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Full Title: Constitutive knockout of Kalirin-7 leads to increased rates of cocaine self- administration

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Running Title:

Altered cocaine self-administration in Kalirin-7 KO mice

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Non-standard abbreviations used: Kalirin-7 (Kal7), Kalirin-7 knockout mouse (Kal7^{KO}), post-synaptic density (PSD), nucleus accumbens (NAc), guanine nucleotide exchange factor (GEF), prefrontal cortex (PFC), progressive ratio (PR), fixed ratio 1 (FR1).

Abstract

Kalirin-7 (Kal7) is a post-synaptic density (PSD) localized Rho-guanine nucleotide exchange factor (GEF) that interacts with the NR2B subunit of the NMDA receptor and regulates aspects of dendritic spine dynamics both *in vitro* and *in vivo*. Chronic treatment with cocaine increases dendritic spine density in the nucleus accumbens (NAc) of rodents and primates. Kal7 mRNA and protein are upregulated in the NAc following cocaine treatment, and the presence of Kal7 is necessary for the normal proliferation of dendritic spines following cocaine. Mice that constitutively lack Kal7 (Kal7^{KO}) demonstrate increased locomotor sensitization to cocaine and a decreased place preference for cocaine. Here, using an intravenous cocaine self-administration paradigm, Kal7^{KO} mice exhibit increased administration of cocaine at lower doses as compared to wildtype (Wt) mice. Analyses of mRNA transcript levels from the NAc of mice that self-administered saline or cocaine reveal that larger splice variants of the *Kalrn* gene are increased by cocaine more dramatically in Kal7^{KO} mice than in Wt mice. Additionally, transcripts encoding the NR2B subunit of the NMDA receptor increased in Wt mice that self-administered cocaine, but were unchanged in similarly experienced Kal7^{KO} mice. These findings suggest that Kal7 participates in the reinforcing effects of cocaine, and that Kal7 and cocaine interact to alter the expression of genes related to critical glutamatergic signaling pathways in the NAc.

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Introduction

Addiction is a disorder marked by persistent compulsive behaviors despite prolonged periods of abstinence and appropriate treatment (Hyman et al., 2006). While a great deal of work has been performed to elucidate the underlying pathophysiology of this disorder, the molecular mechanisms and neural circuits that result in such long standing behavioral changes have yet to be fully clarified (Kalivas, 2009; Kauer and Malenka, 2007; Nestler, 2005). One potential mechanism underlying the chronic nature of addiction is morphological changes in regions of the brain that are important for reward-related learning such as the nucleus accumbens (NAc) and prefrontal cortex (PFC) (Koob and Volkow, 2010). A multitude of animal studies have demonstrated that prolonged treatment with psychostimulants (e.g. cocaine or amphetamine) or opiates (e.g. morphine) cause long-lasting changes in dendritic spine density and morphology in these regions (Lee et al., 2006; Robinson et al., 2001; Robinson and Kolb, 1997; Russo et al., 2010). Changes in spine density and morphology have also been implicated in other chronic psychiatric/neurologic conditions such as schizophrenia, autism and mental retardation (Hutsler and Zhang, 2010; Lin and Koleske, 2010; van Spronsen and Hoogenraad, 2010).

Kalirin-7 (Kal7) is a Rho-GEF that is localized to the postsynaptic density (PSD) of excitatory synapses and plays an important role in the normal formation and maintenance of dendritic spines both *in vitro* (Ma et al., 2008a; Ma et al., 2003) and *in vivo* (Ma et al., 2008b). At baseline, NAc spine density is normal in mice that constitutively lack Kal7 (Kal7[KO]), but spine length and spine area are reduced. In Wt mice, levels of Kal7 protein and mRNA increase in the NAc following chronic administration of cocaine, and both spine density and spine area increase (Kiraly et al., 2010b; Mains et al., 2011). In Kal7[KO] mice, chronic cocaine administration fails to increase NAc spine density and paradoxically decreases spine area (Kiraly et al., 2010b; Mains et al., 2011). Behavioral experiments demonstrate that Kal7[KO] mice exhibit increased locomotor sensitization to cocaine, but decreased cocaine place preference (Kiraly et al., 2010b).

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The glutamate hypothesis of cocaine action posits that many of the short- and long-term behavioral, morphological and biochemical adaptations to cocaine exposure are mediated, in major part, through alterations in glutamatergic neurotransmission (Eipper-Mains et al., 2013; Knackstedt and Kalivas, 2009). Biochemical analyses of Kal7[KO] mice reveal a decrease in the expression, surface localization and function of the NR2B subunit of the NMDA receptor, which interacts directly with Kal7 (Kiraly et al., 2011; Ma et al., 2008b). The literature suggests that NR2B function is important for the strengthening and stabilization of new synapses (Lee and Dong, 2011; Lee et al., 2010). An increase in silent synapses expressing NR2B in the NAc has been noted following cocaine treatment (Huang et al., 2009), and NR2B function is thought to be critical for the development of drug-induced locomotor sensitization and conditioned place preference (Brown et al., 2011; Ma et al., 2011; Pascoli et al., 2011). Similarly, the decrease in NR2B function observed in Kal7[KO] mice is thought to contribute to their decreased conditioned place preference for cocaine. The role of decreased NR2B function in the increased locomotor sensitization response observed in Kal7[KO] mice has not yet been studied (Kiraly et al., 2011).

While these previous studies have provided important information about the role of Kal7 in cocaine-related morphological and behavioral changes, the most directly translational model of addiction is that of drug self-administration (Thomsen and Caine, 2005). Here we demonstrate that Kal7[KO] mice exhibit increased self-administration of cocaine at lower doses, while exhibiting normal responding for a higher dose of cocaine and for food reinforcement. Since Kal7 has been shown to play a role in signaling pathways triggered by receptor tyrosine kinases, G protein coupled receptors, ligand gated ion channels and homophilic adhesion molecules, we used qPCR to search for alterations in the ability of Kal7[KO] mice to regulate the transcriptional response to self-administered cocaine. While a heightened transcriptional response was observed for the *Kalrn* gene, the ability of self-administered cocaine to increase expression of transcripts encoding NR2B was eliminated in Kal7[KO] mice.

Differences in baseline expression of transcripts encoding Vglut1 and Gria1 in the NAc of Wt and

Kal7^{KO} mice may contribute to their different responses to self-administered cocaine.

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Materials and Methods

Animals. Kal7[KO] mice were generated as described previously (Ma et al., 2008b) and have been back-crossed to a C57BL/6 background for ≥15 generations. All experiments were performed using only male littermate controls from 2-5 months of age (25-35g). Animals were shipped from the University of Connecticut Health Center (UCHC) to Arizona State University (ASU) under climate controlled conditions with abundant food and water available. Upon arrival at ASU, animals were quarantined for 6-8 weeks and then transferred to a temperature and humidity controlled colony room on a reversed 12 hour light-dark cycle (lights off at 0700 hr). Animals were given *ad libitum* access to food and water except where specified. All experimental procedures were conducted with the approval of the UCHC and ASU Institutional Animal Care and Use Committees and in accordance with NIH procedures for animal care.

Surgical Procedures. Mice were anesthetized with isoflurane (2% v/v) vaporized in medical grade oxygen at a flow rate of 2 L/min. The skin overlying the dorsum between the scapulae as well as that overlying the jugular vein was shaved and scrubbed with iodine. A 1 cm incision was made to expose the jugular vein, and a sterile silicone catheter (Access Technologies, Skokie, IL; 0.67 mm and 1.0 mm internal diameters at venous and distal ends, respectively) was implanted into the right jugular vein. The catheter was inserted a total of 11 mm into the vein and was sutured in place and to the surrounding tissue. The distal end of the catheter was tunneled subcutaneously to exit the skin between the scapulae via a 1 cm incision. The catheter was attached to a threaded vascular access port (Plastics One, Roanoke, VA) which was then sutured to the surrounding tissue. Both wounds were sutured closed, and mice were treated daily with 10 U/ml heparin (0.05 ml volume) to maintain catheter patency and 6.67 mg/ml Timentin (0.05 ml volume) to protect against infection. The surgery site was treated with topical lidocaine and triple antibiotic ointment to facilitate healing of the wound, and mice were allowed 3-5 days to recover prior to initiation of drug self-administration procedures. All veterinary supplies and pharmaceuticals were obtained from Butler Schein Animal Health (Dublin,

OH).

Behavioral Experiments

Testing chambers. Drug and food self-administration procedures were conducted in operant self-administration chambers (ENV-307; Med Associates, St. Albans, VT). Each self-administration chamber was located inside a sound-attenuating cubicle equipped with a house light and exhaust fan designed to mask external noise and odors, and was interfaced to a computer. Each chamber was also equipped with two infrared nosepoke apertures located approximately 2 cm above the chamber floor. Syringe pumps were located outside of the self-administration chambers, and both were interfaced to a computer. Drug solutions were delivered via the syringe pump through a single-channel liquid swivel mounted atop the chamber, which was connected to the vascular access port.

Cocaine self-administration. Cocaine hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in sterile saline for intravenous self-administration. All self-administration procedures were conducted during the dark phase of the light-dark cycle. Mice were allowed to spontaneously acquire self-administration of cocaine (0.5 mg/kg/infusion) or saline in 2 h daily sessions. Cocaine or saline solution (infusion volume 0.02 ml over a 1 sec period) was delivered to the animal following an active aperture response on a fixed ratio 1 (FR1) schedule of reinforcement. Each infusion was followed by a time-out period of 20 seconds during which additional responses were recorded but did not result in activation of the syringe pump. Self-administration sessions were conducted on consecutive days. Body weights were measured every 5-7 days in order to assess weight loss as a result of drug self-administration and to adjust the concentration of cocaine in the infusion syringe as needed. Responses on the designated inactive aperture were recorded but produced no programmed consequences.

All cocaine self-administration experiments began with 10 daily FR1 sessions at a dose of

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0.5mg/kg/infusion. No shaping or prior operant training was performed to encourage increased responding. Following this initial phase, animals then went through another 10 days of FR1 sessions at either 1.0 or 0.25mg/kg/infusion. Following the 20 days of FR1 training, animals were either given two days of abstinence and then a progressive ratio (PR) session or were sacrificed. For PR sessions, a single cocaine infusion was earned with an escalating number of correct responses (i.e. 1, 2, 4, 8, 16, 32, etc.); the session was terminated when an animal went 60 min without enough responses to initiate an additional infusion.

Due to the vagaries of animal breeding to produce male littermates, and the time and effort-intensive nature of these experiments, it was necessary to use some animals in more than one behavioral assay. **Fig. 1** outlines the different paradigms that were used. With the exception of the PR test, all behavioral procedures were ten days in length. After quarantine, some mice first experienced operant food self-administration. All animals then underwent catheter implantation surgery and 7 days of recovery. The majority of animals were then utilized in **Path A** (indicated by yellow box) wherein they moved from the 0.5 → 1.0mg/kg/infusion training before having a PR test and sacrifice. The other group of mice, **Path B**, moved to a lower dose (0.25mg/kg/infusion) after the first ten days. Animals that started with saline infusions were either utilized in **Path C**, where they then went to cocaine self-administration, or in **Path D**, where they were sacrificed without receiving cocaine to serve as controls for qPCR. For animals with prior operant experience, the active aperture was switched prior to transitioning to cocaine. Additionally, we performed an analysis comparing animals with prior treatment to those who started with cocaine training. RM-ANOVA of the first ten days of cocaine training reveals no difference between the different pathways ($p \geq 0.44$; **Supplemental Figure 1**) so all groups were included in the same analysis. To ensure that results were not biased by animals who never administered enough drug to form an operant association (i.e. low responders) animals that received < 2 infusions/day at the 0.5mg/kg/infusion or 1.0mg/kg/infusion dose or < 1 infusion/day at the 0.25mg/kg/infusion dose were not included in longitudinal behavioral analyses. Similar numbers of

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Wt and Kal7[KO] animals were excluded using these criteria.

Food self-administration experiments. To test for motivation to self-administer a non-drug reinforcer (food), mice were food restricted for 5 days prior to experimentation as described previously (Kiraly et al., 2010b). Daily weights were monitored to ensure the animals did not lose >15-20% of their total body weight. For these experiments animals were placed in the operant conditioning chambers for 30 minutes each day. The active nosepoke hole was programmed to deliver a 20 mg grain pellet (Bio-Serv) on a FR1 schedule with a 20 second timeout. The inactive aperture had no programmed consequences. Animals were given free access to food for 90 minutes/day several hours after the completion of the session. As with cocaine self-administration, very low responders (average of < 1 reward/session) were excluded from longitudinal behavioral analyses. This resulted in considerably more Wt mice being excluded (10 Wt and 3 Kal7[KO]); this issue is addressed in the Results and Discussion sections.

Molecular Biology. Following cocaine self-administration training and progressive ratio tasks, animals were given two additional 2 hr sessions with access to 0.5mg/kg/infusion cocaine and were sacrificed 24 hours after the final exposure. Brains were dissected on ice and a mouse brain block was used to obtain the 2mm slice containing the NAc. Using the anterior commissure as a guide, a tissue punch (d = 1.5 mm) was used to remove the NAc. Samples were homogenized in ice-cold Trizol (Invitrogen) using a Polytron. RNA was prepared as described (Mains et al., 2011) and cDNA was synthesized using iScript (BioRad) with random primers; the 5 min 65°C step before chilling and adding the enzyme gives much more reproducible qPCR data for very long transcripts. Real-time PCR was performed as described (Mains et al., 2011). Data were calculated with respect to GAPDH for each sample within an assay; these values with respect to GAPDH for each transcript were consistent across assays and sets of mice over a three year period. The primer sets all yielded

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products that were ~120 nt long, with melt temperatures of 61 $\pm$ 1°C (Eipper-Mains et al., 2013; Mains et al., 2011).

Statistical analyses. Statistical analysis was performed using Graphpad Prism software. For longitudinal behavioral experiments repeated-measures two-way ANOVA analyses were used. Cumulative distributions were compared using a Kolmogorov-Smirnov test, and progressive ratio data were compared using a two-tailed t-Test. qPCR data were compared using two-way ANOVA analyses for main effects with a Holm-Sidak post-hoc test with correction for multiple comparisons.

Results

Cocaine Self-Administration

All cocaine self-administration experiments were performed with littermate male mice and began with

with 10 daily sessions of 0.5mg/kg/infusion cocaine reinforced on an FR1 schedule (**Fig. 1**). Wt and

Kal7^{KO} mice exhibited similar rates of administration over the first three days of conditioning, but while

Wt mice remained relatively stable in their rate of administration, the Kal7^{KO} mice increased their

number of infusions over time (**Fig. 2a**). A two-way repeated-measures ANOVA examining the

number of infusions revealed main effects of genotype ($F_{(1,48)}$ = 5.34, p=0.025) and day ($F_{(9,432)}$=2.7,

p=0.005) as well as a genotype x day interaction ($F_{(9,432)}$=2.69, p=0.005). Examination of the number

of active responses made by each genotype demonstrated the same pattern as the number of

infusions, with a strong effect of treatment day ($F_{(9,342)}$ = 3.65, p<0.001) but there was no significant

effect of genotype due to increases in variability ($F_{(1,38)}$=2.96, p=0.09) (**Fig. 2b**). Analysis of

responding on the inactive aperture (**Fig. 2c**) and of responding for saline infusions (**Fig. 2d**)

demonstrated low levels of responding for both, with no main effects of genotype, day or any

interactions by RM-ANOVA (p≥0.19 for all analyzed).

After completion of 10 days of training at a dose of 0.5mg/kg/infusion, animals were transferred to a

new dose of cocaine for the next ten days. The first group had their dosage increased to

1.0mg/kg/infusion (**Fig.1 – Path A**). In these animals there was no significant difference between

genotype in the number of infusions earned (**Fig. 3a** - RM two-way ANOVA effect of genotype:

$F_{(1,39)}$=0.76, p=0.39). Likewise, there was no main effect of day for this cohort ($F_{(9,351)}$=0.38, p=0.95) or

a genotype x day interaction ($F_{(9,351)}$=1.02, p=0.42). The animals that were trained at the

1.0mg/kg/infusion dose then went on to a one day progressive ratio task, which also demonstrated no

genotypic difference in the number of infusions earned (graph not shown: Wt 6.92±1.4; Kal7^{KO}

5.49±1.3 - two-tailed t-test t=0.78, df=51, p=0.44). Higher doses of cocaine per infusion were not

tested, as previous studies have shown 1.0mg/kg/infusion to be the dose at which C57/bl6 mice

typically plateau (Thomsen and Caine, 2007).

Given that the Kal7^{KO} mice administered more cocaine at 0.5mg/kg/infusion, but there was no

statistically significant genotypic difference at 1.0mg/kg/infusion, the next cohort of mice were moved

to a lower (0.25mg/kg/infusion) dose following the initial ten days of training (**Fig. 1 – Path B**). While

both Wt and Kal7^{KO} mice exhibited a decrease in the number of infusions earned at this dose, the

Kal7^{KO} mice administered at a significantly higher level than did their Wt counterparts (**Fig 3B;** RM

two-way ANOVA effect of genotype: $F_{(1,11)}$=14.16, p=0.003). As with the 1.0mg/kg dose, there was no

main effect of day ($F_{(9,99)}$=1.46, p=0.17) or genotype x day interaction ($F_{(9,99)}$=0.31, p=0.97).

Population of high responding mice among Kal7^{KO}

Following analysis of the longitudinal group data for cocaine self-administration (**Figs. 2&3**), we

examined the data to determine how the behavior of individual animals contributed to the overall

genotype effect at 0.25 and 0.5mg/kg/infusion. **Fig. 4A** presents a histogram of the total number of

infusions earned by individual mice in the 0.5mg/kg/infusion experiment (all mice including low

responders are included in **Fig. 4**). As can be seen, the Wt animals included a large population

responding in the lower range, with a monotonic decline in the percentage of animals earning higher

numbers of infusions. However, when the corresponding results were plotted for the Kal7^{KO} mice, a

secondary peak representing a population of very high responders was observed. The same data

plotted as a cumulative frequency plot demonstrated a rightward shift in the curve (**Fig. 4B**);

Kolmogorov-Smirnov analysis did not demonstrate statistical significance to this shift (*p*=0.33).

Fig. 4C presents a histogram of the total number of infusions earned by individual mice receiving

0.25mg/kg/infusion. Although the shapes of the Wt and Kal7^{KO} distributions were nearly identical, the

Kal7^{KO} distribution was shifted to the right; 4% of the Wt mice earned 10-29 infusions; none of the

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Kal7^{KO} mice earned fewer than 30 infusions. Fully 67% of the Kal7^{KO} mice earned more total infusions than the highest responding Wt mouse. Cumulative distribution plot and Kolmogorov-Smirnov analysis revealed this to be a statistically significant shift in the curve between genotypes (p=0.039), with more of the Kal7^{KO} mice receiving more total infusions (**Fig. 4D**).

Food Self-Administration

Previous studies have shown that Kal7^{KO} mice exhibited normal radial arm maze learning for a food reward (Kiraly et al., 2010b; Ma et al., 2008b), as well as other food-motivated behaviors (Kiraly et al., 2010b; Ma et al., 2008b). When tested for operant food self-administration, a two-way repeated measures ANOVA revealed that there was an overall increase in responding across days (**Fig. 5A** - $F_{(9,135)}$=3.51; p<0.001) but no significant difference between genotypes ($F_{(1,15)}$=0.02; p=0.90) or a genotype x day interaction ($F_{(9,135)}$=0.99; p=0.45).

As for cocaine self-administration, we examined the individual data for total levels of food responding. Interestingly, far more Wt animals fell into the low responder category (<1 response/session – 10/15Wt, 3/15 Kal7^{KO}) and were excluded from the longitudinal analysis in **Fig. 5A**. This difference was quantified using a cumulative frequency curve of the total number of food rewards earned for all animals followed by Kolmogorov-Smirnov analysis. As can be seen in **Fig. 5B** there was a distinct rightward shift in the Kal7^{KO} curve which was driven by both a population of low responding Wt mice and a population of high responding Kal7^{KO} mice; 27% of the Kal7^{KO} mice earned >100 total rewards, while only 7% of the Wt mice reached this level. Kolmogorov-Smirnov analysis of these data revealed a strongly significant difference between genotypes (p=0.003). While there was no genotypic difference amongst the cohort of mice that responded to the task, the Kal7^{KO} mice had a much greater propensity to show adequate levels of responding for the food reward.

Quantitative PCR analysis of NAc transcripts

Chronic cocaine exposure produces a wide variety of changes in gene expression, and *Kalrn* has

been identified as a cocaine-responsive gene (Kiraly et al., 2010b; Ma et al., 2012; Mains et al.,

2011). *Kalrn* is a large, alternatively spliced gene which yields multiple protein products (Kiraly et al.,

2010a); while Kal7 and ΔKal7 are absent in Kal7^{KO} mice, expression of the larger isoforms, Kal9 and

Kal12, is increased when the single exon unique to Kal7 and ΔKal7 is deleted (Ma et al., 2008b). To

begin to identify the molecular changes underlying the behavioral alterations noted in Kal7^{KO} mice, we

performed quantitative PCR analysis of transcripts from the NAc of Wt and Kal7^{KO} mice which had

self-administered either cocaine or saline (**Fig. 1 – Path A** vs. **Path D**). For these experiments

transcript levels were normalized to GAPDH within each group, and levels were consistent amongst

multiple groups across experiments over a 3 year period; for ease of viewing, these data are

presented here as fold change from Wt Saline levels (**Fig. 6A**); statistics and actual levels of

expression are provided in **Table 1**. As expected based on prior analyses of Kal7 RNA and protein

expression in rodents receiving experimenter administered cocaine (Kiraly et al., 2010b; Ma et al.,

2012; Mains et al., 2011), analysis of Kal7 transcripts in Wt mice revealed an increase after cocaine

self-administration, with no signal in Kal7^{KO} mice (**Fig. 6A & Table 1**). Examination of Kal9 transcript

levels reveals a strong effect of genotype, with Kal7^{KO} mice expressing higher levels of Kal9

transcript, and a main effect of cocaine treatment (**Table 1 & Fig. 6A**). Similarly, Kal12 transcript

levels show a strong effect of genotype and cocaine, as well as a significant genotype x treatment

interaction (**Table 1 & Fig. 6A**). Transcripts encoding Kal9 and Kal12 do not increase significantly in

Wt animals self-administering cocaine. Despite starting at a higher level in Kal7^{KO} mice, levels of

Kal12 transcript increase following cocaine self-administration in Kal7^{KO} mice. Cocaine self-

administration was without effect on expression of transcripts initiated at the ΔKal promoter or at the

rarely utilized Duet promoter.

The heightened effect of cocaine self-administration on *Kalrn* expression in Kal7^{KO} mice led us to

examine several other transcripts known to increase following experimenter-administered cocaine

(Eipper-Mains et al., 2013) (**Fig. 6A & Table 1**). Since Kal7 interacts directly with the NR2B subunit of the NMDA receptor, we examined this transcript. Levels of NR2B mRNA increase in Wt mice self-administering cocaine, but do not increase in cocaine self-administering Kal7[KO] mice; levels of NR2B transcript are indistinguishable in Wt and Kal7[KO] mice self-administering saline. Levels of the mRNA encoding vesicular glutamate transporter 1 (Vglut1) decrease in both Wt and Kal7[KO] mice self-administering cocaine. These changes in NR2B and Vglut1 expression are similar to the changes seen in Wt mice receiving experimenter-administered cocaine (Eipper-Mains et al., 2013).

The fact that self-administration of cocaine affects NR2B transcript levels in the NAc differently in Wt and Kal7[KO] mice (**Fig.6A**) led us to examine gene expression in naïve male Wt and Kal7[KO] mice (**Fig.6B**). As expected, Kal7 transcripts are not detectable in the NAc of Kal7[KO] mice and levels of Kal9 and Kal12 transcripts are significantly increased over Wt levels. NR2B transcript levels in Wt and Kal7[KO] NAc are indistinguishable (**Fig.6B**). We next compared levels of several transcripts previously shown to respond to experimenter-administered cocaine (Eipper-Mains et al., 2013) in the NAc of naive Wt and Kal7[KO] mice (**Fig.6B**). Levels of Vglut1 are diminished more than five-fold in Kal7[KO] NAc. As for NR2B, levels of transcripts encoding NMDA receptor subunits NR1 and NR2A do not differ in Wt and Kal7[KO] NAc. Levels of AMPA receptor subunit AMPA1 (Gria1) transcript are doubled in the NAc of Kal7[KO] mice while levels of kainate receptor subunit kainate 5 (Grik5) and metabotropic glutamate receptor Grm5 transcript are unaltered (**Fig.6B**). Neither Vglut1 nor AMPA1 transcript levels differ in RNA prepared from motor cortex of the same Wt and Kal7[KO] mice (not shown).

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Discussion

Behavioral Results

Previous studies of Kal7[KO] mice demonstrated an altered behavioral response to experimenter administered cocaine. When given a sensitizing dose of cocaine, male Kal7[KO] mice demonstrate a higher degree of locomotor sensitization than Wt mice (Kiraly et al., 2010b). Additionally, Kal7[KO] mice exhibit decreased conditioned place preference for cocaine but normal place preference for food (Kiraly et al., 2010b). The present study represents the first examination of cocaine self-administration in Kal7[KO] mice. While direct comparisons between these three models are difficult, some conclusions can be drawn. Kal7[KO] mice self-administered low doses of cocaine at higher rates than Wt mice; this difference was not observed with higher doses of cocaine (**Figs. 2, 3 & Supplemental Figure 2**). Previous studies have demonstrated a plateau in responding in C57/Bl6 mice at 1.0mg/kg/infusion (Thomsen and Caine, 2007), and these data concur (**Supplemental Figure 2**). In our animals, we saw differences at lower doses that were not present at the 1.0mg/kg/infusion dose, and chose not to escalate dosing beyond this level. It is noteworthy that Kal7[KO] mice maintained a higher level of responding at the 0.25mg/kg/infusion dose that elicited little responding from Wt mice (**Figs. 3&4**). This suggests that Kal7[KO] mice have increased sensitivity to the rewarding properties of cocaine, in line with the increased locomotor sensitization response noted previously (Kiraly et al., 2010b). A connection between failure to upregulate NAc spine density after cocaine and increased locomotor sensitization and food-seeking was noted in Kal7[KO] mice and in mice lacking Cdk5, SynCAM1, Mef2 or Dnmt3a (Benavides et al., 2007; Giza et al., 2013; LaPlant et al., 2010; Meyer et al., 2008; Norrholm et al., 2003; Pulipparacharuvil et al., 2008; Rothenfluh and Cowan, 2013). As other models are examined, it will be interesting to see the possible connection between blunted spine plasticity, heightened locomotor sensitization and increased self-administration.

The fact that Kal7[KO] mice only self-administered at higher rates for lower doses of cocaine helps put the decrease in conditioned place preference into perspective as well (Kiraly et al., 2010b). The

conditioned place preference task is performed with a single large bolus of cocaine – comparable to the total amount administered over two hours in the 1.0mg/kg/infusion experiments (**Fig. 3a**). If Kal7[KO] mice are more sensitive to the behavioral effects of cocaine, the large bolus dosing for the CPP test may be less reinforcing than for Wt mice, whereas response-contingent infusions at low doses may be more motivating for Kal7[KO] mice than for their Wt counterparts.

Examination of data from individual mice revealed that increased self-administration in Kal7[KO] mice was partly driven by a few high responders (**Fig. 4**). This is particularly interesting, as addiction is a disorder in which only a subset of individuals who use a drug escalate to the point of dependency and loss of control (SAMSHA, 2012). Studies in self-administering rats mimicking addiction conditions (e.g. cocaine administration paired with negative consequence such as a footshock) demonstrated that while all rats self-administer cocaine, only ~20% of them meet these "addiction" criteria (Deroche-Gamonet et al., 2004) . Interestingly, approximately the same percentage of very high responders was observed in Kal7[KO] mice at the 0.5mg/kg/infusion dose (**Fig. 4A**). Like many psychiatric disorders, addiction is believed to be a gene x environment interaction – with environmental influences and genetic risks producing pathology (Ducci and Goldman, 2012; Guo et al., 2012). Based on our results, one would predict that decreased Kal7 expression or function would contribute to increased vulnerability to cocaine addiction. Interestingly, a genome-wide linkage scan (Yang et al., 2011) found the region of chromosome 3 containing *KALRN* to have a very high LOD score for African-American families with cocaine dependence distinct from major depressive disorder.

Additional experiments are needed to elucidate the role of Kal7 in cocaine self-administration. The extinction-reinstatement model, which best recapitulates the abstinence-relapse paradigm seen in human addicts (Epstein et al., 2006; Knackstedt and Kalivas, 2009; Schmidt et al., 2005), could determine if lack of Kal7 affects sensitivity to stress, drug, or context-induced reinstatement. Additionally, given the direct interaction of Kal7 and NR2B (Kiraly et al., 2011), the failure of self-

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administered cocaine to increase NAc NR2B expression in Kal7^{KO} mice and the critical role of NR2B

in the behavioral response to cocaine (Brown et al., 2011; Huang et al., 2009; Kiraly et al., 2011; Ma

et al., 2006; Mao et al., 2009; Pascoli et al., 2011), studies examining NR2B inhibition in cocaine self-

administration in Wt and Kal7^{KO} animals will be important.

In addition to cocaine self-administration, we also characterized operant self-administration for food.

Previous experiments showed normal radial arm maze learning (Ma et al., 2008b) and conditioned

place preference for food (Kiraly et al., 2010b) in Kal7^{KO} mice. Food restricted Kal7^{KO} mice had similar

levels of overall responding for food reward pellets, without the population of low responders seen in

Wt mice (**Fig. 5**). The previously studied behaviors are much more dependent on the pairing of an

overall context with food rather than the pairing of an operant response (e.g. nose poke) with food

reinforcement. Instrumental responding for reinforcers is largely dependent on the striatum, while

context pairing is more dependent on the hippocampus (Hyman et al., 2006; Kelley and Berridge,

2002; Shiflett and Balleine, 2011). The effects of eliminating Kal7 expression on spine morphology are

distinctly different in hippocampal CA1 pyramidal neurons and NAc medium spiny neurons (Kiraly et

al., 2010b; Ma et al., 2008b). A systematic characterization of Kal7-mediated changes in dendritic

spine morphology and gene expression in specific cell types is needed in order to determine

mechanisms underlying genotypic differences in food reward and reinforcement place conditioning

and operant self-administration paradigms.

Molecular Biology

In Wt mice, Kal7 protein and transcript increase following experimenter-administered cocaine (Kiraly

et al., 2010b; Mains et al., 2011). After prolonged cocaine self-administration, there was an increase

in Kal7 mRNA levels in Wt mice similar in magnitude to that produced by experimenter-administered

cocaine (**Fig. 6A**). Although the Kal7^{KO} mice do not express Kal7 and ΔKal7, the larger isoforms of

Kalirin (e.g. Kal9 and Kal12) are present in higher amounts at baseline (Ma et al., 2008b) (**Fig.6B**).

While less is known about the functions of these larger isoforms, they promote process outgrowth by sympathetic neurons (May et al., 2002) and are expressed in adult forebrain neurons (Ma et al., 2001; Mains et al., 2011). Interestingly, there was a dramatic increase in Kal9 and Kal12 transcript levels in Kal7[KO] mice (**Fig. 6A**), likely corresponding to the elevated levels in naive animals (**Fig.6B**).

Based on analysis of its role in specific signaling pathways and its extensive phosphorylation, Kal7 is a participant in multiple signaling pathways. While the cortex of Kal7[KO] mice has decreased levels of NR2B protein at the PSD and decreased surface expression of this receptor (Kiraly et al., 2011; Lemtiri-Chlieh et al., 2011; Ma et al., 2008b), this is the first examination of NR2B expression in the NAc of Kal7[KO] mice; NR2B transcript levels did not differ in the NAc of naïve Wt and Kal7[KO] mice. An increase in NAc levels of NR2B mRNA, seen following experimenter-administered cocaine (Eipper-Mains et al., 2013), was also observed in Wt mice self-administering cocaine; strikingly, no increase in NAc NR2B expression was observed in Kal7[KO] mice self-administering cocaine (**Fig. 6A**). While the molecular mechanisms underlying the lack of NR2B regulation in Kal7[KO] mice are unknown, it is clear that NR2B expression is regulated by REST (Rodenas-Ruano et al., 2012), histone acetylation (Fujita et al., 2012; Myers et al., 1999; Qiang et al., 2011), CREB (Brown et al., 2011; Myers et al., 1999; Rani et al., 2005), NRSF (Qiang et al., 2005) and Sp1 (Miyatake et al., 2002; Myers et al., 1999), a transcription factor thought to bind to the *Kalrn* promoter (Mains et al., 2011).

A subset of GABAergic neurons in the NAc express both D1 and D2 dopamine receptors, plus Vglut1 (Gangarossa et al., 2013). Expression of Vglut1, although markedly reduced in the NAc of Kal7[KO] mice, is still diminished in response to self-administered cocaine, as in Wt mice. Vglut1 transcript levels are much higher in motor cortex than in NAc and are independent of genotype (not shown). The doubling of AMPA1 subunit mRNA observed in the NAc of Kal7[KO] vs. Wt mice is another indication that glutamatergic transmission is altered in the NAc of naïve Kal7[KO] mice. Identification of the NAc

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neurons in which Vglut1 and AMPA1 expression is altered in Kal7[KO] mice will be an essential part of elucidating the pathways leading to enhanced cocaine self-administration in these mice.

Conclusions

These studies represent the first behavioral and biochemical examination of Kal7[KO] mice using a cocaine self-administration paradigm. They make it clear that the presence of Kal7 at the synapse plays a critical role in normal self-administration behavior. Given that self-administration of low doses of cocaine is increased substantially in Kal7[KO] mice, our data suggest that augmenting Kal7 function might serve to diminish this response. Additionally, our data reveal a role for Kal7 in baseline glutamatergic signaling in the NAc and in the transcriptional response to cocaine self-administration.

Acknowledgments:

Authorship Contributions:

Participated in research design: DDK, MFO, BAE, REM

Conducted experiments: NEN, TPL, SET, SLY, BAE, REM

Performed data analysis: DDK, TPL, MFO, BAE, REM

Wrote or contributed writing of the manuscript: DDK, TPL, MFO, BAE, REM

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Footnotes:

Figure Legends

Fig. 1. Paradigm for behavioral testing. Due to breeding difficulties and lengths of experiments, some mice were used for multiple tests. Before surgery, some mice received operant conditioning for food reward. After catheter implantation, mice were used in one of four pathways. **Path A** included 0.5mg/kg/infusion for 10 days followed by 1.0mg/kg/infusion for 10 days, a progressive ratio test and sacrifice for dissection. **Path B** received the lower dose (0.25mg/kg/infusion) after the first ten days. **Path C** had ten days of saline self-administration followed by cocaine. **Path D** had ten days of saline self-administration followed by sacrifice and dissection to serve as molecular biology controls for the mice from **Path A**.

Fig. 2. Cocaine self-administration at 0.5mg/kg/infusion and saline. *(A)* When given daily training on an FR1 schedule for cocaine infusions, male Kal7^{KO} mice earn a significantly higher level of infusions than their Wt littermates (RM two-way ANOVA - Genotype $F_{(1,48)}$ = 5.34, *p*=0.025) with additional main effects of day ($F_{(9,432)}$=2.7, *p*=0.005) and a significant genotype x time interaction ($F_{(9,432)}$=2.69, *p*=0.005). *(B)* The pattern of responding on the active aperture (Active Responses) was similar to that seen with infusions, with a main effect of day ($F_{(9,342)}$ = 3.65, *p*<0.001), but no significant effect of genotype due to increased variability ($F_{(1,38)}$=2.96, *p*=0.09). Both Wt and Kal7^{KO} mice exhibit low levels of responding for both the inactive aperture *(C)* and for infusions of saline *(D)*, with no statistically significant differences by RM two-way ANOVA on either measure (*p*≥0.19 for all). [Cocaine - N=23 Wt, 27 Kal7^{KO}; Saline - N= 5 Wt, 5 Kal7^{KO}]

Fig. 3. Cocaine self-administration at other doses. After completing the initial 10 day acquisition period at 0.5mg/kg/infusion, Wt and Kal7^{KO} mice were tested with different doses of cocaine. *(A)* At a dose of 1.0mg/kg/infusion, the Kal7^{KO} mice maintain a trend towards increased infusions, but there were no statistically significant results (RM two-way ANOVA – Genotype $F_{(1,39)}$=0.76, p=0.39; Day $F_{(9,351)}$=0.38, p=0.95; Interaction $F_{(9,351)}$=1.02, p=0.42). *(B)* At the 0.25mg/kg/infusion dose, Kal7^{KO}

mice retain a significantly higher number of infusions (RM two-way ANOVA effect of genotype $F_{(1,11)}$=14.16, p=0.003). There is no main effect of day ($F_{(9,99)}$=1.46, p=0.17) or a genotype x day interaction ($F_{(9,99)}$=0.31, p=0.97). [1.0mg/kg/infusion – N=21 Wt, 20 Kal7[KO]; 0.25mg/kg/infusion – N=7 Wt, 6 Kal7[KO]]

Fig. 4. Analysis of high responder populations in Kal7[KO] mice. The distribution of total levels of responding for cocaine among mice was analyzed. *(A)* Histogram showing the fraction of each genotype earning a set number of infusions. A group of very high level responders is observed for the Kal7[KO] mice, but not for the Wt mice. *(B)* Cumulative frequency plot of the data from *(A)* demonstrates the expected right-shift in the Kal7[KO] curve. Kolmogorov-Smirnov analysis indicates that the shift does not reach statistical significance (*p*=0.33). *(C&D)* Histogram and Kolmogorov-Smirnov plots for 0.25mg/kg/infusion data. A right-shift (higher responding) for the Kal7[KO] mice is apparent on both the histogram and cumulative frequency plots. At this dose, Kolmogorov-Smirnov analysis shows a statistically significant difference between genotypes (*p*=0.039).

Fig. 5. Instrumental responding for food. Cocaine-naïve animals food restricted for 5 days were then tested for operant responding for a grain pellet reward on an FR1 schedule for 10 days. When the number of reinforcers earned is examined by day (*A*), there are no differences in responding by genotype (RM two-way ANOVA - $F_{(1,15)}$=0.02; *p*=0.90). There is a significant increase in responding across days ($F_{(9,135)}$=3.51; *p*<0.001), but no genotype by day interaction ($F_{(9,135)}$=0.99; *p*=0.45). [N= 5 Wt, 12 Kal7[KO]] *(B)* Plot of the total number of reinforcers earned by each animal as a cumulative distribution plot shows a strong right shift of the Kal7[KO] curve, indicating that a larger segment of the Kal7[KO] population garners a greater number of food rewards over time. Additionally, the large population of low responders excluded from the analysis in *(A)* is evident from the course of the Wt line. These results are significant based on Kolmogorov-Smirnov analysis (p=0.002). [N= 15 Wt, 15 Kal7[KO]]

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Fig. 6. Quantitative PCR. *(A)* RNA prepared from the NAc of cocaine and saline self-administering Wt and Kal7^{KO} mice was subjected to qPCR analyses with data averaged across all sets of experiments and expressed with respect to internal GAPDH values. For ease of viewing, all transcripts are graphed as fold-change from Wt Saline; statistical analyses and expression levels are provided in **Table 1**. Kal7, Kal9 and Kal12 reflect the use of different 3'-untranslated regions; ΔKal and Duet measure transcripts derived from alternate promoters. Cocaine self-administration results in an increase in NAc levels of NR2B transcript in Wt, but not in Kal7^{KO} mice; Vglut transcript levels decline in response to self-administered cocaine in both genotypes. No significant changes with genotype or drug treatment are seen for a number of other transcripts known to be regulated by experimenter-administered cocaine (Eipper-Mains et al., 2013): NR1, NR2A, Gria1, Grik5, Grm5, Drd1a, Drd2, Mef2A, Arhgap32, Ppp1r1b (not shown). Symbols represent Holm-Sidak post-hoc results following two-way ANOVAs tabulated in **Table 1**. *Significant compared to Wt-Sal; #Significant compared to Wt-Coc; Significant compared to KO-Sal. *(B)* Comparison of NAc gene expression in naïve male Wt and Kal7^{KO} mice; data are plotted as fold-change from Wt. *Significant compared to naïve Wt NAc. For both (*A*) and (*B*): one symbol $p<0.05$; two symbols $p<0.01$; three symbols $p<0.001$.

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**Table 1. Two-way ANOVAs for qPCR analysis of transcript levels in self-administering mice.**

The full two-way ANOVA datasets for the mRNA analyses from the NAc of cocaine and saline self-administering Wt and Kal7[KO] mice graphed in **Fig. 6** are presented. Transcript levels normalized to GAPDH are presented for saline-administering Wt and Kal7[KO] mice.

Transcript	Test	F(DFn,DFd)	*p* value	Wt sal/ GAPDH	Kal7[KO] Sal/ GAPDH
Kal7	Genotype	(1,39)=107.7	*<0.0001***	0.0311 $\pm$ 0.0057	0.00026 $\pm$ 0.00007
	Treatment	(1,39)=9.37	*0.004***		
	Interaction	(1,39)=9.30	*0.004***		
Kal9	Genotype	(1,54)=12.00	*0.001***	0.0134 $\pm$ 0.0016	0.0277 $\pm$ 0.0041
	Treatment	(1,54)=4.67	*0.04***		
	Interaction	(1,54)=0.76	0.38		
Kal12	Genotype	(1,41)=46.10	*<0.001***	0.0052 $\pm$ 0.0006	0.0074 $\pm$ 0.0006
	Treatment	(1,41)=43.03	*<0.001***		
	Interaction	(1,41)=16.81	*<0.001***		
NR2B	Genotype	(1,52)=1.12	0.29	0.0179 $\pm$ 0.0062	0.0220 $\pm$ 0.0016
	Treatment	(1,52)=3.06	0.09		
	Interaction	(1,52)=4.42	*<0.04**		
Vglut1	Genotype	(1,46)=2.93	0.09	0.0183 $\pm$ 0.0035	0.0118 $\pm$ 0.0052
	Treatment	(1,46)=12.74	*<0.001***		
	Interaction	(1,46)=0.32	0.57		

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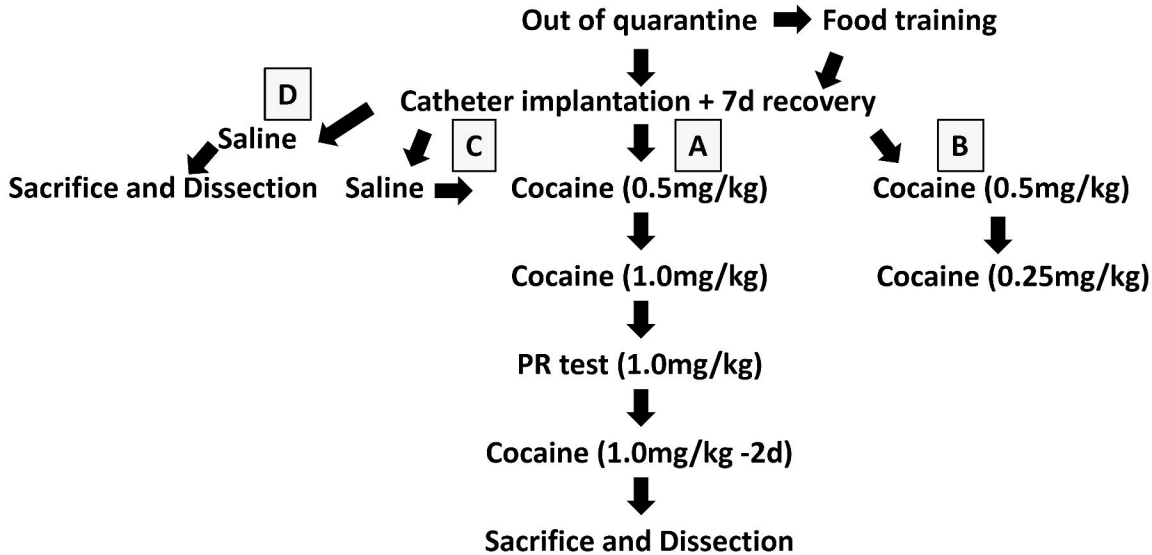
Fig. 1

Fig. 2

Fig. 3

Fig. 4

A)

B)
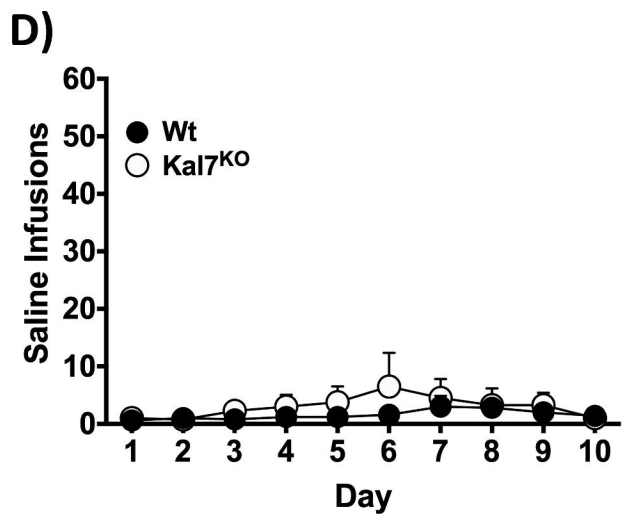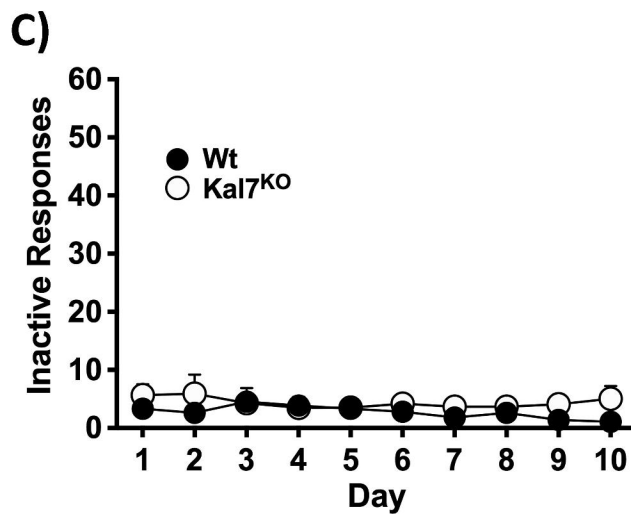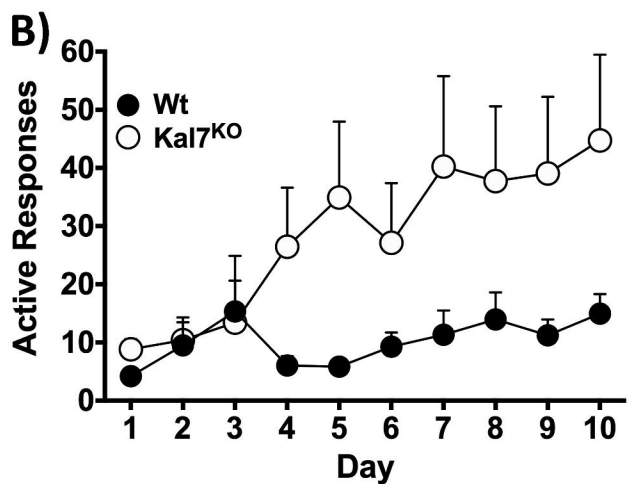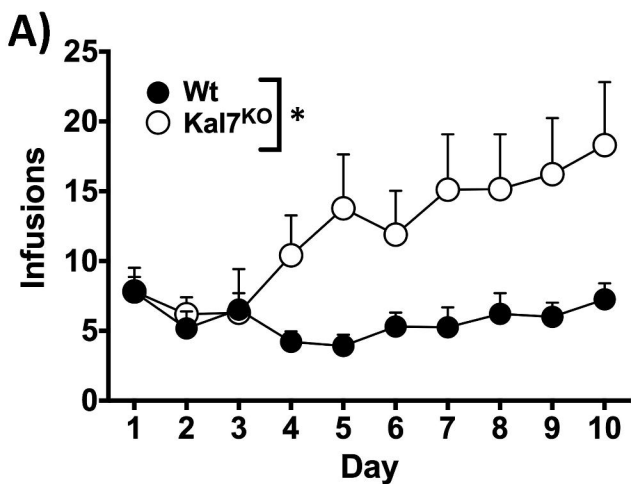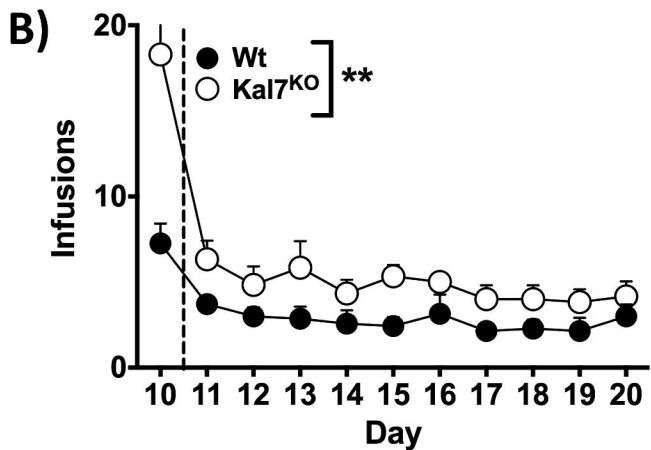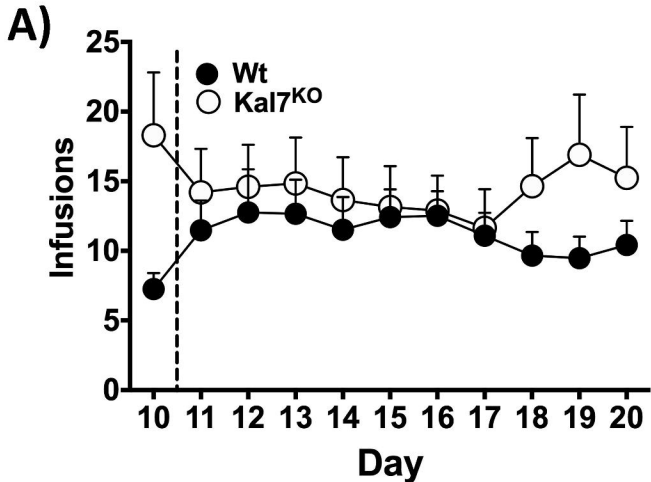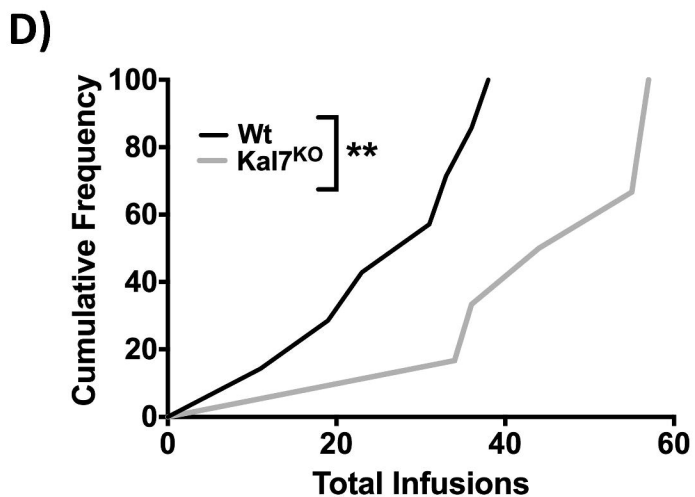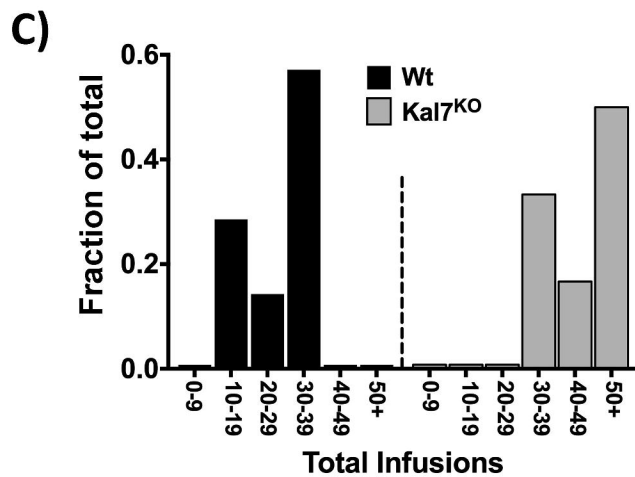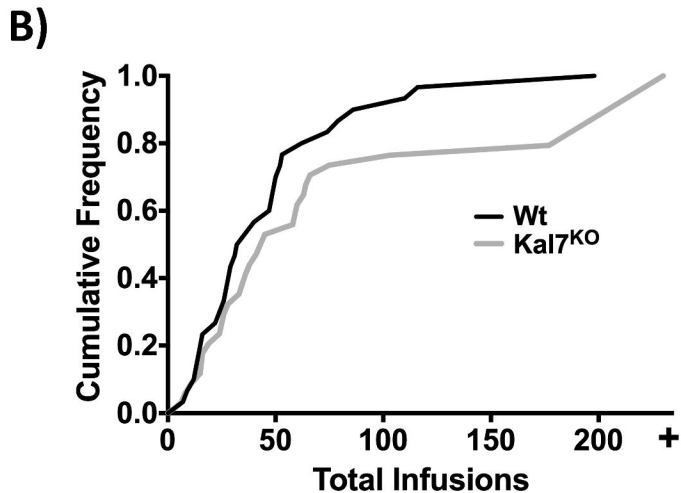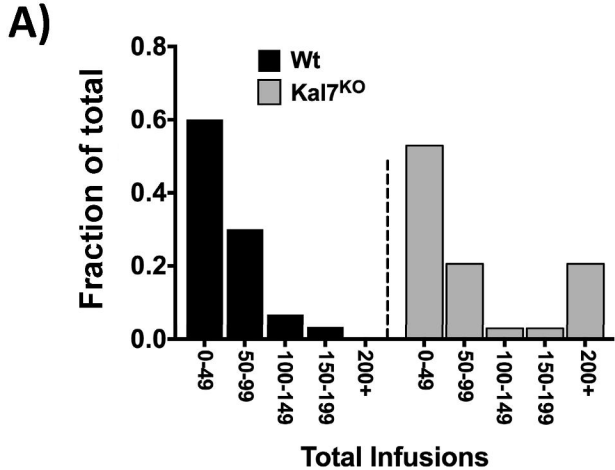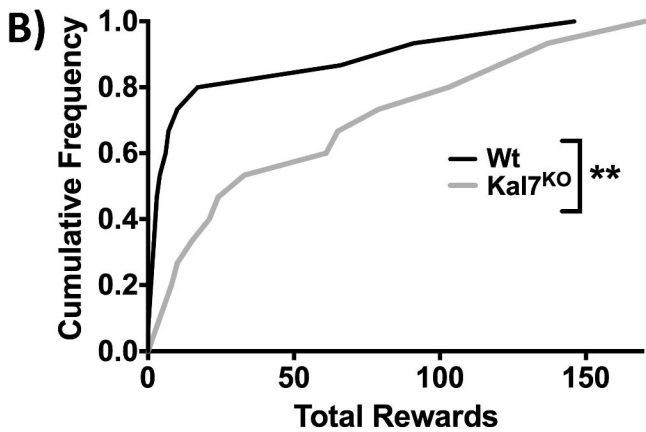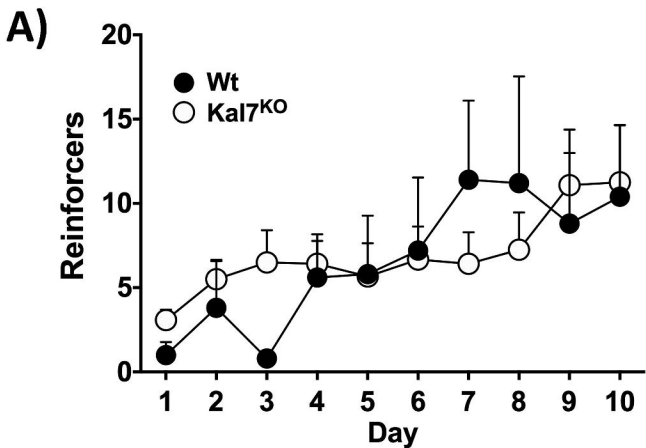
Fig. 5

A. NAc of self-administering mice

Legend: ■ Wt sal ▨ Wt coc ■ Kal7^KO sal ▨ Kal7^KO coc

mRNA levels relative to Wt

Categories: Kal7, Kal9, Kal12, ΔKal, Duet, NR2B, Vglut1

B. NAc of baseline mice

Legend: ■ Wt ■ Kal7^KO

mRNA levels relative to Wt

Categories: Kal7, Kal9, Kal12, NR2B, Vglut1, NR1, NR2A, Gria1, Grik5, Grm5

Fig. 6