CONSTITUTIVE AND CONDITIONAL DELETION OF TYPE 1 DOPAMINE RECEPTOR (Drd1) TO STUDY FOOD ANTICIPATORY ACTIVITY IN MICE

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SIGNATURE PAGE

THESIS: CONSTITUTIVE AND CONDITIONAL DELETION OF TYPE 1 DOPAMINE RECEPTOR (Drd1) TO STUDY FOOD ANTICIPATORY ACTIVITY IN MICE

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ABSTRACT

A structure within the hypothalamus, known as the suprachiasmatic nucleus (SCN), acts as a central clock for the body. It regulates biological rhythms that occur with a near 24-hour period, otherwise known as circadian rhythms. These rhythms, such as sleep, activity, hormone release, and appetite are regulated at the cellular level by a transcriptional-translational feedback loop. The major circadian entraining stimulus is blue wavelengths of light received by a special class of neurons in the retina. Interestingly, circadian rhythms can also be influenced by non-photic stimuli such as feeding. Scavenging for food is a major part of survival for organisms. The activity preceding scheduled mealtimes, or food anticipatory activity (FAA), can be initiated in rodent models when fed a limited amount at the same time daily and is thought to be the output of an as yet unidentified food entrained oscillator. Previous studies, using gene deletion and receptor pharmacology, implicated dopamine type receptor 1 (D1R) signaling in the dorsal striatum as necessary for FAA in mice. To further understand the association of D1R with FAA on a calorie-restricted feeding schedule, we have attempted to create several different cell-type specific conditional deletions of D1R using the Cre-lox system. While the Cre-mediated deletions were being created, we re-tested the initial D1R knockout line and observed only a subtle impairment in FAA compared to our prior results. We also obtained and tested a new D1R KO line created by the Knockout Mouse Project. Interestingly, this line of D1R KO mice had a significant impairment in FAA but overall this impairment was subtle and diminished after 4 weeks of timed, calorie restricted feeding. We were unsuccessful in obtaining conditional deletion of D1R mutants when using a tamoxifen-inducible Cre or a transgenically driven D1R-Cre. Finally, we were successful in
conditionally deleting D1R using a gamma-aminobutyric acid vesicular transporter (vGat)-Cre line. These studies are not yet completed, but preliminary evidence suggests that deletion of D1R by vGat-Cre does not substantially impair FAA. Taken together, our results suggest that the lack of reproducibility of the defect in D1R knockout mice may be due to 1) a spontaneous suppressor mutation, 2) genetic drift of non-isogenic alleles, 3) changes in environmental conditions, or 4) or the fact that D1R is not a major modifier of FAA in mice and that previous results were spurious.
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INTRODUCTION

Circadian Rhythms Require the Suprachiasmatic Nucleus

The daily rising and setting of the sun is one of the most important biological stimulus for all of life. This daily external cue creates an internal rhythm in organisms, most of which are circadian in nature: that is, a biological rhythm with a twenty-four hour cycle that persists even in the absence of the time cue. In mammals particularly, there are photoreceptors in the retina that relay light information to the suprachiasmatic nuclei (SCN), which serves as the body’s main pacemaker for a variety of physiological, psychological, and behavioral processes (Abe et al., 1979; Bass and Takahashi, 2010; Patton and Mistlberger, 2013). Lesion studies, as well as transplant studies (Meyer-Bernstein et al., 1999), have indicated that the SCN is the principal regulator of these processes, controlling the release of many hormones and other chemical messengers in a time-like manner (Stephan and Zucker, 1972; Abe et al., 1979; Edgar et al., 1993). Remarkably, the SCN maintains rhythmic activity even when explanted from a rodent’s brain and maintained in a petri dish (Abe et al., 2002; Yoo et al., 2004).

Circadian Rhythms Are Controlled by a Transcriptional-Translational Feedback Loop

How does the SCN maintain such a robust, light-entrained rhythm? There is a transcriptional-translational feedback loop operating in the SCN that has a period length of approximately twenty-four hours (h), which explains the robust properties of the SCN pacemaker. Several mammalian studies indicate that among circadian core clock proteins, brain and muscle ARNT-like protein (Bmal) and circadian locomotor output cycles kaput (Clock) are pivotal in the regulation of metabolism and behavior cued by light:dark cycles
and are responsible for the further activation of transcription-translation of *Period* (Per) and *Cytochrome* (Cry) family of proteins (Ko and Takahashi, 2006). To begin with, light input initiates Bmal and Clock formation and heterodimerization, launching the transcription-translation of Per and Cry, which will negatively regulate Bmal and Clock over a twenty-four hour oscillation (Gekakis et al., 1998). As the mouse is the most well studied for further understanding of circadian rhythmic regulations, mice with Clock gene mutations have phenotypes with a longer rhythm and even become arrhythmic in constant darkness (Ko and Takahashi, 2006). Studies of Bmal1 gene knockouts (KO) demonstrated that these mice also do not possess twenty-four hour rhythms. Secondly, when accounting for downstream protein mutations, mice with individual mutations in *Per* or *Cry* genes have abnormal rhythms, suggesting that without each other, a circadian rhythm is not maintainable (Ko and Takahashi, 2006). Mice with mutations in *Per* have a period length substantially shorter than 24h, whereas mutations in *Cry* can either cause longer or shorter rhythms depending on the subtype of mutation (Ko and Takahashi, 2006).

An important finding in the last decade was that although the SCN is described as the master clock, other tissues can maintain circadian rhythms in the absence of the SCN, suggesting that peripheral tissues also run the core-clock loop. The laboratory of Ueli Schibler demonstrated that even isolated fibroblasts from human skin have the core period transcription-translation loop running on twenty-four hour cycles (Brown et al., 2005). In a follow up study, researchers constructed tissue-specific rescues of *Period* in *Period* KO mice (Yoo et al., 2004). Interestingly, mice with SCN lesions still maintained circadian rhythms in their peripheral tissues but those rhythms between tissues are desynchronized, showing that the SCN keeps the tissues telling the same time. Per protein is also important
to the maintenance of rhythm in peripheral tissues, such as the liver and cornea, in that Per2 knockins rescue circadian rhythms even with SCN lesions of host animal tissue (Yoo et al., 2004). Altogether, twenty-four hour physiological oscillations synchronized by the SCN are dependent on various protein transcription-translation feedback loops driving the body’s daily rhythms.

What entrains peripheral tissues aside from the SCN? Metabolism is another strong zeitgeber (ZT) and interacts strongly with core circadian clock components. To begin with, Clock is important for its influence on plasma glucose and triglyceride levels, as mice deleted for Clock demonstrate decreased food intake levels during darkness and develop metabolic syndromes such as hyperglycemia, hypoinsulinemia, and have higher susceptibility to development of other diseases, such as sleep disorders and metabolic syndrome, if fed high-fat diets (Turk et al., 2005). Similar observations were made with Bmal KO mice, in addition to defects in locomotor behavior (Rudic et al., 2004). Furthermore, analysis of the gluconeogenesis pathway has many implications of following a diurnal pattern of oscillation occurring over a twenty-four hour period (Ramset et al., 2009). Activation of the major pathway for gluconeogenesis is coordinated via the synthesis of mammalian nicotinamide adenine dinucleotide (NAD+), which is regenerated from the precursor, nicotinamide, through the nicotinamide phosphoribosyltransferase (Nampt) enzyme (Bass and Takahashi, 2010). Fluctuations in Nampt as a result of mutations in the dimerization of Clock and Bmal levels display robust alterations in liver and white adipose tissue (Ramsey et al., 2009). In conclusion, components of the core clock proteins are regulated by many enzymes, and any alterations in the levels of downstream proteins can cause a shift in a variety of core clock and peripheral tissue rhythms (Ramsey
et al., 2009; Bass and Takahashi, 2010). These observations, taken together with other studies on circadian metabolism, suggest the value in understanding the mechanisms and neural circuitry involved with circadian oscillations of feeding and its effect on behavior.

Scheduled Feeding Influences Circadian Rhythms

Food is a strong modifier and is associated with reward, best noted in Pavlovian conditioning. In addition, organisms need to actively seek food sources in order to avoid starvation; this ability to anticipate food is an essential behavior with neural implications necessary for survival. Feeding model organisms, such as mice or rats, at the same time every day creates an increase in locomotor behavior and other physiological changes, such as body temperature which is known as food anticipation (Mistlberger, 1994). While there have been many attempts to study the neural basis of food anticipatory activity (FAA), brain lesions and other genetic manipulations have yielded inconsistent results as to which brain region is responsible for FAA, or if there is even a brain region dedicated solely to the regulation of FAA (Davidson, 2009; Mistlberger et al., 2009a,b; Pendergast et al., 2009; Gunapala et al., 2011; LeSauter et al., 2009). Interestingly, the ability to anticipate food is not governed by the body’s main clock, the SCN (Davidson et al., 2000; Davidson, 2009; Takasu et al., 2012), or even any of its known molecular clock components (Storch and Weitz, 2009; Pendergast et al., 2017). Since there is much difficulty in isolating the neural constituent(s) of FAA, it has been suggested that a particular localized food entrainable oscillator (FEO) is not necessarily attainable through a lesion or even a single deletion study (Davidson, 2009; Mistlberger, 2011).

Most experiments with single core clock proteins, such as Per1, 2, 3 and Bmal1, have normal food anticipatory activity, suggesting circadian entrainment to scheduled
feeding is independent of the known circadian clock (Storch and Weitz, 2009). Follow-up studies with various genetic backgrounds of Per2 KO mice also display food entrainment on restricted food diets and during food deprivation (Pendergast et al., 2017). This further supports that the Per family of genes do not influence the output of FEOs or food anticipation. However, opposing these findings are studies that show a number of Per and Cry double mutants that do not actually exhibit FAA and have desynchronized expectations of mealtimes (Mendoza et al., 2009). Other studies with Cry and Bmal gene deletions do not have activity preceding scheduled meal-times, even under constant dark conditions in which there is no competing environmental cue (Takasu et al., 2012). All in all, there are conflicting findings on what coordinates food anticipation and the primary neural components that dictate FEO(s).

Dopamine and its Interaction with Food Anticipation

Dopamine (DA) is a neuromodulator from a family of catecholamine neurotransmitters associated with reward, motivation, attention, and locomotor behavior (Wise and Rompre, 1989). Dysfunction in dopaminergic neurons can result in numerous abnormalities including psychiatric disorders such as depression, schizophrenia, addictive behaviors, and neurodegenerative diseases such as Parkinson’s Disease (Beaulieu and Gainetdinov, 2011; Beier et al., 2015). DA is synthesized from the amino acid tyrosine, which is derived from another amino acid, phenylalanine, in the liver through the enzyme phenylalanine hydroxylase, and is transported to the brain via active transport (Ayano, 2016). The conversion of tyrosine to dopamine requires the enzyme tyrosine hydroxylase (TH), which produces L-DOPA, a rate-limiting step in the catecholamine biosynthesis process (Ayano, 2016). L-DOPA is immediately converted to DA by the enzyme DOPA

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decarboxylase in the cytoplasm of dopaminergic (DAergic) neurons, at which point it is taken into vesicles by a vesicular monoamine transporter. Once packaged into vesicles, DA can be released into the synaptic cleft in response to action potentials, leading to the fusion of the vesicle containing DA with the plasma membrane (Ayano, 2016).

DAergic neurons integrate several parts of the brain, including the hypothalamus, olfactory bulb, and the striatum, and primarily extend from the ventral tegmental area (A10), the substantia nigra pars compacta (A9), and the retrorubral area (A8) (Beier et al., 2015; Bjorklund and Dunnett, 2007). These neurons co-release either GABA, glutamate, and/or combinations of these two compounds thus allowing distinct firing patterns (Beier et al., 2015). Furthermore, DA neurons activate a class of G-protein coupled receptors (GPCR) with five possible receptors subtypes that are classified into two main types: D1-like and D2-like (Beaulieu and Gainetdinov, 2011). D1-like neurons include type 1 dopamine receptor (D1R) and type 5 dopamine receptor (D15) due to their similarity in activation and biochemistry; whereas D2-like receptors include type 2, 3, and 4 dopamine receptors (D2R, D3R, and D4R) due to their similarities in inhibitory function (Beaulieu and Gainetdinov, 2011). In fact, D1-like receptors do not have introns in their genes, unlike D2-like receptors which include three to five introns (Beaulieu and Gainetdinov, 2011). GPCRs are crucial for understanding much of physiology, as they serve as receptors for many stimuli, such as hormonal, ionic, other neurotransmitters, and photic and non-photic environmental stimuli (Rosenbaum et al., 2009). Typically, a receptor ligand will bind to the GPCR and activate specific heterotrimeric G proteins that will lead to the activation of the downstream protein, adenylyl cyclase, which releases cyclic AMP (cAMP) (Rosenbaum et al., 2009). This in turn activates protein kinases, thereby leading to the
activation of transcription factors that will ultimately lead to genetic modification (Rosenbaum et al., 2009). D1-like receptors function with an increase in cAMP, leading to activation of transcription factors in contrast with D2-like receptors, which are inhibitory and lead to a decrease in cAMP and inactivation of downstream transcription factors (Beaulieu and Gainetdinov, 2011).

As DA is involved with many physiological and psychological processes, it is possible that this neurotransmitter can be under circadian regulation and potentially the SCN’s synchronization. Supporting this idea, dopamine tone in the striatum has proved to be diurnal (Mendoza and Challet, 2014); however, dopamine cell firing is unrelated to the diurnal tone as midbrain DAergic neurons do not display diurnal variation (Ferris et al., 2014). A study with REV-ERBα, a circadian nuclear receptor involved with negative regulation of the feedback-loop targeting Bmal mRNA, implies that REV-ERBα protein impacts midbrain DA production by suppression of TH mRNA levels (Chung et al., 2014). TH levels were found to be highest at night while REV-ERBα levels were lowest, suggesting an inverse relationship exists between the two proteins. In addition, mice with REV-ERBα gene deletion indicate higher DA production and firing rates than controls (Chung et al., 2014). Another finding reports that extracellular levels of DA are controlled by the dopamine transporter protein (DAT or Slc6a3) and are highest during the night while lower during the day, suggesting the activity of DAT also follows a diurnal rhythm (Ferris et al., 2014). Therefore, further understanding of the timing and release of DA by DAergic neurons is essential for the treatment of many disorders related to DA.

Midbrain DAergic neurons respond more to appetitive stimuli rather than aversive stimuli that often lead to addictive behaviors (Mirenowicz and Schultz, 1996). In addition,
anatomical identification of the circuitry of these DA neurons has many implications with learned-addictive behaviors associated with food (Beier et al., 2015; Fields et al., 2007). Daily access to a reward stimulus, such as food in a calorie-restricted (CR) paradigm or even mating, leads to anticipatory behavioral activity prior to the scheduled event, thereby activating reward pathways (Opiol et al., 2017). Several studies indicate that there is direct influence of these pathways stemming from the central brain region for reward, the striatum, in circadian food anticipatory behavior (Gallardo et al., 2014; Smit et al., 2013; Opiol et al., 2017; Skov et al., 2017). In other words, DA circuits in the striatum are critical for processing of reward stimuli and expression of activity (Smit et al., 2013). Therefore, these external stimuli, or zeitgebers (ZT), acting on reward pathways in a circadian fashion could potentially be serving as input signals to clock genes, such as Per2, regulating circadian locomotor activities including FAA (Opiol et al., 2017). At the same time, mice with mutations in Cry, Bmal, and Per2 have been shown to retain FAA rhythms, but are deficient in clock proteins in peripheral tissues (Takasu et al., 2012). Likewise, a study conducted on liver-specific deletion of Per2 showed a reduction of FAA by inhibiting production of a substrate necessary for signaling metabolism in the brain (Chavan et al., 2016). Therefore, exploring clock genes’ effect(s) on FAA has not been completely ruled out and analysis of Per2 or other proteins are still accounted for in many FAA studies (Gallardo et al., 2014; Opiol et al., 2017). Nevertheless, additional studies on the importance of DA in entrainment are necessary to further understand the role of downstream proteins in this circuitry (e.g. Per2).

In a recent experiment exploring the impact of dopaminergic neurons in the regulation of FAA, prenatal and constitutive deletion of D1R attenuated FAA appreciably,
whereas D2R deletion did not (Gallardo et al., 2014). Furthermore, viral reintroduction of dopamine production in the dorsal striatum of dopamine-deficient mice was permissive for FAA, while daily-timed D1R agonist injection caused behavioral entrainment even in mice fed *ad libitum* (AL) (Gallardo et al., 2014). Altogether, these results suggest that FEOs may indeed be embedded in reward pathways of the striatum more closely associated with D1R rather than D2R. However, the specific D1R-DA circuitry of FAA is yet to be fully resolved and further fine-mapping is necessary to understand the mechanisms of this interaction.

**Hypothesis**

As previous work was done with a constitutive, prenatal deletion of D1R resulting in impairment of food anticipation, further identification of role of D1R in promoting FAA is made possible by other genetic and conditional deletion methods. The use of Cre/lox is used to determine if a mutated genome, like KO studies of DA receptors, are essential for producing phenotypes of interest, such as FAA. This technology also allows for region-specific or cell-type specific experiments, promoting investigation of DA in many regions of the brain.

Therefore, our aim in these experiments is to understand the role of D1R by using several different mouse lines with mutations in *Drd1*. To begin with, mice from the experiments of Gallardo *et al.* (2014) were tested for FAA on two diets: formulated and unformulated chow. In addition, another constitutive KO of D1R from the Knockout Mouse Project (KOMP) was also analyzed for FAA, as this mouse line used a different method for knocking out D1R. Last, we attempted three conditional deletion strategies for D1R using the Cre/Lox method: 1) an endogenous promoter deletion for *Drd1* (Tg(Drd1a-
Cre$^{120MXu}$, 2) temporal and post-natal deletion using a tamoxifen-inducible Cre (CAGGCre-ERT$^{TM}$), and 3) a conditional deletion of D1R in vesicular GABA transporter (vGAT) neurons of the striatum ($Slc32a1^{tm2(cre)Lowl}$). We hypothesized, in congruence with past findings of our lab, that mice with mutations for $Drd1$ will have an impairment in FAA over a twenty-eight day CR study.
MATERIALS AND METHODS

Ethics Statement

The experiments described herein were approved by the California State Polytechnic, Pomona Animal Care and Use Committee (ACUC) under protocols: 16.029 and 17.003.

Mouse Husbandry

All mice were maintained on a 12:12 light:dark cycle throughout all experiments unless otherwise indicated. ZT 0 was defined as lights on, while ZT 12 was designated as lights-off, by convention. Mice were housed in static microisolator cages with temperatures ranged between 22-24°C and humidity between 20-45%. Home cages contained sani-chip bedding (Envigo, 7090) and a cotton nestlet. On video recording days, mice home cages only contained a minimum of sani-chip bedding (100ml). Dim red lighting was provided with High Power 42 SMT RED LED PAR38 from LEDwholesalers.com. Only mice in figures 4-5 were fed formulated chow (Research Diets, D12450K), whereas mice in all other experiments were fed unformulated chow mouse chow (2018 Teklad Diet Envigo). The two different chow types had overall similar macronutrient content, except for carbohydrate amounts, in which the formulated chow had about 20% more carbohydrates and used only complex sugars, such as corn starch and maltodextrin, rather than simple sugars like sucrose or glucose.

Mice in figures 13-15 were housed individually and placed on constant light for about 10-12 weeks before measuring for day:night activity. Using HomeCageScan© video
recording software, daily high activity (sum of rearing, walking, jumping, and hanging) was analyzed to determine if usual night-time mouse activity (ZT 12-24) was relatively the same as normal day time activity (ZT 1-11). Mice with a 1:1 ratio of day:night activity were deemed to have broken nocturnal rhythms, and were then placed on a CR study for about 50 days.

Calorie Restriction Conditions

Food intake tests were conducted prior to starting CR with mice at an age of about 9-10 weeks. To calculate designated food amounts that will serve as the 60% CR food allotment per cohort, food intakes were measured per mouse by placing approximately 35-40g of formulated or unformulated chow in food bins, then measuring the remaining chow mass after 48h. These values were then averaged per group and 60% of the daily average was determined as the daily CR value.

All mice entering CR were at least 10 weeks of age. Mice were individually housed on ad libitum (AL) for 5-7 days prior to beginning CR with food intake studies conducted at 2-4 days preceding CR. Mice were then fed 60% of their daily caloric intake every day for 28 days at ZT 6 or 7 (seen in arrow on each graph) on Day 0, or the start of CR. Body weights were measured every 7th day prior to scheduled mealtime, beginning from Day -7 and ending at Day 28. Mice were euthanized via a standardized CO₂ method after CR studies.

Genomic DNA Isolation and Genomic PCR for Genotyping

Mice from the Drd1tm1Jcd, Tg(Drd1a-Cre)120MXu, CAGGCcre-ER™, and Slc32a1tm2(cre)Lowl lines were genotyped using DNA obtained from tail clippings. Digestion
of tails occurred with proteinase K (VWR Biotechnology), stored and prepared at 20°C, then incubated in tail lysis buffer at 55°C in a water bath overnight. DNA extraction occurred via an isopropanol precipitation method and DNA was stored at room temperature in Tris-EDTA buffer (ThermoFisher). To genotype the D1R genomic locus on Drd1\textsuperscript{tm1Jcd} mice, the following primers were used: TCC TGA TTA GCG TAG CAT GGA and GGT GAC GAT CAT AAT GGC TAC. To test for PGK-Neo, the following primers were used: CAC TTG TGT AGC GCC AAG TGC. Mice from lines Tg(Drd1a-Cre)\textsuperscript{120MXu}, CAGGCre-ER\textsuperscript{TM}, and Slc32a1\textsuperscript{tm2(cre)Lowl} required the use of Cre and D1R primers to test for KO alleles. For Cre recombinase, the following primers were used: GTT CGC AAG AAC CTG ATG GAC A and CTA GAG CCT GTT TTG CAC GTT C. Testing for D1R Flox required the use of 3 different primers: 1) CTT CCT CAT TTC AGG GAA TTG CAG GG 2) GCA CAG GGT AAA ACC CTC AGG and 3) CAA ATG ACC TCT GTG TAC CAG and CC. The Drd1\textsuperscript{tm1e(KOMP)Wtsi} mouse DNA was genotyped through a third party using qPCR methods (Transnetyx).

Genomic DNA of Drd1\textsuperscript{tm1Jcd} and Drd1\textsuperscript{tm1e(KOMP)Wtsi} mice was assessed using single polymorphic nucleotides (SNPs) for differences between genetic background comparable to C57BL/6J and C57BL/6N WT mice (Table III).

\textit{Breeding Dopamine Receptor 1 Knockout Mice}

Drd1\textsuperscript{tm1e(KOMP)Wtsi} mice were obtained from the KOMP repository (Skarnes et al., 2011), whereas Cre-driver mice were all obtained from the Jackson Laboratory (Bar Harbor, ME) (Table I and II). Drd1\textsuperscript{tm1Jcd}/J heterozygote (+/-) mice were bred for 3-5
generations before being tested for FAA, with only WT/WT (+/+) and KO/KO (-/-) mice used for CR experiments.

Tg(Drd1a-Cre)$^{120MXu}$ and Slc32a1$^{tm2(cre)Lowl}$ KO for D1R were all created in-house through a D1R-floxed LoxP transgenic mouse cross with the mouse strain Drd1$^{tm2.1Sd/J}$; these mice were also obtained from Jackson Labs (Stock#025700). These D1R-floxed transgenic mice were crossed to each line of Cre-driver mice for 2-3 generations before experimental use. For CR experiments with Drd1$^{tm1et(KOMP)Wtsi}$ and Slc32a1$^{tm2(cre)Lowl}$ mice, both WT/WT and WT/KO mice were used as controls, whereas KO/KO mice were used as experimentals. In all other experiments, WT/WT were used as controls while KO/KO mice were used as experimentals.

For post-natal deletion of D1R, the Cre-driver CAGGGCre-ER$^{TM}$ was crossed with D1R-floxed transgenic mice to create D1R;CAGGGCre-ER$^{TM}$. F2 mice with floxD1R/floxD1R; D1R-Cre$^{+}$ and floxD1R/floxD1R; D1R-Cre$^{-}$ genotypes were then housed individually for CR experiments. Tamoxifen (Sigma, T5648) was first dissolved in 10% ethanol and sesame oil (Sigma, S3547) at 10 mg/mL. Solutions were prepared the night before then stored at 4°C overnight. After which, mice were injected intraperitoneally at 10-11 weeks of age with a dose of 10 mg/kg for 5 consecutive days. D1R;CAGGGCre-ER$^{TM}$ were then placed on CR approximately 10-14 days after the last day of tamoxifen administration.

**Testing Dopamine Receptor 1 Knockout Mice**

In-home cage behavior measurements were obtained by video recording mice from a perpendicular angle to their home cages and analyzing these videos using an automated behavior recognition software, HomeCageScan 3.0 (Steele et al., 2007; Hsu et al., 2010)
which annotates for the following behaviors: remain low, pause, twitch, awaken, distance traveled, turn, sniff, groom, food bin entry, chew, drink, stretch, hanging, jumping, rearing, walking, and unassigned behaviors. This data was output by HomeCageScan into twenty-four one-hour bins to quantify the temporal structure of activity. The behaviors hanging, jumping, rearing, and walking are designated as high activity. Total high activity was then determined by summation of high activity bins, while FAA ratios were calculated by dividing the final 3h of high activity over total high activity of each mouse. For in-home cage behavior measurements, mouse behavior was recorded on Day -7 at about 9-10 weeks of age.

For immunochemistry, brains were immersion-fixed in 10% buffered formalin (Sigma) or were perfusion-fixed using 4% paraformaldehyde (Sigma) for at least 24h before being sectioned at room temperature using a vibratome (Leica Instruments). Antibody staining was performed using a rat monoclonal D1R antibody 1:250 (Sigma) and a chicken polyclonal tyrosine hydroxylase (TH) 1:500 (Aves). An Alexa Fluor 647-conjugated AffiniPure Goat Anti-Chicken IgY 1:500 and an AffiniPure Goat Anti-Rat 488 1:500 were used as fluorotags, along with DAPI (4’, 6-Diamidino-2-Phenylindole, Dihydrochloride) 1:1000 for nuclear staining. For immunohistochemistry staining, a Vector ABC immunoperoxidase staining kit (Vector Labs) was utilized. Brain imaging was conducted on a Nikon Eclipse TE2000-U light microscope linked to a computer with NIS-Elements BR 3.0 software or a Nikon Eclipse Ti-E inverted microscope system linked to a computer system with NIS-Elements Imaging Software.
Statistical Analysis

Statistical tests for behavior were calculated using GraphPad Instat or Prism. Food intakes, which fell on a normal distribution, were tested for significance using an Unpaired T Test. Body weights, which also fell on a normal distribution, were analyzed for significance using a One-Way ANOVA with Tukey’s post-test. On the other hand, home-cage behavior data was not normally distributed, therefore we used the nonparametric test, Mann-Whitney. To analyze a contingency table for mouse genotypes, a Chi-Square Test was performed to determine the dependency of genotype to viable offspring (Table IV). P values on all figures are defined as follows: *≤0.05, **≤0.01, ***≤0.001.
RESULTS

*Drd1tm1Jcd* Knockouts Show Food Anticipatory Activity on a Standard Chow Diet

To study the role of D1R on FAA in response to a scheduled CR, we tested *Drd1tm1Jcd* KO mice from a previous study within our lab that initially did not display FAA (Gallardo et al., 2014). To begin with, we verified D1R deletion using fluorescent immunostaining techniques by obtaining brains from adult mice. TH-antibody staining was used to mark the striatum, which is innervated by DA neurons (Wall et al., 2013), and is visible in both WT and KO forebrain tissue (Fig 1A & D). D1R staining is abundant in the striatum, consistent with prior findings (Araki et al., 2007) (Fig 1B). However, D1R KO mice do not appear to have any D1R staining in this region, as depicted by the lack of green fluorescence, confirming that the *Drd1tm1Jcd* KO mice are missing detectable levels of D1R protein (Fig 1E). An overlap of both TH- and D1R-staining further confirms that *Drd1tm1Jcd* KO mice do not have D1R protein in their striatum (Fig 1C & F).

*Drd1tm1Jcd* mice were then individually housed and measured for daily food intakes while on an AL diet. Mice fed unformulated chow had mean food intakes with no major differences between KO and WT groups at 3.6g for WT (+/-0.84g) and 3.0g for KO mice (+/-0.73g) (Fig 2A). Body weights measured weekly indicate that WT mice are greater than KO mice on all days of the study, before and after CR (P<0.0001 on all days, Unpaired T test) (Fig 2B). However, fraction of initial weight for both KO and WT groups exhibit virtually superimposable trend lines, indicating mean losses were more or less equal on all days (Fig 2C). Consistent with the D1R KO phenotype, KO mice on unformulated chow displayed generally more hyperactivity than their WT counterparts (Fig 2D). Interestingly, on Day 0 and 7, *Drd1tm1Jcd* KO mice were substantially more active than WT groups.
(P=0.0055 Day 0 and 0.0013 Day 7, Two-Tailed T test). Total high activity, or the sum of walking, cuddling, hanging, and jumping, appear similar between groups (Fig 2D). When accounting for the total high activity in the 3h preceding scheduled meal-times both on and before CR, KO and WT mice indicate no major differences. Lastly, to check for FAA in these mice, we then divided the total high activity in the 3h preceding scheduled-mealtime by the total high activity per mouse on all days of the study as fraction of high activity, where both groups did not display any differences in FAA on all days (Fig 2F).

Drd1\textsuperscript{tm1Jcd} mice on unformulated chow clearly displayed activity prior to scheduled meal-times. These mice are also clearly hyperactive before CR (Fig 3A-B) and throughout the night during ZT hours 12-24 (Fig 3). Peak nighttime activity remained higher for Drd1\textsuperscript{tm1Jcd} KO mice on most days of CR but did not exceed 15% of activity on all days (Fig 3). By Days 21 and 28, KO mice were able to reach approximately equal peak levels to nighttime by ZT 5 in fraction high activity (Fig 3H and J).

\textit{Drd1\textsuperscript{tm1Jcd} Knockouts Show Food Anticipatory Activity on a Formulated Chow Diet}

Since we obtained an unexpected result in our study using standard, unformulated chow diet, we examined diet variables that might have caused Drd1\textsuperscript{tm1Jcd} mice to show FAA. Since diet has potential effects on activity and metabolism (Di Giorgio et al., 1962), and we used a standard, unformulated chow, we sought to test an alternative food source that does not contain contaminants, such as phytoestrogen, that may have an effect on behavior. In an unpublished study from our laboratory, a formulated chow diet led to a slightly high amplitude of FAA in C57BL/6J male mice in comparison to unformulated chow (unpublished data). Overall, the formulated and unformulated chow have similar macronutrient percentages for protein and fat, but formulated chow had about 70% of kcal

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derived from carbohydrates whereas unformulated chow contains about 44% of carbohydrates only. The formulated chow was also distinct in that it was comprised primarily of complex sugars, such as the two starch types: corn starch and maltodextrin, whereas unformulated used a variety of carbohydrates, not specific to simple or complex.

We then tested the Drd1
tm1Jcd mice on formulated chow, individually housing mice aged 9-11 weeks and measuring their food intake levels on AL. The mean daily food intake of both WT and KO groups on formulated chow did not differ between groups averaging 3.7g for WT (+/- 0.81g) and 3.0g for KO (+/-1.5g) (Fig 4A). Mice were administered 60% of their daily average food intake when placed on CR beginning Day 0. Weekly body weight measurements were congruent with our study above using the unformulated chow diet: Drd1
tm1Jcd KO also had significantly lower body weights prior to starting CR (P<0.0001 Day -7, Unpaired T test) and on all days of CR (P=0.00021 on Day 0, P=0.0001 on Day 7, P=0.0005 on Day 14, P=0.0013 on Day 21, and P=0.0023 on Day 28, Unpaired T test) (Fig 4B). There was no difference in normalized weight loss (each value is divided by Day 0 weight) throughout the study (Fig 4C).

Mouse behavior was monitored weekly throughout experiments via video recordings in which individually housed mice were recorded every 7 days for 24h at a time. On Day -7, Drd1
tm1Jcd KO mice had substantially greater total high activity when compared to their WT counterparts (P=0.0433, Two-Tailed T test) and on the first day of CR (P=0.0111, Two-Tailed T test; Fig 4D). This can be expected as D1R gene deletion causes mice to be hyperactive. However, all days following Day 0 indicate no major differences of total high activity between KO and WT groups fed on a formulated diet. To analyze the activity preceding scheduled meal-times, we summed high activity in the final 3h of video
recording (Fig 4E) and normalized these values in respects to total high activity (Fig 4F). The sum high activity preceding scheduled mealtime are similar between means of activity in WT and KO groups except on Day 21, in which Drd1^tm1Jcd WT mice were much more active than KO mice (P=0.0021; Two-Tailed T test) (Fig 4E). In general, WT mice exhibited a trend towards greater food anticipation than KO groups on all days except Day -7 and Day 0 (Fig 4F). However, no substantial differences in the means of activity between these groups existed on these days.

When analyzing daily activity of mice during the 24h period of recording indicate that the Drd1^tm1Jcd KO achieve respectable FAA levels by Day 28 (Fig 5A). Mice typically started off with greater nocturnal activity, seen in Day -7 for both sum high activity and fraction of high activity (Fig 5A-B). However, by Day 28 nocturnal activity is diminished and mice are unable to achieve 10% of activity (Fig 5C-J). Allocating enough energy for FAA typically results in a loss of nocturnal activity in mice, and this is apparent in Drd1^tm1Jcd KO on formulated chow (Fig 5).

An Additional D1R Knockout (Drd1^tm1e(KOMP)Wtsi) has a Modest Impairment in FAA

We next employed a second strain of mice, Drd1^tm1e(KOMP)Wtsi from the KOMP, as another D1R deletion mechanism to study the effects of D1R on FAA. Testing Drd1^tm1e(KOMP)Wtsi mice, a different prenatal KO of D1R, allows for careful analysis of the interaction between D1R and food anticipation. D1R deletion in this line varied in that incorporated an IRES: lacZ trapping cassette and a floxed promoter-driven neo cassette which disrupts gene function when inserted into an intron of the gene of interest (Skarnes et al., 2011). This innovative technique allows for conditional deletion of D1R using Cre
and/or Flp restriction enzymes. However, for our experiments we only used mice with global, D1R gene deletions to study if gene manipulation techniques have any effect on FAA on mice.

Confirmation of D1R deletion in the brains of Drd1\textsuperscript{tm1e(KOMP)Wtsi} WT and KO mice was also completed through mouse brain extraction and staining for D1R-antibodies. TH staining is apparent on both WT and KO forebrain tissue, seen in the striatum and hypothalamus areas of the brain (Fig 6A and D), whereas D1R does not appear to stain in KO mouse forebrain tissue (Fig 6E). In contrast, Drd1\textsuperscript{tm1e(KOMP)Wtsi} WT appear to show D1R in the striatum and other areas within the forebrain (Fig 6B). An overlap image for both TH and D1R markers WT and KO can be seen in Fig 6C and F.

Food intake measurements were conducted on mice during AL. Average daily food intake of Drd1\textsuperscript{tm1e(KOMP)Wtsi} WT mice was 3.1g (+/- 0.9g), while KO mice ate on average of 3.0g (+/- 0.42g), with no overall statistical differences between means of both groups (Fig 7A). Both groups of mice experienced a similar trend of weight loss in grams, with KO mice starting at a lower average body weight than WT (Fig 7B). Between Day 0 and 7, mice dropped nearly 20% of body weight, but stabilized throughout the rest of the study. Normalizing body weights with respect to Day 0 indicate no major differences between groups on all days (Fig 7C).

While Drd1\textsuperscript{tm1e(KOMP)Wtsi} KO mice were generally hyperactive, congruent with Drd1\textsuperscript{tm1Jcd} studies using both chow types, there were no major differences for total high activity between WT and KO groups during CR, except on Day 28, where Drd1\textsuperscript{tm1e(KOMP)Wtsi} KO mice showed significantly less mean activity than WT (P=0.0053, Mann-Whitney) (Fig 7D). Summation of high activity preceding scheduled mealtime
indicate that KO mice also experienced significantly less high activity on Days 21 and 28 (P=0.0035 and P=0.0053 respectively, Mann-Whitney) but were not of statistical value on all other days (Fig 7E). Next, Drd1tm1e(KOMP)Wtsi KO mice appear to be unable to reach WT FAA levels on Days 7-21 of CR (Fig 7F). WT mice had much higher fraction of high activity on Day 7, 14, and 21, with all reporting statistical significance (P=0.0167 on Day 7, P=0.0183 on Day 14, and P=0.0035 on Day 21, Mann-Whitney), indicating that KO mice are surely unable to exhibit comparable FAA.

Plotting bin behavior for each 24h video recording shows slight impairment of fraction of high activity on Days 7 and 21, during ZT hours 3-5 (Fig 8D and 8H), but no other obvious impairments of fraction of high activity can be seen on other days (Fig 8). KO mice have a trend towards greater nocturnal activity, seen in ZT hours 12-24 (Fig 8A-J). Taken together, these results indicate that Drd1tm1e(KOMP)Wtsi KO mice have some impairment of FAA during CR.

*Conditional Deletion of D1R using Drd1-Cre May Be Embryonically Lethal*

Next, the broad Tg(Drd1a-Cre)120MXu Cre-driver, which uses the endogenous Drd1 promoter, was crossed to floxed Drd1 mice in attempt to achieve deletion of D1R with the most exact spatial and temporal precision as possible. This cross did not produce viable progeny with the genotype: floxD1R/floxD1R; D1R-Cre++; of 47 progeny examined, 0 had this genotype (Table IV). To determine whether the lack of floxD1R/floxD1R; D1R-Cre++ was statistically significant, we performed a Chi-Square test which resulted in a P value of statistical significance (P<0.0001, Chi-Square). The lack of floxD1R/floxD1R; D1R-Cre++ progeny suggests embryonic lethality of this deletion; however, we would need to test the genotypic ratios in early stage embryos in order to confirm this. It could also be possible
that the transgene is genetically linked with the Drd1a locus and therefore, does not recombine during meiosis. Since the insertion site for the D1R-Cre transgene is unknown, we cannot rule out the possibility that genetic linkage is causing the lack of floxD1R/floxD1R; D1R-Cre+ progeny.

Tamoxifen-Inducible Cre Does Not Post-Nataly Delete D1R in Mice Aged 10 Weeks

Since prenatal deletion of D1R using the endogenous promoter for Drd1 did not yield viable progeny for experiments, a post-natal deletion using a tamoxifen-inducible Cre-driver was employed. Both control and conditional knockout (cKO) mice were injected at 10 weeks of age, with a subset utilized for D1R labeling. Both CAGGCre-ERT™ WT and cKO mouse brains exhibited abundant amounts of the D1R protein in expected parts of the brain, including the striatum (Fig 9A and B). Therefore, D1R importance in the development and sustenance of FAA cannot be determined using this Cre-driver line.

Conditional Deletion of D1R with a Vesicular GABA Transporter Cre-Driver to Study FAA

Given our failure to conditionally delete D1R using either a D1R-Cre line or a post-natal tamoxifen-inducible strategy, we sought to delete D1R using a different Cre-expressing line. To that end, we obtained a well-characterized line of mice that expresses Cre in all GABA neurons, with Cre inserted downstream of the vesicular GABA transporter gene (Vong et al., 2011). We chose this line because previously we demonstrated that virally rescued DA-deficient mice with restoration of dopamine solely in the dorsolateral striatum displayed normal levels of FAA (Gallardo et al., 2014). The only known D1R-expressing neurons in the dorsolateral striatum are GABAergic medium-
sized spiny neurons (Gangarossa et al., 2013). Therefore, D1R should be deleted throughout the striatum in floxD1R/floxD1R; Slc32a1<sup>tm2(cre)Lowl</sup> (vGat-Cre) mice.

We verified deletion of D1R protein using antibody labeling using the same conditions as described for the lines above. D1R staining can be seen in control forebrain tissue (Fig 10B), with overlap of TH-antibody staining in Fig 10C. D1R protein is absent in vGat-Cre cKO forebrain tissue (Fig 10E), while overlap of TH- and D1R-antibody staining visible in Fig 10F.

We measured daily 24h food intake levels for control and cKO mice, and found no difference in mean food intakes at an average of 4.2g (+/-1.1g) for both groups (Fig 11A). Since this is a preliminary study, we were not able to conduct statistical tests for behavior due to small sample sizes on Days 21 and 28. Weekly recorded body weights in grams indicate that cKO body weights are much lower than their control counterparts (Day -7 P=0.0001; Day 0 P=0.0057; Day 7 P=0.002; Day 14 P=0.0015; Day 21 P=0.001; and Day 28 P=0.0109, Unpaired T test; Fig 11B). However, when normalized, trend lines are almost superimposable and exhibit no differences (Fig 11C). Next, analyzing high activity behaviors for both groups display similar means of total high activity and 3h pre-meal high activity on all days (Fig 11E), except Day 7 of total high activity (P=0.0031, Mann-Whitney), in which cKO mice were much more active than controls (Fig 11D). cKO mice exhibit lower fraction of high activity on Day 0 (P=0.031, Mann-Whitney) and Day 14 of CR (P=0.004, Mann-Whitney; Fig 11F). These preliminary results suggest a slight impairment of FAA exists using this Cre-driver, however, more mice are needed on Days 21-28 to determine if D1R deletion in GABA neurons surely interacts with FAA.
When analyzing hourly behavior bins, cKO mice appear more active during the nocturnal period on most days of CR (Fig 12C, 12E, 12G, 12I). However, normalized behavior bins indicate mostly similar high activities in both groups of mice throughout a 24h recording period (Fig 12). By Day 28, cKO mice have a trend towards lesser activity in ZT 3-6, prior to their scheduled mealtimes (Fig 12J).

To test if D1R cKO mice are still entrainable to scheduled mealtimes without a competing zeitgeber, we took a set of vGat-Cre cKO (n=6) and control (n=10; both Cre and Flox allele controls were used such that genotypes for controls were either Flox/Flox; Cre−, WT/Flox;Cre+ or WT/Flox; Cre−) mice, and placed them on 24h constant light conditions for 12 weeks to break circadian rhythms cued by light. During this time, mice were fed food and water AL, and housed individually. To test for arhythmicity, we measured activity over a 2-4 day period after 12 weeks of constant light conditions and compared behavioral output during intended day and night hours (Fig 13). ZT hours 1-12 were deemed day activities, while 13-24 were deemed night activities, as these were the initial day:night hours for all other experiments. While vGat-Cre cKO mice exhibited greater total seconds of day activity (P=0.0160, Mann-Whitney) and total seconds of nighttime activity (P=0.0312, Mann-Whitney; Fig 13A and 13B) on average, a comparative analysis of normalized night and day behaviors indicate relatively similar fraction of high activity (Fig 13C and 13D). In addition, we analyzed behaviors of individual mice (data not shown) and removed any mouse that did not exhibit a 1:1 ratio of night and day activity from proceeding with the CR study.

We then began daily, timed 60% CR feeding (as previously described) at what would have been ZT 7 in their former LD cycle. Food intake measurements indicate that
Slc32a1 tm2(cre) Lowl cKO mice eat relatively similar amounts to controls at 4.6g (+/-1.4g) and 4g (+/- 0.78g) for controls (Fig 14A). Slc32a1 tm2(cre) Lowl cKO mice initially began at a lower body weight than controls: mean weights were statistically significant at Day 7, 14, 21, and 28 of CR (Day 7, P=0.0061; Day 14, P=0.0041; Day 21, P=0.003; and at Day 28, P=0.0075, Unpaired T test; Fig 14B). However, when normalizing body weights, both groups indicate comparable fraction of initial weights (Fig 14C).

Rather than test home cage behavior weekly, we waited until mice were on timed CR feeding for 5 weeks then measured behavior on 4-5 consecutive days. Interestingly, vGat-Cre cKO mice were more active than controls when accounting for mean total high activity (P=0.0127, Mann-Whitney; Fig 14D). However, these differences were diminished when summing the 3h pre-meal activity in seconds and normalizing this behavior (Fig 14E and 14F). vGAT-Cre cKO mice exhibited more than 20% of FAA prior to scheduled meal-times (Fig 14F). Furthermore, analysis of high activity hourly bin behavior over a 4-day recording period revealed that vGat-Cre cKO mice are relatively similar to controls (Fig 15A and 15B). Taken together, vGat-Cre cKO mice on 12:12 L:D conditions appear to have blunted FAA although the study needs a greater sample size, while cKO mice in constant light conditions appear to anticipate food adequately (Fig 11J and 15B).
Table I: Mouse strains used in this study. A list of the various D1R-flanked mouse strains along with their corresponding references used in this study. Drd1\textsuperscript{tm1Jcd/J} is referred as Drd1\textsuperscript{tm1Jcd} throughout this paper.

<table>
<thead>
<tr>
<th>Strain</th>
<th>D1R allele</th>
<th>Area of expression/deletion</th>
<th>Reference</th>
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<td>Constitutive, Null Allele</td>
<td>Global</td>
<td>Drago et al., 1994</td>
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<td>Drd1\textsuperscript{tm1e(KOMP)Wtsi}</td>
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<td>Skarnes et al., 2011</td>
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<td>Drd1\textsuperscript{tm2.1Stl/J}</td>
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Table II. Cre-Driver mouse strains used in this study. List of Cre-drivers and their corresponding references. Slc32a1<sup>tm2(cre)Lowl</sup> is also referred to as vGat-Cre throughout this paper.

<table>
<thead>
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<th>Strain</th>
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<td>D1R;CAGGCre-ER&lt;sup&gt;TM&lt;/sup&gt;</td>
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<td>Hayashi and McMahon, 2002</td>
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<td>Slc32a1&lt;sup&gt;tm2(cre)Lowl&lt;/sup&gt;</td>
<td>Conditional</td>
<td>vesicular GABA transporter-expressing cells</td>
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Table II: Single nucleotide polymorphism in genomic DNA from Drd1\textsuperscript{tm1Jcd} and Drd1\textsuperscript{tm1e(KOMP)Wtsi} mouse line to assess purity of genetic background. Drd1\textsuperscript{tm1Jcd} mice (n=3) were tested at 144 positions across the genome for the presence of C57BL/6J or 129S1/SvImJ-FVB/NJ alleles. Drd1\textsuperscript{tm1e(KOMP)Wtsi} line (n=4) was tested for the presence C57BL/6J or C57BL/6N strain at 144 positions across the genome. Percentages were in respects to SEM values.

<table>
<thead>
<tr>
<th>Mouse Strain</th>
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<th>Relative to C57BL/6N</th>
<th>Relative to 129S1/SvImJ-FVB/NJ</th>
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<td>83.34%</td>
<td>16.66%</td>
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Table IV: Observed and expected genotypes for the Tg(Drd1a-Cre)120MXu mouse line. Four possible genotypes were expected as a result of crossing WTD1R/floxD1R; D1R-Cre+ and floxD1R/floxD1R; D1R-Cre– mice. Of 47 offspring observed, an expected offspring of 11.75 for each genotype did not match observed, yielding a Chi-square statistic of 24.064 (P<0.0001, Chi-Square).

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<th>F2 Possible Genotypes</th>
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<tr>
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<td>9</td>
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<td>floxD1R/floxD1R; D1R-Cre +</td>
<td>11.75</td>
<td>0</td>
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Fig 1: Confocal image of immunofluorescence antibody staining of TH and D1R in forebrain tissue of Drd1<sup>tm1Jcd</sup> WT and KO mice on both chow types. A) TH (pink-orange color) staining is restricted to striatum, olfactory tubercle, and hypothalamus regions of WT mouse brain, B) D1R antibody staining (green) in striatum, and C) an overlap image of TH and D1R antibody staining. An overlap of images is shown in Panel F.
Figure 2: Food intake, body weight, and behavior analyses for Drd1<sup>tm1Jcd</sup> WT (black) and KO (teal) mice on unformulated chow. A) Mice have similar 24h food intake means (3.7g +/- 0.84g WT, n=10 and 3.0g +/-0.73g KO, n=16). B) Body weights of mice were recorded throughout studies, with fraction of initial weight seen in Panel C. D) Total high activity in seconds, E) 3h pre-meal activity (sec), and F) fraction of high activity of weekly recordings. Food intake values were analyzed using Unpaired T test and body weights employed One-Way ANOVA with Tukey’s Post-test, while behavior was analyzed using Mann-Whitney nonparametric statistical test. WT sample size is 9 on Day -7, 0, 7, and 28 with n=10 on Day 14 and 11 on Day 21. KO sample size n=16 Day -7; Day 0 and 14 n=14; Day 7 n=12; Day 14 n=8; Day 28 n=11. Asterisks were assigned to P values as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
Figure 3: Summed high activity in seconds and normalized for Drd1<sup>tm1Jcd</sup> WT (black) and KO (teal) mice fed unformulated chow. CR began Day 0 (not displayed). KO mice appear to have relatively higher nocturnal activity than WT but display overall less activity in the 3h preceding scheduled mealtimes. A) sum of high activity (sec) and B) normalized high activity on Day -7 prior to CR. C) Day 7, E) Day 14, G) Day 21, and I) Day 28 depict summed high activity in seconds. D) Day 7, F) Day 14, H) Day 21, and J) Day 28 display normalized high activity levels for both groups of mice. WT sample size is 9 on Day -7, 0, 7, and 28 with n=10 on Day 14 and 11 on Day 21. KO sample size n=16 Day -7; Day 0 and 14 n=14; Day 7 n=12; Day 14 n=8; Day 28 n=11.
Figure 4: Food intake, body weight, and behavior analyses for Drd1<sup>tm1Jcd</sup> WT (black) and KO (teal) mice on formulated chow. A) 24h food intake means for WT is 3.6g (+/- 0.84g) and 3.0g (+/- 0.73g) for KO. B) Body weights of WT and KO mice in grams, while C) fraction of initial weight. Day -7 represents AL diet while CR began Day 0. D) Mean total seconds of high activity of mice, and E) 3h pre-meal sum high activity. Normalized behavior is seen in Panel F. Food intake was analyzed using Unpaired T test and One-Way ANOVA with Tukey’s Post-test for body weights, while behavior was analyzed using Mann-Whitney nonparametric statistical test. n=10 for WT and KO on all days except Day 0: WT=8, KO=9 and Day 28, WT=10 and KO=9. Asterisks were assigned to P values as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
Figure 5: Summed high activity in hourly bins displayed as seconds of activity (left) and normalized by dividing by total activity (right) for Drd1tm1Jcd WT (black) and KO (teal) mice on formulated chow. CR began Day 0 (not displayed). Mean seconds of high activity (+/- SEM) behaviors for WT and KO A) Day -7, C) Day 7, E) Day 14, G) Day 21, and I) Day 28 throughout CR experiments. Normalized activity is seen in B) Day -7 on AL, D) Day 7, F) Day 14, H) Day 21, and J) Day 28 of CR. Behavior was tested for significance using Mann-Whitney test. n=10 for WT and KO on Day -7, 7, 14, and 21. On Day 28, WT=10 and KO=9.
Figure 6: Confocal image of immunofluorescence staining of WT and KO Drd1<sup>tm1e(KOMP)Wtsi</sup> mouse forebrain tissue using antibodies for D1R and TH. A) TH staining (pink-orange color) primarily in striatum region and lower hypothalamus area, B) D1R (green color) stain in striatum, and C) overlap image of D1R and TH antibody staining of a WT mouse brain. Panel D) depicts a TH stain in a KO mouse brain, while E) is a D1R stain within a KO mouse brain. Panel F is an overlap of TH and D1R images.
Figure 7: Food intake, body weight, total high activity, and FAA analyses for Drd1tm1e(KOMP)Wtsi WT (black) and KO (red) mice. A) WT mice have an average food intake of 3.1g (+/- 0.9g), while KO mice averaged 3.0g (+/- 0.42g). B) Body weights in grams and C) fraction of initial weight throughout CR. AL is indicated by Day -7 and CR begins on Day 0. D) Total seconds of high activity, E) mean total seconds in the 3h preceding scheduled mealtimes, and F) normalized food anticipatory behavior. Days -7, 14, 21, and 28 have n=17 for WT and n=9 for KO, on Day 7 n=14 WT and n=6 KO. Food intake was analyzed using Unpaired T test and body weights utilized a One-Way ANOVA with Tukey’s Post-test, while behavior was analyzed using Mann-Whitney nonparametric statistical test. Asterisks were assigned to P values as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
Figure 8: Summed high activity behaviors in seconds and normalized for Drd1\textsuperscript{tm1e(KOMP)Wtsi} WT (black) and KO (red) mice. CR began Day 0 (not displayed). (A) Mean (+/- SEM) seconds of high activity behaviors in the home cage environment prior to CR (A) Day -7, (B) normalized data from A, (C) Day 7, (D) Day 14, (E) Day 21, and (I) Day 28 of CR. Days -7, 14, 21, and 28 n=17 for WT and n=9 for KO; Day 0 n=16 WT and n=7 KO; Day 7 n=14 WT and n=6 KO.
Figure 9: D1R antibody labelling (DAB-Peroxidase). (A) WT floxD1R/floxD1R; CAGGCre-ER<sup>TM</sup> and (B) cKO CAGGCre-ER<sup>TM</sup> forebrain tissue.
Figure 10: Confocal image of Slc32a1<sup>tm2(cre)Lowl</sup> control (left) and cKO (right) forebrain tissue staining for D1R deletion. Panels A and D depict TH-antibody (pinkish-red) staining, B) and E) show brain tissues stained with D1R-antibody (green), and C) and F) are overlap images of both antibodies for control and cKO brain tissue.
Figure 11: Slc32a1<sup>tm2(cre)Lowl</sup> controls (black) and cKO (purple) mice on 12:12 L:D conditions. Panel A) depicts mean 24h food intakes (g) (n=12 for both groups), B) represents mouse body weight (g) throughout the course of the experiment, with C) normalized body weights. Total high activity (s) is seen in Panel D), with 3h pre-meal high activity (s) in E), and FAA bar graph in F. WT sample sizes on Day -7 and 7: n=10, Day 0: n=11; Days 14: n=9; Day 21: n=8; and 28: n=5. KO sample sizes on Day -7 and 0: n=9, Day 7: n=7; Days 14, 21 and 28: n=3. Asterisks were assigned to P values as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
Figure 12: High activity hourly bins in seconds (left) and normalized (right) for S\text{cl}32a1\text{tm2(cre)Lowl} control (black) and cKO (purple) mice in 12:12 L:D conditions. CR began on Day 0 (not displayed). A) seconds of high activity and B) normalized behaviors prior to CR. Hourly bin behavior accounting for high activities is seen in Panels C) for Day 7, E) for Day 14, G) for Day 21, and I) for Day 28. Whereas, normalized behaviors for those days are seen in D) Day 7, F) Day 14, H) Day 21, and J) Day 28. WT sample sizes on Day -7 and 7: n=10, Day 0: n=11; Days 14: n=9; Day 21: n=8; and 28: n=5. KO sample sizes on Day -7 and 0: n=9, Day 7: n=7; Days 14, 21 and 28: n=3.
Figure 13: Slc32a1^{tm2(cre)Lowl} were housed in constant light conditions for about 12 weeks before tested for arhythmicity. ZT hours 1-12 were considered to be day behaviors, as these ZT hours were initially lights on, while 13-24 were deemed subjective night behaviors. A) mean seconds of high activity behavior for vGat-Cre WT (n=10) and cKO (n=6) mice to compare day:night output with normalized seen in C). A side by side comparison of mean high activity behaviors summed for intended day and night hours in panel B and normalized behavior in D). Mice that did not indicate equal amounts of activity during intended day:night hours were taken out of the study. Asterisks were assigned to P values as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
Figure 14: Food intake, body weight, average total high activity, and average FAA analyses for Slc32a1^{tm2(cre)Lowl} controls (black) and cKO (purple) mice in constant light conditions. A) cKO mice ate on average more than controls at 4.6g (±1.4g) and 4g (±0.78g). B) Body weights for Slc32a1^{tm2(cre)Lowl} cKO mice with significance on days 7-28 (P=0.0061, P=0.0041, P=0.003, and P=0.0075 respectively, Unpaired T test, n=10 controls and n=4 cKO). Fraction of initial weight is seen in Panel C. On average, D) total high activity (s), E) 3h pre-meal high activity (s), and F) fraction of high activity (from 7 days of recordings throughout CR) is represented in bar graphs with no major differences between controls and cKO groups. Asterisks were assigned to P values as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.
Figure 15: Average sum high activity in seconds A) and average normalized B) for Slc32a1<sup>tm2(cre)Lowl</sup> control (black) and cKO (purple) mice on constant light. Mice were video recorded for four days continuously and fed at ZT 7, as designated by the arrow. Both control and cKO mice display activity prior to scheduled meal-times, however, control mice reach levels of 20% by the end of the 24h recording. Mice appear to lack circadian rhythms, indicated by the lack of nocturnal activity during the hours 12-24, when lights would normally be off.
DISCUSSION

D1R gene deletions did not have a major impact on FAA when using two D1R null alleles and one conditional allele on similar genetic backgrounds (C57BL/6J). As these results do not replicate our initial study in Gallardo et al. (2014), one or more possibilities may have occurred: 1) a spontaneous suppressor mutation surfaced and propagated through generations of inbreeding; 2) genetic drift of non-isogenic alleles played a more important role in FAA repression; 3) changes in environmental conditions, such as intensity of lighting or diet composition; or 4) or D1R is not a major modifier of FAA in mice and that previous results were an artifact. We found that our prior results are unrepeatable, and therefore we tentatively conclude that D1R is not a major modifier of FAA in mice.

The originally studied deletion strain, Drd1\(^{tm1Jcd}\), had a small trend towards impairment in FAA while the novel deletion strain, Drd1\(^{tm1e(KOMP)Wtsi}\), had a small but significant impairment of FAA when compared to WT mice on most days (Fig 2F, 4F, and 7F). Given that Drd1\(^{tm1Jcd}\) KO mice have FAA comparable to controls, we tested these mice using a formulated chow in order to rule out any effect(s) due to contaminants, such as phytoestrogens. Interestingly, both groups of mice had a lesser induction of FAA on unformulated chow in comparison to formulated chow (Fig 2F and 4F) but KO mice lost relatively the same fraction of weight as WT on both diets (Fig 2C and 4C). Therefore, the diet formulation does not appear to be a likely explanation as to why D1R KO mice showed robust FAA in comparison to our previously published study (Gallardo et al., 2014). We next sought to test an alternative D1R gene deletion mouse produced by the KOMP. Although not a major deficit, Drd1\(^{tm1e(KOMP)Wtsi}\) KO mice do appear to have impaired FAA, and exhibit a slower buildup of FAA. Eventually, these mice reach control levels of FAA.
by Day 28 but were lower at Day 7, 14, and 21 (Fig 7F). Consequently, these results suggest that this strain of mice has a delayed acquisition of FAA in comparison to Drd1\textsuperscript{tm1Jcd} KO mice.

The use of the Tg(Drd1a-Cre)\textsuperscript{120MXu} Cre-driver to delete D1R with its endogenous promoter appeared to affect mice embryonically or developmentally, such that no experimental mice were produced (Table IV). These results implicate that mice may require D1R embryonically for development thereby prohibiting this cross from producing any viable progeny with the necessary genotype for testing. It is not likely that the importance of D1R in fetal growth and development could account for the lack of viable progeny with the floxD1R/floxD1R; D1R-Cre\textsuperscript{+} genotype, as Drd1\textsuperscript{tm1Jcd} and Drd1\textsuperscript{tm1e(KOMP)Wtsi} employs a prenatal deletion of D1R. It is also possible that this Cre-transgene might be genetically linked to the D1R locus because the chromosomal integration site of the transgene is unknown. In addition, creating a global, post-natal deletion of D1R using CAGGCre-ERT\textsuperscript{TM} was unattainable, as mouse brain tissue still retained much of their D1R protein despite 5-consecutive days of tamoxifen injections (Fig 9B).

Lastly, our preliminary study with a conditional deletion of D1R in Slc32a1\textsuperscript{tm2(cre)Lowl} cKO mice appears to modestly diminish FAA in 12:12 L:D conditions (Fig 11F) but not in constant light conditions (Fig 14F). vGat-Cre cKO placed on 24h light also were hyperphagic (Fig 14A). While more mice are needed to determine if there are significant differences due to genetic deletion in these mice, presently, D1R deletion does not appear to make a drastically diminish FAA, although the deletion of D1R does weaken acquisition of FAA.
**Nutrition and Circadian Rhythms**

Diet plays a large role in metabolism and has effects on feeding behavior. Binge-eating behaviors lead to obesity and often stem from not only the addictive behavior of eating at a specific time during the day but even just from the sight or smell of palatable food (Hambly and Speakman, 2015). As such, we expected our Drd1<sup>tm1Jcd</sup> KO mice to show some variation when we employed two different types of chow, formulated and unformulated. The main difference between these two diets was the lack of a simple sugar in unformulated or standard mouse chow, whereas formulated chow had sucrose. During CR, these mice experienced relatively similar sum high activity (Fig 2D and 4D), sum of 3h preceding mealtime (Fig 2E and 4E), and food anticipation (Fig 2F and 4F). The only obvious difference between groups of mice on different diets is seen in KO mice fed unformulated chow that lost less weight than their WT mates throughout the study (Fig 2C and 4C). Regardless, this ability to achieve FAA on both diets corresponds with another macronutrient restrictive food anticipatory study in which subsequent restriction of protein, carbohydrate, and fat nutrients in rats did not substantially affect locomotor behavior prior to reward stimulus (Mistlberger et al., 1990). Rats in this study were deprived of either protein, carbohydrates, or high fat diet for about two weeks, then introduced to the missing macronutrient and tested for locomotor behavior using tilt cages (Mistlberger et al., 1990). In other words, a palatable meal offered during caloric restriction is sufficient as an entrainment cue for food anticipation. These findings suggest an independence exists between a single macronutrient and FEO(s), thus diet is not a sufficient cue to entrain any FEO(s).
Analyzing D1RKO Mice on Different Genetic Backgrounds

Genetic impurity of mouse models may interact with certain genetic disorders and/or behaviors (Simon et al., 2013). Prior research has found both major and minor variations in the genomes of commonly used strains of mice: C57BL/6J (B6/J) and C57BL/6N (B6/N). For example, a common mutation in nicotinamide (NAD) nucleotide transhydrogenase (Nnt) gene, which is found solely in the B6/J strain, appears to negatively impact mice in metabolic studies (Nicholson et al., 2010). Mice with the Nnt mutation have an increased chance of diet-induced obesity (DOI) due to glucose intolerance, as Nnt is necessary for the maintenance of ATP synthesis (Nicholson et al., 2013 and Ronchi et al., 2013). On the other hand, B6/N mice carry a frameshift mutation, retinal degeneration 8 (rd8), in the cell polarity complex component (Crb1) (Mattapallil et al., 2012). This mutation causes subsequent impairment of vision and multiple light-colored spots in the fundus of the eye eventually leading to degeneration (Mattapallil et al., 2012). The effects of these mutations have not been fully studied, and are not tested on mice for FAA.

In an attempt to identify any genomic differences between the two strains, Drd1tm1Jcd and Drd1tm1e(KOMP)Wtsi, we conducted a genome-wide scan assessing SNPs of these mice (Table III). Interestingly, the Drd1tm1e(KOMP)Wtsi mice resulted in a 83% (±SEM) similarity to B6/J WT mice, whereas Drd1tm1Jcd were nearly 99.7% (±SEM) similar to B6/J WT mice. We are not sure if the 16% similarity to B6/N mice seen in Drd1tm1e(KOMP)Wtsi could account for the difference in impairment of FAA between the two strains. Taken together, future experiments with mice need to account for genetic background to further understand the neural circuitry responsible for FAA.
Sex Differences in Food Anticipation

While there are clear distinctions between male and female individuals, the extent to which neural circuitry varies between both sexes has yet to be fully resolved. Prior experiments with mice indicate that males and females experience asymmetrical circadian food anticipatory behavior, with males generally exhibiting more activity. In a study using restricted-feeding (RF) to regulate feeding, food intake, body weight, and food anticipation were clearly different between the sexes, with males experiencing greater wheel-running activity (Li et al., 2015). Gonadectomized male and female mice were also compared for differences in FAA, providing the idea that gonadal hormones may play a role for this dichotomy (Li et al., 2015). However, Aguayo et al. (2018) showed that singular manipulations to gonadal hormones, sex chromosomes, and developmental patterning in wild-type mice are not enough to explain the sex.

A sexual dimorphism exists within the structure and functional properties of midbrain DAergic neurons, which primarily has been thought of as part of the underlying reason(s) for various addictive disorders seen in humans and animal models (Fattore, 2015; Gillies et al., 2014; Michalik et al., 2015). In a study conducted with D1R KO mice, females were found to display significantly lesser FAA than males when on RF by as much as 71% (Michalik et al., 2015). Even controls displayed differences between males and females, with females exhibiting 32% lesser FAA (Michalik et al., 2015). Therefore, to further refine the neural circuitry for food anticipation, future studies must take into account both sexes when analyzing FAA, as there are clear differences between males and females.
REFERENCES


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