotonic buffered sucrose. A crude synaptosomal fraction was collected and hypotonically lysed. Lysed synaptosomes were purified on a discontinuous sucrose gradient and the fraction from the 1.0 M/1.2 M sucrose interface was incubated with 1% Triton X-100. The Triton-insoluble fraction was taken as purified PSDs and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis. Antibodies used included JH2958 [Kal7-specific rabbit polyclonal; (Ma et al., 2008)], NR2B (mouse monoclonal N59/20; NeuroMab), and βIII-tubulin (mouse monoclonal; Covance).

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**Serum E2 Assay:** An ELISA for 17β-estradiol (IBL International) was used in accordance with the manufacturer's instructions. This assay kit reports a sensitivity of 9.7 pg/ml, an intra-assay variability of 6.8% and an inter-assay variability of 7.3% for the range of E2 levels used in this study.

**Statistical analyses:** Statistical analyses were calculated using Sigma Plot 11. Comparisons between serum E2, uterine weights, and behavioral differences in intact Wt and Kal7$^{KO}$ mice were evaluated using t-tests. Differences between ovariectomized and E2-replaced Wt and Kal7$^{KO}$ female mice were compared using a two-way ANOVA in a 2x2 design to test for main effects of genotype and E2-treatment, as well as genotype by treatment interactions, as detailed in the figure legends. PSD levels of NR2B were compared using a two-way ANOVA, while PSD levels of Kal7 in Wt mice were compared using a t-test.

## RESULTS

**Female Kal7$^{KO}$ mice.** We evaluated the role of Kal7 in intact, randomly cycling female Wt and Kal7$^{KO}$ mice and in ovariectomized Wt and Kal7$^{KO}$ mice receiving a placebo- or E2-pellet at the time of surgery. Serum E2 levels were measured two weeks following surgery in order to select an E2-pellet that produced levels similar to those occurring during proestrus, the peak of the estrus cycle (50-100 pg estradiol/ml) (Walf and Frye, 2010;Morgan and Pfaff, 2002;Tomihara et al., 2009). Sixty-day release pellets containing 0.01 mg of E2 produced serum E2 levels in the target range (**Fig. 1A**). When tested at least two weeks after surgery, mice implanted with a placebo pellet had serum E2 levels far below those of randomly sampled intact female mice. Sixty-day release pellets containing 0.10 mg of E2 produced serum E2 levels far above the target range (560 ± 64 pg/ml; data not graphed). As another measure of estrogen levels, uterine wet weight was recorded at the time of sacrifice (Walf and Frye, 2010;Morgan and Pfaff, 2002;Tomihara et al., 2009). As expected, ovariectomy resulted in dramatically decreased uterine weight, while 0.01 mg E2-pellets maintained uterine weight at a level slightly greater than that observed in intact females (**Fig. 1B**). To ensure experimentation that could yield a biologically relevant result, 0.01 mg E2 pellets were used for subsequent studies.

**Estrogen-treated Wt and Kal7$^{KO}$ mice show decreased locomotor activity.** Since altered locomotor activity would affect many of the planned behavioral tests, we first examined the behavior of intact female mice in the open field; no genotypic difference was observed (**Fig. 2A**). We next examined the behavior of ovariectomized Wt and Kal7$^{KO}$ females receiving placebo or E2 pellets in the open field (**Fig. 2B**). As with intact male and female mice, the ambulatory activity of ovariectomized females was not affected by Kalirin genotype (**Fig.2 B, left**). However,E2-treatment decreased ambulatory activity to a similar extent in ovariectomized Wt and Kal7$^{KO}$ mice (**Fig. 2B, right**).

**Kal7$^{KO}$ females show decreased anxiety-like behavior.** Previous work demonstrated that male Kal7$^{KO}$ mice exhibited decreased anxiety-like behavior in the elevated zero maze compared with Wt male littermates (Ma et al., 2008). When intact females were placed in an elevated zero maze for a single five-minute trial, the Kal7$^{KO}$ females spent significantly more time in the open arm than Wt females (**Fig. 3A**). Similarly, the Kal7$^{KO}$ females emerged from a dark chamber into the light in less time than Wt females (**Fig. 3B**). To assess the effect of estrogen, ovariectomized Wt and Kal7$^{KO}$ female mice treated with placebo or E2 pellets were tested in the same manner (**Fig. 3C**). Based on both time spent in the open area of the elevated zero maze and number of entries into the open area, genotype exerted a significant effect on this behavior. Kal7$^{KO}$ females spent more time in the open area and made more entries into the open area, indicative of a decrease in anxiety-like behavior. Estrogen treatment had no effect on anxiety-like behavior in either genotype. Importantly, estradiol replacement did not affect the number of open arm entries, suggesting that the decreased locomotor activity observed in the open field did not affect the elevated zero maze results. These results provide evidence that loss of Kal7 results in a decrease in anxiety-like behavior in both female and male mice.

**Estrogen enhances passive-avoidance fear conditioning.** Passive avoidance fear conditioning is dependent on both the hippocampus and amygdala for consolidation of contextual and fear memories, respectively (Morgan and Pfaff, 2002;Lewis et al., 2008;Orr et al., 2012;Zhao et al., 2012). Male Kal7$^{KO}$ mice exhibited impaired fear conditioning in this test (Ma et al., 2008). In contrast, intact Wt and Kal7$^{KO}$ females showed no differences on the training or 24 hour test day (**Fig. 4A**).

To evaluate the ability of estrogen to affect this behavior, ovariectomized Wt and Kal7$^{KO}$ mice implanted with placebo or E2-pellets were tested (**Fig. 4B**). On the training day, neither genotype nor E2 treatment affected latency to cross into the dark compartment. On the test day, latency to cross into the dark compartment was unaffected by genotype, while E2-treatment produced a robust increase in latency to cross in both Wt and Kal7$^{KO}$ mice. While male Kal7$^{KO}$ mice showed diminished fear conditioning compared to Wt mice, female Kal7$^{KO}$ mice did not. Kal7$^{KO}$ mice remained responsive to E2-treatment, as seen by increased latency.

**Kal7$^{KO}$ females show altered behavior in novel object recognition task.** Given that estrogen treatment improved acquisition of the passive avoidance task in both Wt and Kal7$^{KO}$ mice, we wanted to measure performance in a strictly hippocampal-dependent task. Based on time spent exploring a novel object, male Wt and Kal7$^{KO}$ mice were equally capable of distinguishing a novel object from a familiar object (Ma et al., 2008). Intact Wt and Kal7$^{KO}$ female mice were allowed to explore a familiar object, seen 24 hours previously during the training session, and a novel object. On the test day, neither Wt nor Kal7$^{KO}$ intact female mice spent significantly more time exploring the novel object than the familiar object (**Fig. 5A**); however, Kal7$^{KO}$ females spent substantially more time exploring both objects than Wt females, whether the objects were novel or familiar. Total time spent exploring objects is shown in **Fig. 5B**.

The ability of estrogen to affect this behavior was assessed by testing ovariectomized Wt and Kal7$^{KO}$ mice, with placebo or E2 pellets (**Fig.5 C,D**). Regardless of genotype, neither ovariectomized nor hormone replaced ovariectomized female mice spent more time with the novel object (**Fig.5C**). As with intact females, ovariectomized female Kal7$^{KO}$ mice spent more time exploring both the familiar and the novel object than female Wt mice. In addition, estrogen treatment reduced object exploration time for both Wt and Kal7$^{KO}$ mice on the test day, perhaps due to reduced ambulatory behavior (**Fig.2**).

**Estrogen affects the response of both Wt and Kal7$^{KO}$ female mice to cocaine.** Given that male Kal7$^{KO}$ mice showed enhanced locomotor sensitization to repeated administration of cocaine (Kiraly et al., 2010), we evaluated female Kal7$^{KO}$ mice using a similar paradigm. The cocaine sensitization paradigm is outlined in **Fig.6A**: i.p. saline injections were given on three days to habituate mice to the injections and to the testing chambers. As expected from the open field test (**Fig.2**), E2-treatment reduced locomotor activity in both genotypes. Administration of the first dose of cocaine (10 mg/kg on cocaine day 1, C1), when compared with baseline locomotor activity on the third day of saline (C1/S3), produced the expected increase in locomotor activity in all the mice (**Fig. 6B**). E2-treatment enhanced the initial locomotor response to cocaine for both Wt and Kal7$^{KO}$ female mice (**Fig. 6B**).

On each of the following five days (C2→C6), mice received 20 mg/kg cocaine. On the seventh day of cocaine treatment, mice again received the lower dose of cocaine. All groups showed an enhanced locomotor response to the 10mg/kg dose on this day (C7/S3 vs. C1/S3), indicating that locomotor sensitization was achieved. The degree of sensitization was not significantly affected by genotype (**Fig. 6B**); estradiol treatment did not significantly increase sensitization to the 10 mg/kg dose of cocaine. Following a 9-day period of withdrawal, mice received a challenge dose of 10 mg/kg cocaine; all four groups remained sensitized and their responses did not differ from each other (**Fig. 6B**). The increased locomotor sensitization observed in male Kal7$^{KO}$ mice compare to Wt mice (Kiraly et al., 2010) was not observed in female Kal7$^{KO}$ mice.

**Effects of E2-treatment on protein expression in Kal7$^{KO}$ females**. One of the few biochemical changes observed in male Kal7$^{KO}$ cortex was a decrease in PSD-localized NR2B levels (Ma et al., 2008). Kal7 was subsequently shown to interact directly with the NR2B subunit of the NMDA receptor and pre-treatment with ifenprodil, an NR2B-specific blocker, largely abrogated the differences observed between Wt and Kal7$^{KO}$ male mice in passive avoidance behavior and conditioned place preference for cocaine (Kiraly et al., 2011). PSDs were purified from the hippocampus of E2-treated and control ovariectomized Wt and Kal7$^{KO}$ female mice. Levels of NR2B were reduced in hippocampal PSDs purified from Kal7$^{KO}$ female mice (**Fig. 7A,B**). Estrogen treatment had no effect on NR2B levels in hippocampal PSDs. Kal7 levels in hippocampal PSD preparations were not altered by E2-treatment (**Fig. 7A,C**).

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## DISCUSSION

### Kal7 plays an essential role in selected behaviors in female mice

One of the most striking differences observed in female Kal7$^{KO}$ vs. Wt mice was a decrease in anxiety-like behavior as assessed in the elevated zero maze; this effect was observed in intact females as well as in ovariectomized females with placebo or E2 pellets and was not altered by E2 treatment (**Fig. 3**). Kal7$^{KO}$ males exhibited a similar decrease in anxiety-like behavior relative to Wt controls (Ma et al., 2008). E2 is usually reported to be anxiolytic or without effect on anxiety-like behavior in mice (Koss et al., 2004;Walf and Frye, 2010;Pandaranandaka et al., 2006;Morgan and Pfaff, 2002). Using subcutaneous pellets with varying doses of E2, higher doses of E2 (2 µg/day) produced increased anxiety-like behavior in a light to dark transition task, while lower doses (0.2 µg E2/day) produced decreased anxiety-like behavior (Tomihara et al., 2009). The slow release pellets used in our studies would be expected to deliver about 0.165 µg E2/day, and it would appear that this dosage is without effect on anxiety-like behavior.

The novel object recognition test revealed a Kal7-dependent behavior unique to female mice. Kal7$^{KO}$ females spent significantly more time exploring objects than Wt mice on both the training and test days (**Fig.5B,D**). Estrogen treatment decreased total object exploration time on the test day, perhaps reflecting the decrease in open field activity associated with E2 treatment (**Fig.2**). Neither the intact, hormone-replaced nor hormone-deprived Wt or Kal7$^{KO}$ females showed a preference for the novel object compared to a familiar object (**Fig.5A,C**). Previous object recognition studies with female mice have yielded variable results, but ovariectomized mice failed to show a significant preference for novel objects in several earlier studies (Walf et al., 2008;Li et al., 2004;Fernandez et al., 2008;Lewis et al., 2008). Time since ovariectomy, time between presentation of familiar and novel objects, and the timing and mode of delivery of E2 are all important variables (Li et al., 2004;Walf et al., 2008;Walf and Frye, 2009;Walf and Frye, 2010;Fan et al., 2010;Lewis et al., 2008;Bettis and Jacobs, 2009;Capettini et al., 2011;Orr et al., 2012;Phan et al., 2011;Siegel et al., 2011;Win-Shwe and Fujimaki, 2012). By contrast, both Wt and Kal7$^{KO}$ male mice showed a clear preference for the novel object (Ma et al., 2008), as usually reported for male mice (Fan et al., 2010;Fernandez et al., 2008;Lewis et al., 2008;Zhao et al., 2012;Li et al., 2004;Walf et al., 2008). Our studies with male and female Wt and Kal7$^{KO}$ mice used the same objects and experimental set-up.

### Chronic E2 has similar effects on the behavior of Wt and Kal7$^{KO}$ female mice

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The inability of rat hippocampal neurons transfected with Kal7shRNA to form dendritic spines in response to estradiol suggested an essential role for Kal7 in this response (Ma et al., 2011). However, the estrogen-sensitive behaviors examined in this study were largely unaltered in Kal7$^{KO}$ vs. Wt mice. E2 treatment produced a significant decrease in open field mobility in Wt and Kal7$^{KO}$ mice (**Fig.2**). This response was expected based on most earlier studies of ambulatory activity in E2-treated ovariectomized female rodents (Segarra et al., 2010;Morgan and Pfaff, 2002;Morgan et al., 2004;Morgan and Pfaff, 2001). The fact that some studies did not find an inhibitory effect of E2 on open field locomotion in ovariectomized rodents may reflect the treatment paradigms used and E2 levels obtained (Ogawa et al., 2003;Walf et al., 2008;Hiroi et al., 2006). The decrease in mobility caused by E2 treatment would be expected to have an impact on several behavioral tests.

While the ability of ovariectomized mice to acquire a passive avoidance task was not altered by the absence of Kal7, both Wt and Kal7$^{KO}$ females treated with E2-pellets exhibited increased latency to cross into the dark chamber on the test day (**Fig. 4**). Estrogen treatment did not alter the latency to cross to the dark compartment on the training day, indicating that the decreased locomotor activity in the open field does not confound these results. E2 treatment lead to increased acquisition of the passive avoidance task in both genotypes. E2 was previously reported to improve fear-based learning by ovariectomized mice (Farr et al., 1995;Lewis et al., 2008;Hiroi et al., 2006;Morgan and Pfaff, 2002;Morgan et al., 2004;Morgan and Pfaff, 2001), with a few exceptions (Mora et al., 1996). The performance of E2-treated ovariectomized Wt and Kal7$^{KO}$ mice most closely resembled the performance of Wt males in this test. In male mice, the absence of Kal7 produced a substantial impairment in passive avoidance acquisition (Ma et al., 2008). Administration of an NR2B antagonist (Ro 25-6981) impaired acquisition of conditioned fear behavior in male mice (Mathur et al., 2009). Consistent with the idea that Kal7 plays a role in signaling downstream of NR2B-containing NMDA receptors, pre-treatment of male wildtype and Kal7$^{KO}$ mice with a selective NR2B antagonist abrogated the difference observed in their fear-learning behavior (Kiraly et al., 2011). With no genotypic difference in fear conditioning in female mice, it will be interesting to compare the effects of an NR2B antagonist on ovariectomized mice with E2- and placebo pellets.

### Role of Kal7 in response to cocaine differs in female and male mice

As expected, E2 treatment increased the initial locomotor response of ovariectomized Wt mice to cocaine (C1/S3; **Fig.6B**) (Festa et al., 2004;Segarra et al., 2010;Parylak et al., 2008;Sell et al., 2002;Walker et al., 2001); locomotor activity increased roughly 3-fold relative to the saline response in E2-treated animals, while placebo-treated animals showed only a 2-fold increase in ambulatory activity. Deletion of Kal7 did not alter the initial response of female mice to cocaine (**Fig.6B**). Similarly, the acute response of male mice to cocaine was unaltered in Kal7$^{KO}$ mice (Kiraly et al., 2010). Following repeated cocaine administration, Kal7$^{KO}$ females, unlike males, did not show increased locomotor sensitization relative to Wt mice (**Fig.6B**) (Kiraly et al., 2010). In male mice, the sensitized response to cocaine was accompanied by an increase in dendritic spine density in the NAc of Wt, but not Kal7$^{KO}$ males (Kiraly et al., 2010). It will be interesting to determine whether E2 treatment of female mice alters dendritic spine density in the same brain region. In hippocampus, rats showed increased Kal7 and increased dendritic spines in response to E2 (Ma et al., 2011;Woolley et al., 1990;Gould et al., 1990;Yankova et al., 2001), while mice showed no increase in Kal7 in hippocampus in response to E2 (**Fig.7**) and no increase in dendritic spine density (Li et al., 2004).

The effects of chronic cocaine treatment on female mice were maintained after 9-days of withdrawal; Wt and Kal7$^{KO}$ female mice remained equally sensitized to the challenge dose (**Fig. 6B**). These results differ from published studies in rats reporting that chronic E2-treatment enhances the sensitization response in ovariectomized rats (Becker, 1999;Festa et al., 2004;Sell et al., 2002;Segarra et al., 2010;Parylak et al., 2008). In rats that were ovariectomized and E2 replaced via a subcutaneous pellet, E2 enhanced the acute locomotor response to cocaine, as observed here and there was no increase in cocaine sensitization (Sell et al., 2002). As observed with alterations in dendritic spine density, it is possible that the role of E2 in the response to cocaine differs between species.

### The Kalirin/NR2B/estrogen interaction

The first pleckstrin homology domain of Kalirin, which is present in all of the major isoforms of Kalirin, interacts directly with NR2B (Kiraly et al., 2011). As in male mice, PSDs prepared from the hippocampus of female Kal7$^{KO}$ mice contained less NR2B than PSDs prepared from the hippocampus of Wt mice. In addition to cell-specific and developmental changes in the expression of Kalirin and NR2B, it is expected that both estrogen and cocaine will affect interactions between NR2B and Kalirin. Chronic cocaine treatment of male mice altered *Kalrn* promoter and 3'-terminal exon usage in the striatum, yielding isoforms that produce different structural changes in spines (Mains et al., 2011). While the Kal7$^{KO}$ mice cannot produce Kal7 or

∆-Kal7, production of Kalirin9 and Kalirin12 is increased (Ma et al., 2008;Kiraly et al., 2011); these isoforms of Kalirin are capable of interacting with NR2B.

In some systems, expression of both Kal7 and NR2B is sensitive to E2-treatment. There is widespread agreement that NR2B function increases in hippocampal CA1 pyramidal neurons after E2 treatment, but the mechanism is controversial, with reports of increased NR2B mRNA, protein, and protein phosphorylation (Nebieridze et al., 2012;Raval et al., 2012;Xu et al., 2011) *in vivo*, decreased NR2B phosphorylation in response to E2 using cultured cortical neurons (Liu et al., 2012), or enhanced recruitment of NR2B receptors to synapses but no change in protein or phosphorylation levels (Snyder et al., 2011). E2 treatment is known to increase extracellular signal-regulated kinase (ERK) phosphorylation in hippocampus in an NMDA-dependent manner (Lewis et al., 2008). Although estrogen increased Kalirin expression in the rat hippocampus *in vivo* and in culture (Ma et al., 2011), perhaps by interacting with estrogen-responsive elements in the *Kalrn B and C* promoters (Ma et al., 2011), it did not do so in mouse hippocampus. Through the use of NR2B-selective antagonists in Wt and Kal7$^{KO}$ mice, it should be possible to identify the behaviors in which the interaction of NR2B with Kalirin plays a key role.

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**Disclosure:** The authors declare no conflict of interest.

## AUTHORSHIP CONTRIBUTION

Participated in research design: CM, DK, BE, RM

Conducted experiments: CM, TL, BE, RM

Performed data analysis: CM, TL, DK, BE, RM

Wrote or contributed to writing the manuscript: CM, TL, DK, BE, RM

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## FOOTNOTES

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## FIGURE LEGENDS

**Figure 1: Serum Estradiol and Uterine Weight: (A)** Serum E2 levels depended on treatment group. Placebo pellet treated mice had lower estrogen levels than cycling intact females ($p < 0.001$, t-test) and 0.01 mg E2 pellet treated mice had higher E2 levels than intact females or placebo (both $p < 0.001$, t-test). N: 15-intact, 15-placebo, 12- E2. **(B)** Uterine wet weight varied depending on E2 replacement. Ovariectomized mice receiving a placebo pellet had decreased uterine weights relative to intact females ($p < 0.001$, t-test). Ovariectomized mice receiving an E2 pellet had uterine weights that were larger than both placebo-treated and intact females ($p < 0.001$, t-test). **$p < 0.001$. N: 15-intact, 67-placebo, 65- E2. Error bars represent SEM.

**Figure 2: Estradiol treatment decreases locomotor activity in the open field:** Mice were placed into an open field apparatus and their ambulatory activity was monitored in five-minute time bins for a single one-hour session. (**A**) There was no difference in locomotor activity between intact Wt and Kal7$^{KO}$ females (t-test) N = 5-6/group. (**B**) Ovariectomized animals that received an E2-pellet showed fewer ambulatory counts than those that received a placebo treatment ($F_{(1,55)} = 21.858$; $p < 0.001$, two-way ANOVA). There was no main effect of genotype ($F_{(1,55)} = 0.735$; $p = 0.395$, two-way ANOVA), and no genotype x treatment interaction ($F_{(1,55)} = 1.065$; $p = 0.307$). N=12-17 per group. **$p<0.001$. Error bars indicate SEM.

**Figure 3: Kal7$^{KO}$ females display decreased anxiety-like behavior in the elevated zero maze, independent of estradiol treatment: (A)** Intact females were tested in the elevated zero maze (t-test, *$p<0.05$; N = 5-8/group). **(B)** Intact females were tested in the dark-light box (t-test, *$p<0.05$; N = 6/group). **(C)** Ovariectomized mice that received an E2 or placebo pellet were tested in the same manner. Kal7$^{KO}$ females spent more time in the open arms of the elevated zero maze than Wt female mice over a five-minute trial ($F_{(1,35)} = 5.110$; $p=0.03$, two-way ANOVA). Kal7$^{KO}$ females made more entries into the open arms than Wt female mice ($F_{(1,35)} = 12.002$; $p = 0.001$, two-way ANOVA). There were no main effects of E2 or genotype x E2 interactions. N=9-10 per group. *$p < 0.05$; **$p < 0.01$. For **C**, asterisks indicate main effect of genotype. Error bars indicate SEM.

**Figure 4: Estradiol treatment enhances passive avoidance fear conditioning: (A)** Intact females were tested on the training day and 24 hours later; genotype had no effect on latency to cross on either day; N = 5-8/group. **(B)** Ovariectomized mice that received an E2 or placebo pellet were tested in the same manner. On the training day, there were no differences in the

latency to cross to the dark compartment with respect to genotype ($F_{(1,33)}$ = 0.0798; *p* = 0.779, two-way ANOVA) or treatment ($F_{(1,33)}$ = 0.769; *p* = 0.387, two-way ANOVA). Twenty-four hours later, there was no main effect of genotype on latency to cross to the dark compartment ($F_{(1,33)}$ = 0.0942; *p* = 0.761, two-way ANOVA), but estradiol treatment increased latency to cross ($F_{(1,33)}$ = 5.958; * *p* < 0.05). There was no genotype x treatment interaction ($F_{(1,33)}$ = 0.0518; *p* = 0.821). N = 6-11/group. Error bars indicate SEM.

**Figure 5: Novel Object Exploration: (A)** Novel object recognition was evaluated in intact Wt and Kal7$^{KO}$ females; Kal7$^{KO}$ females spent more time exploring both the familiar and the novel object. **(B)** Total object exploration time was significantly higher in Kal7$^{KO}$ females than in Wt females on both Day 1 (training day) and Day 2 (test day). **(C)** Ovariectomized Wt and Kal7$^{KO}$ mice with placebo or E2 pellets were evaluated. Female mice did not spend significantly more time exploring the novel object than the familiar object with respect to either genotype or E2-treatment (two-way ANOVA). **(D)** On both the training and test day, Kal7$^{KO}$ mice spent more time than Wt mice exploring both objects ($p<0.05$, two-way ANOVA). While there was no main effect of E2 treatment on object exploration during the training day ($F_{(1,36)}=2.155$, $p=0.151$, two-way ANOVA), E2 treatment decreased total object exploration time on the test day ($F_{(1,36)}$ = 7.239; *p* = 0.011). There was no genotype x treatment interaction on either day (two-way ANOVA). N: 10 per group. **p* < 0.05 for main genotype effect; #$p<0.05$ for main E2 effect. Error bars represent SEM.

**Figure 6: Locomotor Sensitization to Cocaine: (A)** Locomotor activity was assessed for one hour immediately following injection of saline or cocaine. After 3 days of saline injections (S1-3), mice received an i.p. injection of 10 mg/kg cocaine (C1). On the following five days (C2-6), mice received injections of 20 mg/kg cocaine. On the next day (C7), mice received 10 mg/kg cocaine. Following nine days of withdrawal, mice received a single injection of 10 mg/kg cocaine (Challenge). **(B)** The initial locomotor response of each mouse to 10 mg/kg cocaine was compared to its response to saline (C1/S3). There was a main effect of estradiol treatment ($F_{(1,25)}$ = 12.92; p = 0.001, two-way ANOVA; ******, p<0.01). However, there was no main effect of genotype or a genotype by treatment interaction. Sensitization to 10 mg/kg cocaine was observed, with no effect of genotype or hormone treatment (C7/S3 vs. C1/S3), but a clear effect of prolonged cocaine administration (*, p<0.05). While all groups showed an enhanced response to the 10mg/kg dose, there were no statistically significant differences between groups. Long-term sensitization to 10 mg/kg cocaine was evaluated after withdrawal for 9 days

(Challenge). Interestingly, chronically E2-treated animals showed no significant alteration in sensitization maintenance compared to placebo-treated animals ($F_{(1,21)}$=2.687; *p*=0.116,two-way ANOVA). There were no differences in genotype ($F_{(1,21)}$=0.292; *p*=0.594, two-way ANOVA) or genotype by treatment interactions ($F_{(1,21)}$=0.662; *p*=0.425) on the challenge day. N: 6-9 per group for C1/S3 and C7/S3. N: 4-9 per group for Challenge/S3. Error bars represent SEM.

**Figure 7: Decreased NR2B Expression in Kal7$^{KO}$ Hippocampal PSDs.** PSDs were purified from the hippocampi of ovariectomized mice treated with placebo- or E2-pellets for 14-15 days. Tissue from 3-4 mice was pooled for analysis. **(A)** Western blot showing bands of Kal7, NR2B, and βIII-tubulin. **(B)** PSD levels of NR2B were decreased in Kal7$^{KO}$ mice ($F_{(1,4)}$ = 8.703; *p* = 0.042, two-way ANOVA). There was no effect of estrogen treatment ($F_{(1,4)}$ = 0.154; *p* = 0.715), nor a genotype x treatment interaction ($F_{(1,4)}$ = 0.009; *p* = 0.929). **(C)** Estrogen treatment did not alter PSD levels of Kal7 (P=0.69, t-test). Signals for Kal7 and NR2B were normalized to βIII-tubulin. N: 2 per group. *$p < 0.05$ for main effect of genotype. Error bars indicate range of values.

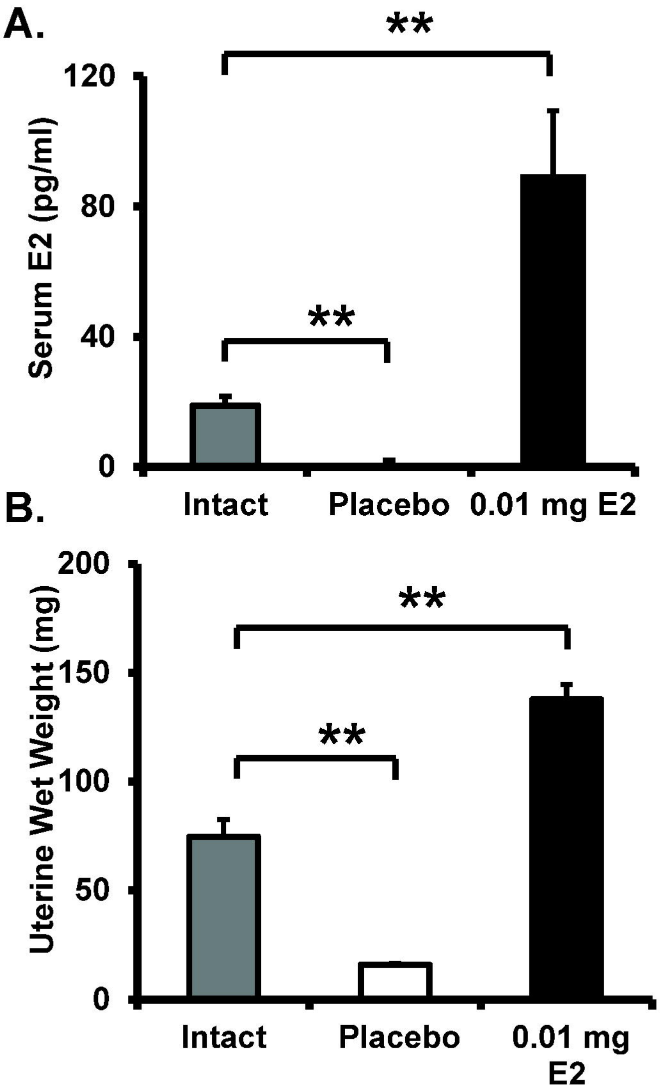
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Figure 1

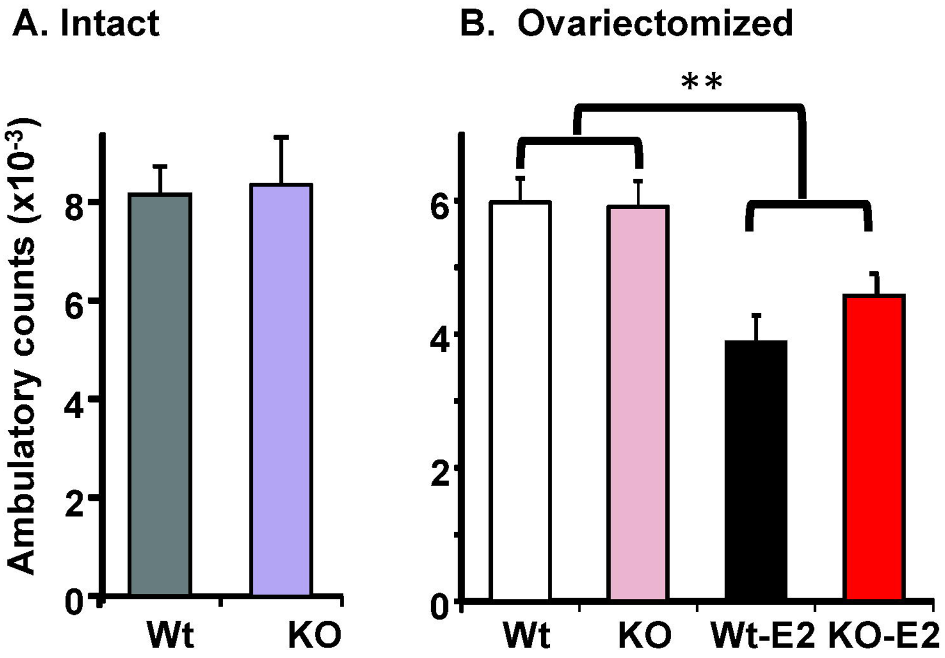
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Figure 2

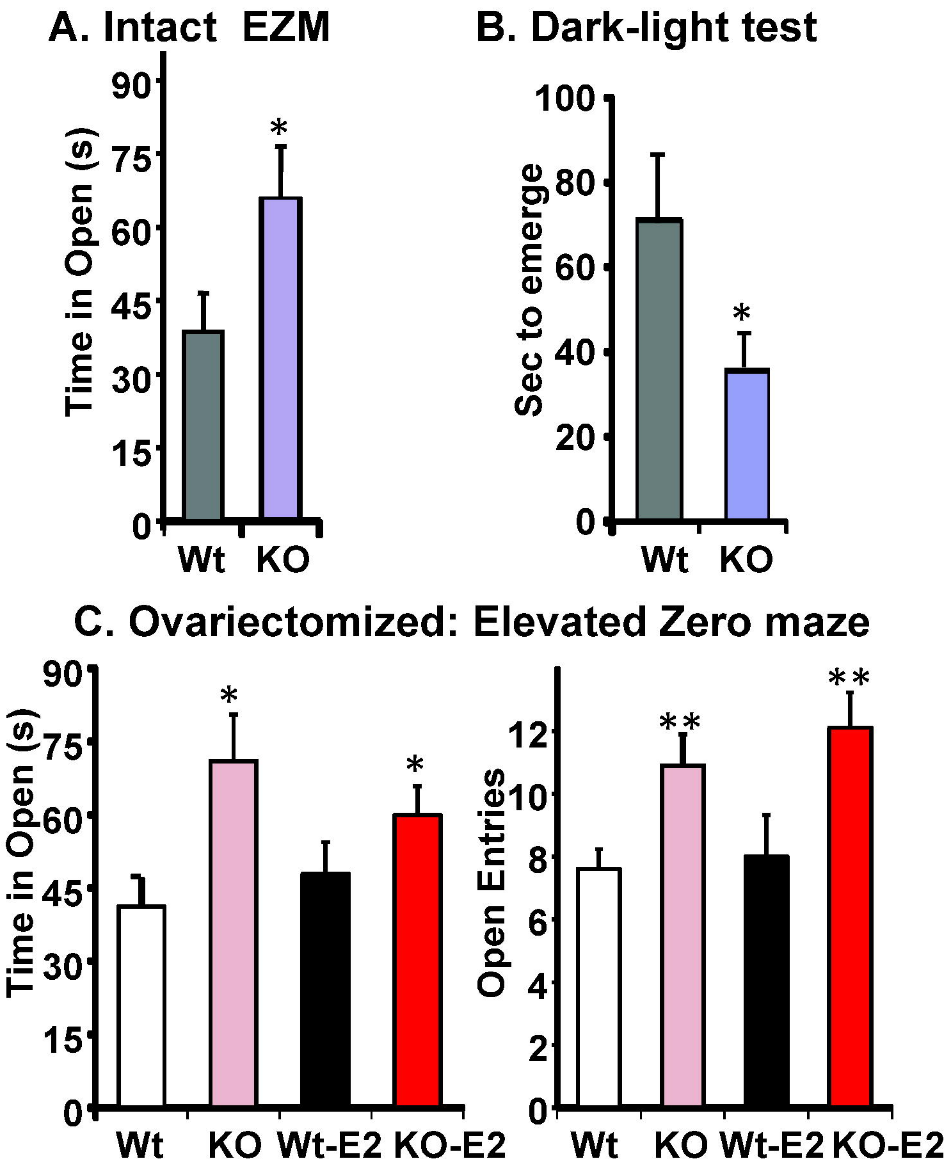
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Figure 3

Molecular Pharmacology Fast Forward. Published on September 18, 2012 as DOI: 10.1124/mol.112.080838

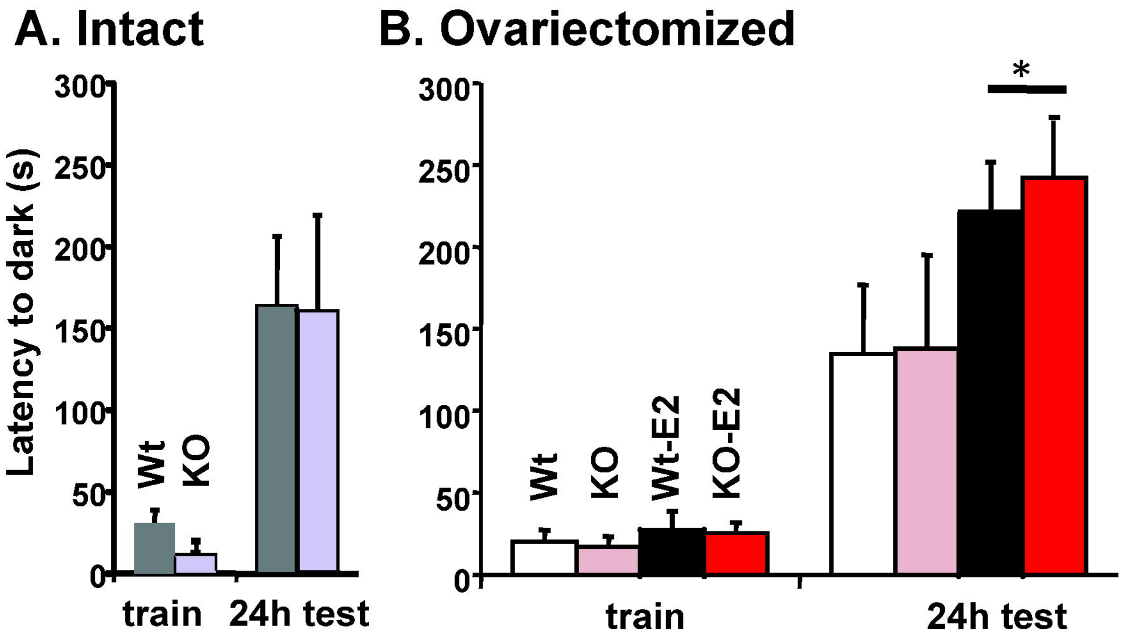

Figure 4

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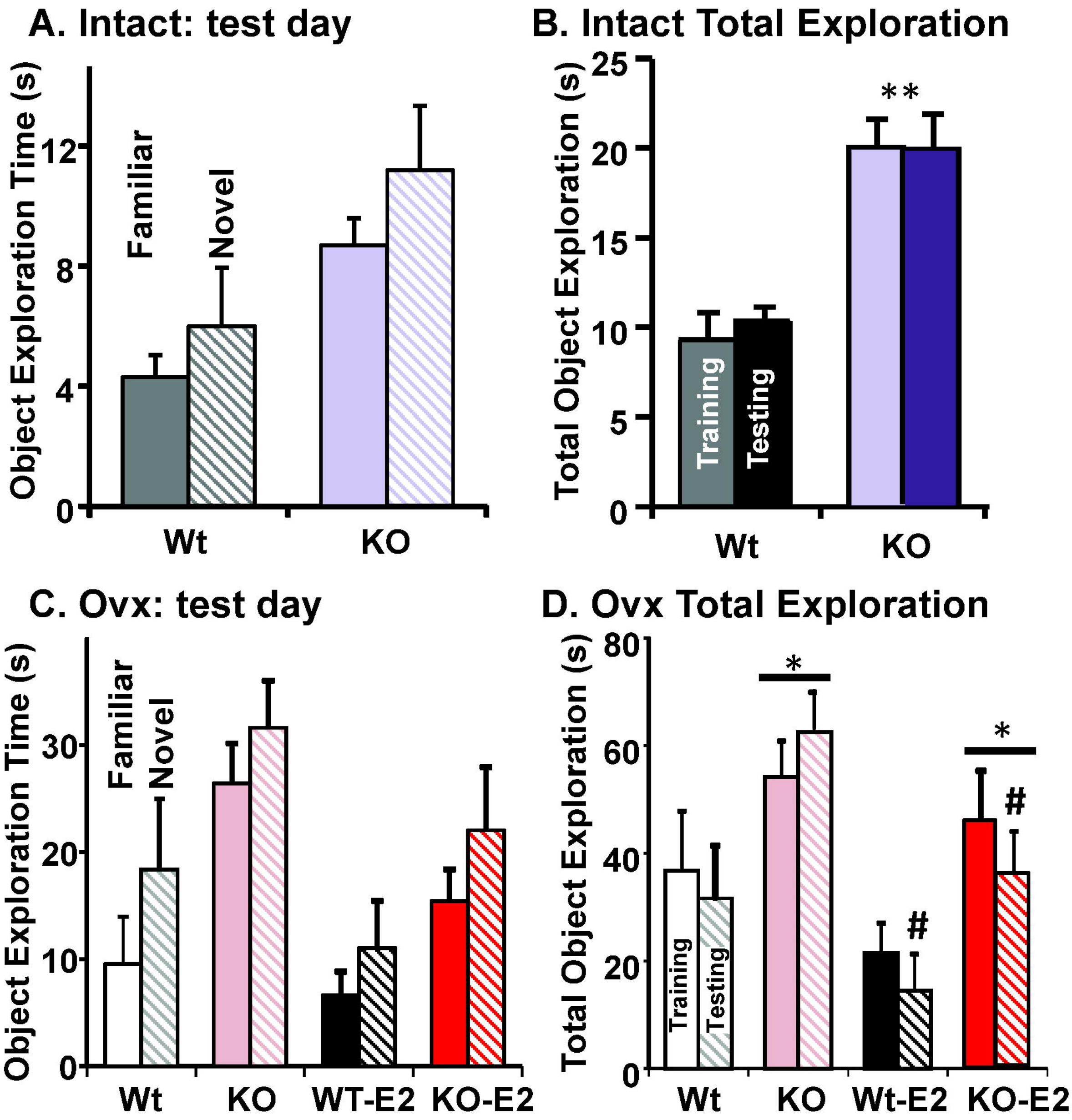

Figure 5

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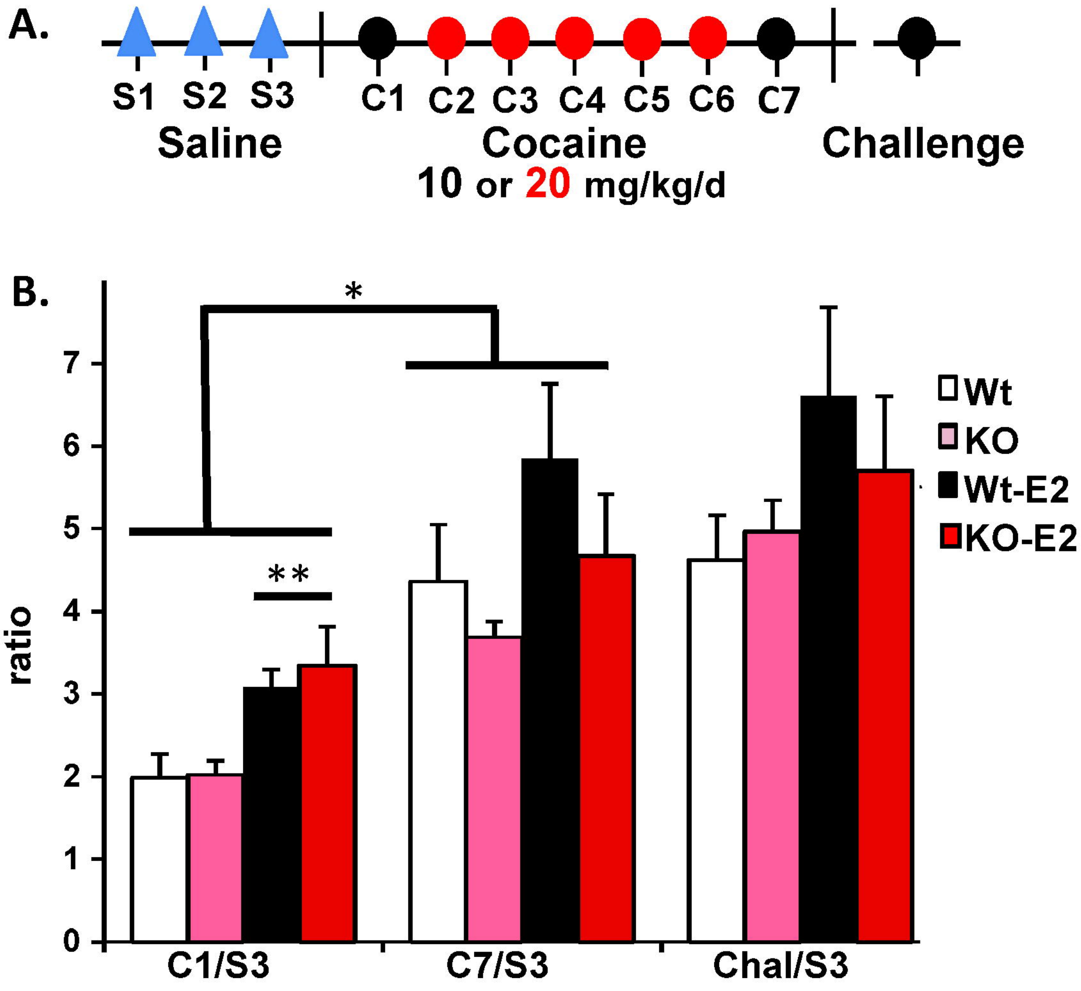

Figure 6

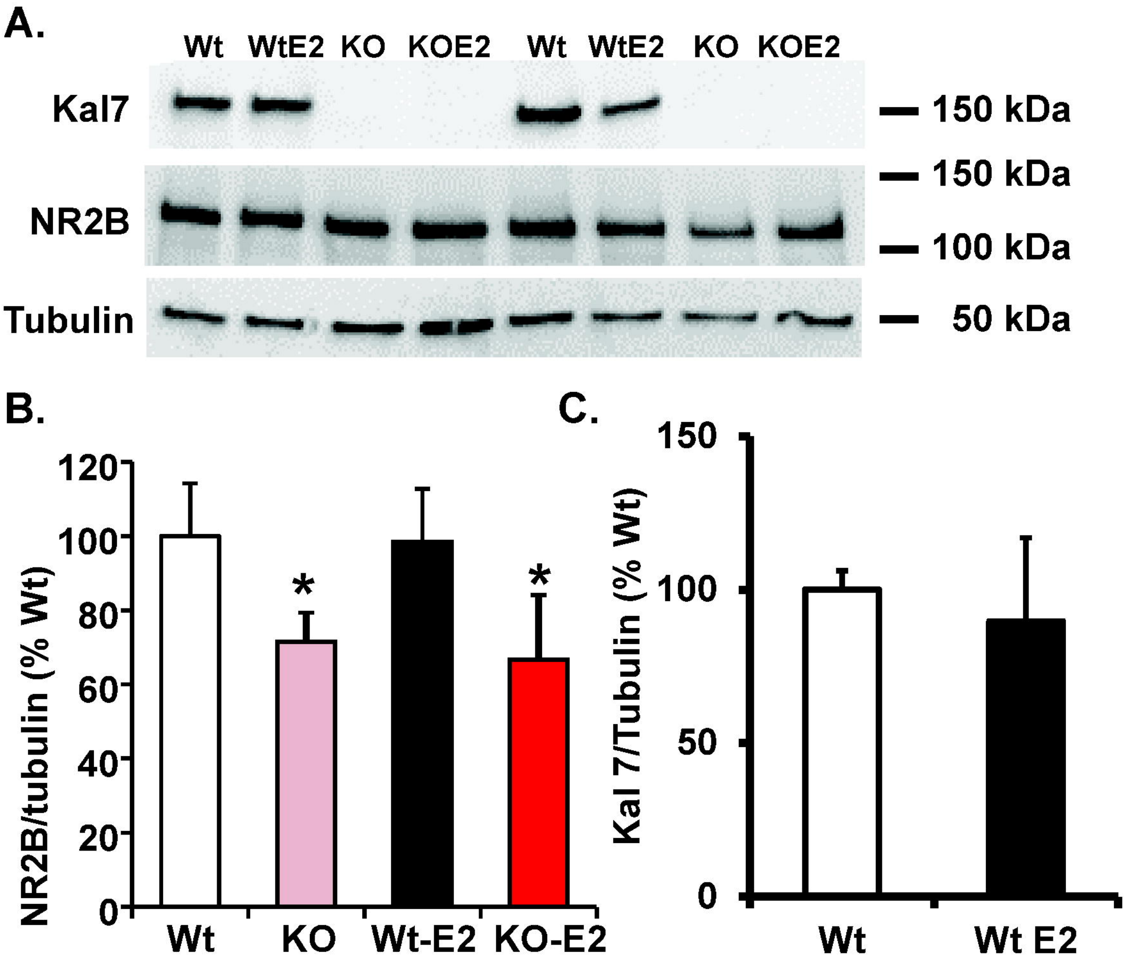
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Figure 7