

A functional 3'UTR polymorphism (rs2235749) of prodynorphin alters microRNA-365 binding in ventral striatonigral neurons to influence novelty seeking and positive reward traits

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Running title: Prodynorphin-microRNA interaction affects novelty seeking and reward choice

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ABSTRACT

Genetic factors impact behavioral traits relevant to numerous psychiatric disorders and risk-taking behaviors, and different lines of evidence have indicated that discrete neurobiological systems contribute to such individual differences. In this study, we explored the relationship of genetic variants of the prodynorphin (*PDYN*) gene, which is enriched in the striatonigral/striatomesencephalic pathway, a key neuronal circuit implicated in positive 'Go' behavioral choice and action. Our multidisciplinary approach revealed that single nucleotide polymorphism (SNP) rs2235749 (in high linkage disequilibrium with rs910080) modifies striatal *PDYN* expression via impaired binding of miR-365, a microRNA that targets the *PDYN* 3'-untranslated region (3'UTR), and is significantly associated to novelty- and reward-related behavioral traits in humans and translational animal models. Carriers of the rs2235749G allele exhibited increased levels of *PDYN* 3'UTR *in vitro* and had elevated mRNA expression in the medial nucleus accumbens shell (NAcSh) and caudate nucleus in post-mortem human brains. There was an association of rs2235749 with novelty seeking trait and a strong genotype-dose association with positive reinforcement behavior in control subjects, which differed in cannabis-dependent individuals. Using lentiviral miRZip-365 constructs selectively expressed in *Pdyn*-neurons of the NAcSh, we demonstrated that the *Pdyn*-miR365 interaction in the NAcSh directly influences novelty seeking exploratory behavior and facilitates self-administration of natural reward. Overall, this translational study suggests that genetically determined miR-365-mediated epigenetic regulation of *PDYN* expression in mesolimbic striatonigral/striatomesencephalic circuits possibly contributes to novelty seeking and positive reinforcement traits.

INTRODUCTION

Vulnerability to psychiatric disorders is influenced by a complex relationship between hereditary behavioral traits, genetics, as well as environment. Of the heritable traits, reward sensitivity and novelty/sensation seeking (Cloninger, 1987; Zuckerman, 1993) have been strongly linked to goal-directed and risk-taking behaviors. These traits are considered significant contributors to the development of drug abuse (Belin *et al*, 2008; Blanchard *et al*, 2009, Jupp and Dalley, 2014, Piazza *et al*, 1989), are found at high levels in gamblers (Janiri *et al*, 2007) and are positively associated with depressive symptoms and suicidal behavior in adolescents (Ortin *et al*, 2012). A key component of the neural circuitry shown to be critical for reinforcement learning and goal-directed behavior is the striatonigral/ventral striatomesencephalic (SN/VSM) pathway, which has also been shown to innervate the pallidum (Kupchik *et al*, 2015). Medium spiny SN and VSM neurons in the dorsal and ventral striatum, respectively, are well established to constitute the “Go” (positive reward choice) pathway facilitating behavior (Lobo *et al*, 2010, Hikida *et al*, 2010, Kravitz *et al*, 2012). Human studies (Frank *et al*, 2007) have documented that genetic polymorphisms of the *DARPP-32* gene, used as a non-selective proxy of SN neurons, are associated with impaired positive reinforcement learning. The SN/VSM neurons are, however, more specifically characterized by the predominant expression of the opioid neuropeptide prodynorphin (*PDYN*) (Heiman *et al*, 2008). Individual differences within the 3′ un-translated region (3′UTR) of the *PDYN* gene have been shown to be associated with the abuse of various drugs, specifically cocaine, alcohol and heroin (Clarke *et al*, 2009; Taqi *et al*, 2011; Yuferov *et al*, 2009). However, no study has yet addressed whether such differences in *PDYN* are associated with behavioral endophenotypes relevant to psychiatric disorders and risk-taking behaviors and no information exists regarding the association with cannabis dependence, a drug which is now widely used by teens and young adults.

Here we present converging molecular, *in vitro* and *in vivo* evidence suggesting a central role of *PDYN*-expressing SN/VSM neurons in behavioral traits related to psychiatric vulnerability. We demonstrate that a functional polymorphism in the 3′UTR of *PDYN*, rs2235749, impairs the binding of miR-365 as well as *PDYN* expression in the human striatum. In addition, we show that the miR-365-*PDYN* interaction, regulated by rs2235749, is specifically associated with positive reinforcement and novelty seeking traits in rats and humans.

MATERIALS AND METHODS

Participants and Personality Traits Assessment

Healthy subjects were recruited from a population (18-27 years old) participating in neurocognitive and genetic studies (Jutras-Aswad *et al*, 2012). They had no history of major psychiatric or medical disorders and did not report current or sustained (more than one month)

past psychotropic medication intake. Subjects were evaluated using an array of questionnaires to obtain information on health and sociodemographic characteristics. Subjects were excluded for major psychiatric disorder based on the Mini International Neuropsychiatric Interview (Sheehan *et al*, 1998). Fifty normal subjects were enrolled as well as fifty subjects with a lifetime cannabis dependence diagnosis determined using the Structured Clinical Interview for DSM-IV (defined as a maladaptive pattern of substance use, leading to clinically significant impairment of distress, as manifested by 3 or more criteria of substance dependence occurring in the same 12-month period at any time in the past). Participants had a mean age of 22.7 years old. Two thirds of the sample population were males from a racially diverse but mainly Hispanic background. A vast majority of subjects (93%) had a high school diploma or higher level of education and were single (91%) at the time of the study. Cannabis-dependent subjects and controls had similar racial breakdowns and only differed in the percentage of Hispanic origin (52 vs. 30%, $p < 0.05$) and daily cigarette use (54 vs. 4%, $p < 0.001$). Subjects abstained from alcohol or drugs (except nicotine) on the day of the testing session as assessed by urine toxicology (or by self-report in cannabis dependent individuals in relation to cannabis use). In addition, participants were assessed for symptoms of acute intoxication and were directly observed for two hours before testing to ensure that they were not intoxicated during the study.

Personality traits were studied using the revised edition of the Temperament and Character Inventory (TCI-R), a self-report questionnaire designed to assess 4 temperament and character dimensions (Cloninger, 1999). Our study focused on Novelty Seeking and Reward Dependence subscales, which were used as a measure of temperamental proneness to positive reward sensitivity. We also administered the Zuckerman-Kuhlman Personality Questionnaire (ZKPQ) (Zuckerman, 2002) with a specific focus on the Impulsive Sensation-Seeking subscale to differentiate novelty seeking from the overall, less specific level of impulsivity.

Probabilistic Reward Learning Task

A cognitive probabilistic learning task (Frank *et al*, 2007) was used to examine positive and negative reinforcement-based decision-making. The task involved a training phase in which participants learned to discriminate between feedback probabilities (positive feedback: green check mark; negative feedback: red cross) associated with different pairs of symbolic stimuli. At the end of the training phase (128 trials), participants should have learned that symbol A is the most favorable choice and the one most likely to get positive feedback, whereas symbol B is the least favorable one and should be avoided. In the course of the testing phase, during which no feedback was given, symbols A and B were paired with the other four symbols to create 8 new pairs for a total of 96 trials. Performance on "Choose A" pairs was used as a measure of positive reinforcement learning, whereas performance on "Avoid B" pairs reflected negative

reinforcement learning. Evaluation of behavior was carried out by investigators blinded to genotype.

Genotyping

Genotyping of DNA (from saliva samples or cerebellar brain tissue) was performed using Taqman predesigned single nucleotide polymorphism (SNP) genotyping assays (Applied Biosystems, Foster City, CA) and ABI 7900HT. *PDYN* SNPs rs2235749 and rs910080 (assay IDs 2507439 and 2507541, respectively) were chosen based on published data that specifically implicated the 3'UTR SNPs in substance dependence (Xuei *et al*, 2006; Yuferov *et al*, 2009). Linkage disequilibrium (LD) analysis was also carried out for SNPs in the *PDYN* gene +/-10 kb using the 1000 Genomes data based on racially mixed reference populations and with a minor allele frequency of at least 0.05. Samples were also analyzed for eighty ancestry indicative markers using an enhanced 34-plex assay (Fondevila *et al*, 2013) in order to confirm genetic ancestry.

mRNA expression in the post-mortem brain

Fresh-frozen striatal specimens were obtained from normal adult Caucasian subjects without head trauma from our brain bank collection (specimens collected at the Department of Forensic Medicine, Semmelweis University, Hungary) (Drakenberg *et al*, 2006). The specimens were collected under the guidelines approved by the local Human Ethical Committee within 24 hours after death. The demographic characteristics of the subjects (N=16) were: 35.9±3.2 years old; 14 males/2 females, 6.71±0.05 brain pH; postmortem interval within 22.7±1.3hrs; cause of death being cardiac failure (N=12), electric shock (N=2), viral infection (N=1), pulmonary embolus (N=1).

In situ hybridization histochemistry (ISHH) was performed on 20µm-thick cryosections to measure *PDYN* mRNA expression levels with discrete anatomical specificity, as described previously (Drakenberg *et al*, 2006; Fagergren *et al*, 2003). For microRNA assessment, small RNAs were isolated using miRVana miRNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA; cat #AM1560), cDNA was synthesized using the qScript microRNA cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD; cat. #95107) and measured by qPCR using pre-designed assays from Quanta Biosciences (HSMIR-0365A-3P, HSMIR-0135A, HSMIR-022-3P).

Stereotaxic surgeries and rat behavioral model

Adult (70-85 days) male Long-Evans rats, maintained on reversed 12hr dark/light cycle with food/water *ad libitum*, were stereotaxically infused (0.5µl) with lentiviral vectors expressing GFP, as well as miRZip-365 under the control of the *Pdyn* promoter (n=9) or empty control vectors (n=9; **Fig. S1A**; custom cloned by Systems Biosciences, Mountain View, CA). Vectors were bilaterally infused at 0.1µl/min into the NAcSh (10° angle from midline relative to bregma: AP+1.7 mm; ML+2.3 mm; DV-6.8 mm from dura). Behavioral paradigms began 12 days later to allow for

optimal expression of the vector as determined by pilot studies (**Fig. S2**). Anatomical validation of the vector location was conducted after the behavioral procedures examining GFP fluorescence (control) and Nissl staining (general needle track and morphology). Increased *Pdyn* mRNA expression in the target brain region was validated using ISHH.

To determine novelty seeking behavior (locomotor activity in a novel environment), animals were placed in an Open Field apparatus (16"×16"; Accuscan, Columbus, OH) and movements automatically tracked by beam breaks during 60 min.

To assess food self-administration behavior, animals were food-restricted overnight prior to testing. Animals were placed in an operant chamber (29.5cm x 32.5cm x 23.5cm) housed in sound-attenuating boxes (MED Associates Inc., St. Albans, VT) with two levers; depression of one (designated the active lever) resulted in the delivery of one standard food chow pellet under a fixed-ratio schedule of reinforcement, whereas depression of the other (designated the inactive lever) had no programmed consequences. To test palatable food self-administration assessment, animals had ad lib to normal chow and depression of reinforcement lever resulted in the delivery of a chocolate pellet. All animal testing was carried out using a counter-balanced experimental design at a similar time of the day.

Cell culture and luciferase assays

Trizol was used to extract mRNA from putamen punches of two post-mortem human subjects that differed in their rs2235749/rs910080 genotype. Subsequently, we synthesized cDNA (qScript cDNA SuperMix, Quanta Biosciences, Gaithersburg, MD; cat #95048), amplified the region containing rs2235749 (forward primer: CAGTTCCTCAGCCCTCATT, reverse primer: ACTCCAGAAGCAGCAGCTAG) and confirmed the genotype by Sanger sequencing. 3'UTR segments corresponding to the A and G alleles were then ligated into pFRL2 vector (Malo *et al*, 2003) attached downstream of firefly luciferase, driven by a CMV promoter (**Fig. S1B**). The constructs were used to transform HEK 293 cells that were subsequently incubated at 37°C for 3 days. Protein levels were subsequently measured using the Dual-Glo luciferase assay system (Promega, Madison, WI, cat #E2920). Firefly luciferase(-PDYN) signals were normalized to renilla luciferase, expressed from a separate promoter within pFRL2, to account for potential differences in transfection efficiency. Co-transfection experiments were carried out using hsa-miR-365a-3p mirVana miRNA mimic (Life Technologies, Carlsbad, CA; cat #4464066) and baseline levels were assessed using dsRNA with no known endogenous targets from SwitchGear Genomics (Active Motif, Carlsbad, CA; cat #MIM9001).

Statistical Analysis

mRNA expression (DPM/mg) from ISHH data was normalized using natural logarithm. General linear stepwise regression analysis was used to evaluate genotype group differences with

covariates: e.g., age, postmortem interval, brain pH, and sex. Statistical evaluations were performed using the JMP 12 (SAS Institute, Cary, NC) software package. The relationship between genotype, behavioral traits and cannabis use outcomes were analyzed using ANOVA and Pearson correlation, while group (based on a cannabis-dependence diagnosis) and gene x group interaction effects were calculated using general linear models. Genotyping patterns with phenotype were assessed with Fisher's exact test. For the rat analyses, repeated measures ANOVA with Holm-Sidak post-hoc multiple comparisons were conducted.

RESULTS

3'UTR polymorphisms of the *PDYN* gene are associated with behavioral traits related to novelty seeking and reward learning

To assess the role of *PDYN* variants with behavioral endophenotypes related to novelty seeking and reward learning, SNPs within the 3'UTR of *PDYN* were genotyped. Since novelty seeking and reward learning are important risk conferring factors with respect to neuropsychiatric disorders, especially addiction, we focused on two SNPs (rs2235749, rs910080) implicated in substance use disorders (Xuei *et al*, 2006; Yuferov *et al*, 2009). All genotypes conformed to Hardy-Weinberg equilibrium. Based on the 1000 Genome data analysis of the *PDYN* gene (+/- 10 kb), rs2235749 was in strong LD with several 3'UTR SNPs including rs910080 and was thus a good proxy for the 3'UTR (**Fig. S3**). Since there was a near complete overlap of the rs2235749 and rs910080 polymorphisms in each subject consistent with the strong LD ($D' > 0.9$) between these SNPs (Wei *et al*, 2011), only the genotype data of the rs2235749 variant is presented below for the post-mortem and clinical cohorts. Racial composition did not differ among the different genotypes and self-reported race showed a complete overlap with ancestry informative markers

There was a significant overall association between novelty seeking and the rs2235749 variant such that G/G homozygous individuals had decreased novelty seeking compared to other groups (**Fig. 1A**, $p < 0.05$). In addition, there was a significant overall difference for novelty-seeking ($F_{5,94} = 5.76$, $p = 0.001$) with group ($p = 0.0001$) and a group x *PDYN* rs2235749 interaction ($p = 0.042$). Cannabis subjects had higher novelty seeking trait as compared to controls that was driven by carriers of the rs2235749G allele who had higher novelty-seeking scores in the cannabis dependence group as compared to controls ($p < 0.01$). No association was evident based on SNPs for the age at first use of cannabis or age at onset of dependence. Impulsive risk-taking and Reward dependence traits that were more prevalent in cannabis subjects ($p = 0.013$) were not predicted by the SNPs studied although there was a significant correlation between novelty seeking and impulsive risk taking ($r = 0.6355$, $p < 0.0001$).

Performance on the cognitive probabilistic learning task examined in regard to positive (Choose A) and negative (Avoid B) reward behaviors revealed a significant group x *PDYN* rs2235749 interaction for Choose A behavior ($p = 0.0242$; **Fig. 1B**). A genotype dose effect was

apparent such that control homozygous G/G subjects had higher Choose A behavior than heterozygous A/G ($p=0.037$) and homozygous A/A individuals ($p=0.0002$) (**Fig. 1B**). However, there was no association of genotype in cannabis subjects. There was no significant genotype relationship with Avoid B avoidance behavior in any group (not shown).

3'UTR *PDYN* polymorphisms correlate to *PDYN* mRNA levels *in vivo* and *in vitro*

In order to investigate the functional relevance of rs2235749 and to assess whether the polymorphism relates to gene expression, we measured *PDYN* mRNA levels in the human striatum of post-mortem specimens from normal subjects. Only three subjects for the SNP rs2235749 were A/A genotype and thus were combined with A/G subjects since their mRNA levels completely overlapped the A/G group. *PDYN* mRNA expression levels were increased 1.5-fold in the medial NAcSh of homozygous G allele subjects compared to rs2235749A carriers ($F_{1,14}=23.24$, $p=0.0003$; **Fig. 1C**). No significant association was detected for this polymorphism in the NAc core ($p=0.81$) or putamen subregions ($p=0.12$). However, there was a significant association with *PDYN* mRNA expression in the associative caudate nucleus region ($F_{1,14}=6.37$, $p=0.025$).

In order to elucidate the mechanism by which *PDYN* 3'UTR polymorphisms contribute to differential expression, we studied the causal relationship of the allelic differences on *PDYN* levels *in vitro*. 3'UTR sequences corresponding to either the A or the G allele of rs2235749 were inserted into a pFRL2 vector downstream of a CMV promoter driven firefly luciferase, and a dual luciferase assay was performed to assess the expression of the two constructs in HEK 293 cells. Interestingly, levels of the rs2235749G construct were significantly higher compared to rs2235749A (fold change 1.49, $p=0.016$; **Fig. 2A**). These data are in line with our post-mortem gene expression data and together suggest an important functional role for the rs2235749 SNP.

rs2235749 affects the binding of miR-365 to *PDYN* mRNA

MicroRNAs are known to target the 3'UTR of transcribed mRNAs and thereby to regulate mRNA levels. One possibility to explain the differential *PDYN* expression in relation to rs2235749 is that the polymorphism might affect the binding of one or more microRNAs. To identify microRNAs potentially targeting the 3'UTR *PDYN* region, we performed *in silico* analyses on predicted gene targets from multiple databases. Intriguingly, rs2235749 is located just 5 base pairs upstream from a predicted target site of miR-365 (TargetScanHuman 6.2). Evidence supporting a potential functional interplay between rs2235749, miR-365 and the *PDYN* gene expression was apparent in our post-mortem cohort. Striatal *PDYN* mRNA levels were negatively correlated to miR-365 expression in rs2235749G/G subjects ($r=-0.69$; $p=0.039$), but not in A allele carriers ($r=0.03$, $p=0.866$) (**Fig. 2B**). Two other microRNAs, miR-22 and miR-135, with poorly conserved predicted binding sites in the *PDYN* 3'UTR (relatively close to rs2235749) showed no correlation to *PDYN* mRNA,

irrespective of rs2235749 (for miR-22, $r=-0.51$ and $p=0.16$ in G/G subjects and $r=0.17$ and $p=0.40$ in A allele carriers; for miR-135, $r=-0.057$ and $p=0.89$ in G/G subjects and $r=-0.074$ and $p=0.72$ in A allele carriers; **Fig. 2C/D**). The same was also true for four additional microRNAs (miR-223, miR-335, miR-485 and miR-873) with poorly conserved predicted binding sites located near rs910080 (not shown).

In order to assess whether rs2235749 indeed affected miR-365 binding, HEK 293 cells were co-transfected with pFRL2 constructs corresponding to either A or G allele containing *PDYN* 3'UTRs, as well as a miR-365 mimic or a non-binding scrambled double stranded RNA (negative control). Using a dual luciferase assay, we found that the miR-365 mimic significantly decreased luciferase intensity, confirming the binding of this microRNA to the *PDYN* 3'UTR. Strikingly, this effect fully depended on rs2235749 genotype: the stability of G allele-corresponding constructs was significantly decreased in the presence of the microRNA, while no such effect was observed with the A allele (**Fig. 2A**).

miR-365 regulates *Pdyn* expression, novelty seeking behavior and positive reinforcement-based decision making *in vivo*

To confirm the functional relevance of the proposed *PDYN*-miR-365 interaction *in vivo*, a lentiviral construct expressing miRZip-365 or empty vector was infused into the NAcSh of adult male Long-Evans rats. miRZips are synthetic single-stranded anti-microRNAs designed to suppress microRNA function. By expressing a miRZip construct specifically targeting miR-365, our aim was to inhibit the binding of the microRNA to *Pdyn* mRNA molecules and to assess how disrupting this system affects *Pdyn* expression as well as novelty seeking and positive reinforcement learning behaviors. Importantly, the expression of our lentiviral construct was driven by the *Pdyn* promoter, thus ensuring specificity to the striatonigral/striatomesencephalic pathway. We validated the constructs using immunohistochemistry and *in situ* hybridization histochemistry: viral expression was determined to be localized to the NAcSh, and miRZip-365 animals had increased *Pdyn* mRNA beginning 12 days after stereotaxic surgery ($p=0.026$).

Open Field locomotor testing demonstrated that NAcSh miRZip-365 infusion increased novelty seeking behavior apparent in both time spent moving ($p=0.05$) and total distance traveled ($p=0.026$) as compared to controls (**Fig. 3A**). Positive reinforcement behavior was subsequently assessed using a food self-administration paradigm. In line with our hypothesis, miRZip-365 expression significantly increased active lever pressing for normal food reward ($p=0.006$), palatable chocolate pellets ($p=0.01$) and during progressive ratio to determine motivated behavior ($p=0.003$), confirming enhanced positive reinforcement upon down-regulation of miR-365 (**Fig. 3B**). Importantly, animals did not differ in inactive lever pressing during the food self-administration sessions emphasizing the specificity of the reward-directed behavior, and did not show differences in general locomotor activity.

DISCUSSION

We propose a novel, genetically influenced microRNA-mRNA regulatory relationship between *PDYN* and miR-365 that contributes to behavioral endophenotypes relevant to addiction. Our *in vitro* and *in vivo* data, including direct study of human subjects revealed that SNPs in the 3'UTR of the *PDYN* gene strongly affect the binding of miR-365 to this region, relate to *PDYN* mRNA expression levels in the human NAcSh and are associated with novelty seeking and positive reinforcement-based decision-making. These findings suggest that a genetically determined *PDYN* tone within the ventromedial striatal circuitry, established through impaired targeting of miR-365 to the *PDYN* 3'UTR, may contribute to individual differences linked to behavioral traits relevant to reward sensitivity.

PDYN in the striatum is expressed in a ventromedial-to-dorsolateral gradient with most abundant levels in the NAcSh (Hurd and Herkenham, 1995). While a significant relationship was apparent between *PDYN* mRNA expression in the associative subdivision of the human caudate nucleus and SNP genotype, the genetic association with mRNA expression levels was most pronounced in the NAcSh. In addition to the well-documented involvement of the SN 'direct' pathway of the dorsal striatum in 'Go' behavior, a similar organization exists in the ventral striatum for reinforcement behavior (Lobo *et al*, 2010, Hikida *et al*, 2010, Kravitz *et al*, 2012). The ventral striatal 'direct' output VSM pathway is organized such that mesencephalic projections of *Pdyn* cells in the NAc core primarily target the substantia nigra pars compacta, whereas those in the shell subdivision innervate the ventral tegmental area (VTA) (Zhou *et al*, 2003). The striato-VTA output of the NAcSh would be predicted to directly modulate mesolimbic neurons relevant to reward and goal motivated behavior. Several human and primate studies have documented that *in vivo* NAc activity is correlated with individual differences in sensation and novelty seeking (Abler *et al*, 2006; McClure *et al*, 2003; Montague *et al*, 2004). Interestingly, rats with increased vulnerability for alcohol intake and novelty seeking behavioral traits have enhanced *Pdyn* in the NAcSh, but not NAc core or dorsal striatum (Guitart-Masip *et al*, 2006). Moreover, high responder rats to novelty stressor show enhanced drug self-administration and are characterized by elevated NAc *Pdyn* mRNA expression (Lucas *et al*, 1998). Consistently, animals in our study with increased *Pdyn* in the NAcSh, induced by the lentiviral miRZip-365 construct, exhibited enhanced novelty-induced locomotor behavior. An association between the *PDYN* rs2235749 SNP and novelty seeking-trait was also observed in our human study. Homozygous carriers of the rs2235749G allele, which related to enhanced NAcSh *PDYN* mRNA expression, had reduced novelty seeking scores, but interestingly, novelty seeking was enhanced in G-allele cannabis-dependent subjects. Novelty seeking is a multifaceted behavioral construct, thus further studies are needed to examine its different dimensions in relation to *PDYN* and the interaction with cannabis use and dependence.

The relationship of the *PDYN* rs2235749 variant in relation to positive reinforcement behavior was well matched in both human subjects and our animal model. Human subjects showed a significant genotype-dose relationship to Choose A behavior, in line with the expected increase in positive decision-making choice among carriers of the genotypes associated with higher NAcSh *PDYN* mRNA expression. Using the same neurocognitive task known to dissociate vulnerability in probabilistic reward 'Go' from avoidance learning 'No/Go' based on discrete cortico-striatal circuits (Frank *et al*, 2007), our results did, however, demonstrate that *PDYN* is specifically associated with decisions related to positive, but not negative, reinforcement. The fact that various lines of evidence from animal models have validated that the 'direct' and 'indirect' striatal pathways dichotomize reward 'Go' and avoidance 'No/Go' behaviors (Hikida *et al*, 2010; Lobo *et al*, 2010) strengthens the human findings. Indeed, rats injected with miRZip-365 demonstrated increased self-administration of natural and palatable food and were more motivated to obtain the reward.

Interestingly, in contrast to controls, the association between *PDYN* genotype with performance on the cognitive probabilistic learning task was not present in cannabis subjects. Whether these differences predated the onset of cannabis dependence in at-risk individuals or are due to a possible residual effect of recent cannabis use on testing measures is difficult to determine in the current study. Cannabis dependent participants were directly observed for a few hours before performing cognitive tasks and did not show symptoms of acute intoxication. Nevertheless, residual effects may have masked the effect of genotype variation on learning task performance and novelty seeking behavior in the cannabis dependent group. A number of studies have shown acute and short term effects of cannabis on various domains of cognition (reviewed in Broyd *et al*, 2015), which further underscores the need for future research that could disentangle pre-existing, acute and residual cognitive deficit in cannabis dependent populations.

An important aspect of the current study was the unique mechanistic insights provided about the functional relationship between 3'UTR SNPs and microRNA regulation of *PDYN* that contribute to behavior. microRNAs, small non-coding RNAs that regulate mRNA levels by binding to the 3'UTR, have been shown to play critical roles in neurodevelopment and survival, as well as adult brain function including synaptic plasticity and memory formation (Kenny, 2014, McNeill and Van Vactor, 2012). microRNAs have also been implicated in reward and addiction such that striatal deficiency of Argonaute-2, a master regulator of the maturation and function of all microRNAs, directly alters cocaine self-administration behavior (Schaefer *et al*, 2010). The previous study of Argonaute-2 directly targeted dopamine D2 receptor-expressing cells that are abundant within the striatopallidal pathway. Our study now emphasizes the direct involvement of microRNAs in the VSM circuit in regulating addiction-related phenotypes. Very limited publications currently exist about miR-365 with most related to cancer and none regarding brain function. Here, we propose a novel, genetically influenced regulatory relationship between

PDYN and miR-365 that contributes to behavioral endophenotypes associated with positive valence that may be relevant to addiction and related psychiatric disorders.

Whether the association between individual differences in *PDYN* variants and behavioral traits do contribute to addiction disorders in humans remains to be established. Kreek and colleagues have shown that the 3'UTR *PDYN* SNPs currently studied are in high LD and together with the rs910079 SNP comprise a haplotype block associated with striatal *PDYN* mRNA levels as well as cocaine dependence and combined cocaine/alcohol codependence (Yuferov *et al*, 2009). 3'UTR *PDYN* SNPs have also been observed to be associated with heroin abuse (Clarke *et al*, 2009). Moreover, the rs2235749 SNP currently shown to affect miR-365 binding to *PDYN* mRNA was correlated to impaired DNA methylation and *PDYN* expression in the dorsolateral prefrontal cortex of alcohol-dependent individuals (Taqi *et al*, 2011). Although insight is still lacking regarding the functional mechanisms of 3'UTR SNPs, it is clear that the 3'UTR plays a significant role in the stability of the mRNA, as well as translation and transcriptional regulation. Importantly, our data demonstrate a causal role of the 3'UTR SNP on mRNA levels and specific behaviors associated with the VSM pathway.

As with all human studies, and considering the limitations of mixed ethnicity and small sample size, replication in another population will be important to validate the results of our association studies. Nevertheless, the human findings provided the foundation for the experimental models that did support the suggestion that the *Pdyn* striatonigral/VSM circuit and, more specifically, the miR-365-*Pdyn* relationship is directly relevant to novelty seeking and positive reinforcement decision-making. How cannabis exposure factors in to this equation will need to be addressed in follow-up studies that assess the drug's effect on *Pdyn* expression and behavior in translational animal models. Another important consideration is the population admixture of the sample population. It has been shown that the *PDYN* SNPs evaluated in our study are in high LD, but are not related to ethnicity (Yuferov *et al*, 2009), and there was no evidence in the present study to suggest that the genetic effects observed were due to ethnicity. Nevertheless, future studies involving larger populations will help clarify these issues especially in relation to novelty seeking traits and allow the possibility to examine other markers specifically enriched in VSM neurons. Moreover, although we focused on the 3'UTR based on our initial hypothesis, other polymorphisms of *PDYN* within the promoter region have also been shown to be relevant for gene expression (Zimprich *et al*, 2000, Nikoshkov *et al*, 2008) and in relation to substance abuse (Saify *et al*, 2014, Clarke *et al*, 2012, Ray *et al*, 2005). The contribution of different variants within the *PDYN* gene to discrete behaviors needs to be addressed in larger populations that would provide the statistical power to examine such questions.

Overall, the current translational study reveals a significant contribution of 3'UTR *PDYN* polymorphisms to mesolimbic NAcSh *PDYN* expression and positive reward sensitivity traits in humans. Our *in vitro* data causally demonstrates that this interaction is regulated by impaired binding of miR-365 to the *PDYN* 3'UTR dependent on rs2235749 allele. Furthermore, direct

manipulation of miR-365 activity in *Pdyn*-expressing neurons in the NAcSh directly relates to novelty seeking, reward and motivated behavior. These findings emphasize a novel mechanism involving miR-365–*PDYN* interaction relevant to individual behavioral traits that might enhance vulnerability for addiction and other psychiatric disorders.

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FIGURES

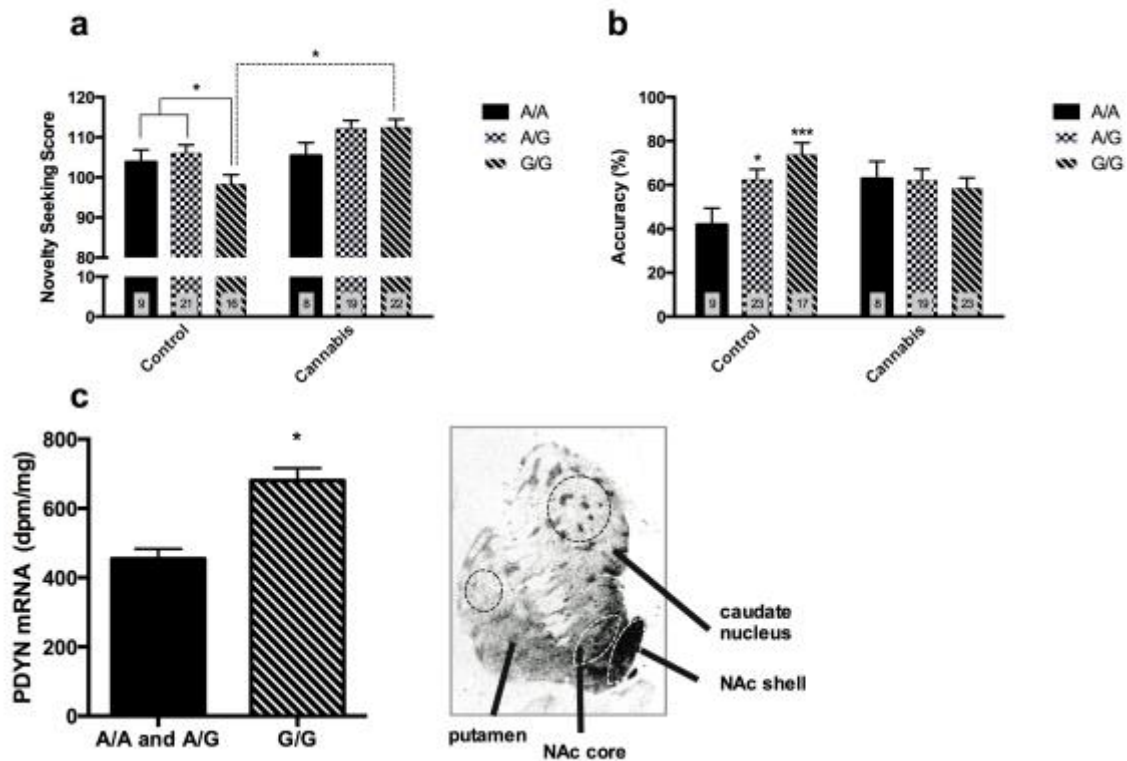


Figure 1: *PDYN* 3'UTR SNP rs2235749 is associated to novelty seeking, positive reward learning and striatal *PDYN* mRNA levels in control human subjects.

(a) Novelty seeking scores in relation to *PDYN* rs2235749 genotype in control and cannabis dependence groups. (b) *PDYN* SNPs in relation to the accuracy of Choose A (positive reinforcement) performance in controls and cannabis users. (c) *PDYN* mRNA expression levels (dpm/mg) in the nucleus accumbens shell (NAcSh) of normal human subjects. *PDYN in situ* hybridization histochemistry micrograph with dashed lines representing sampled striatal regions. Error bars indicate SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

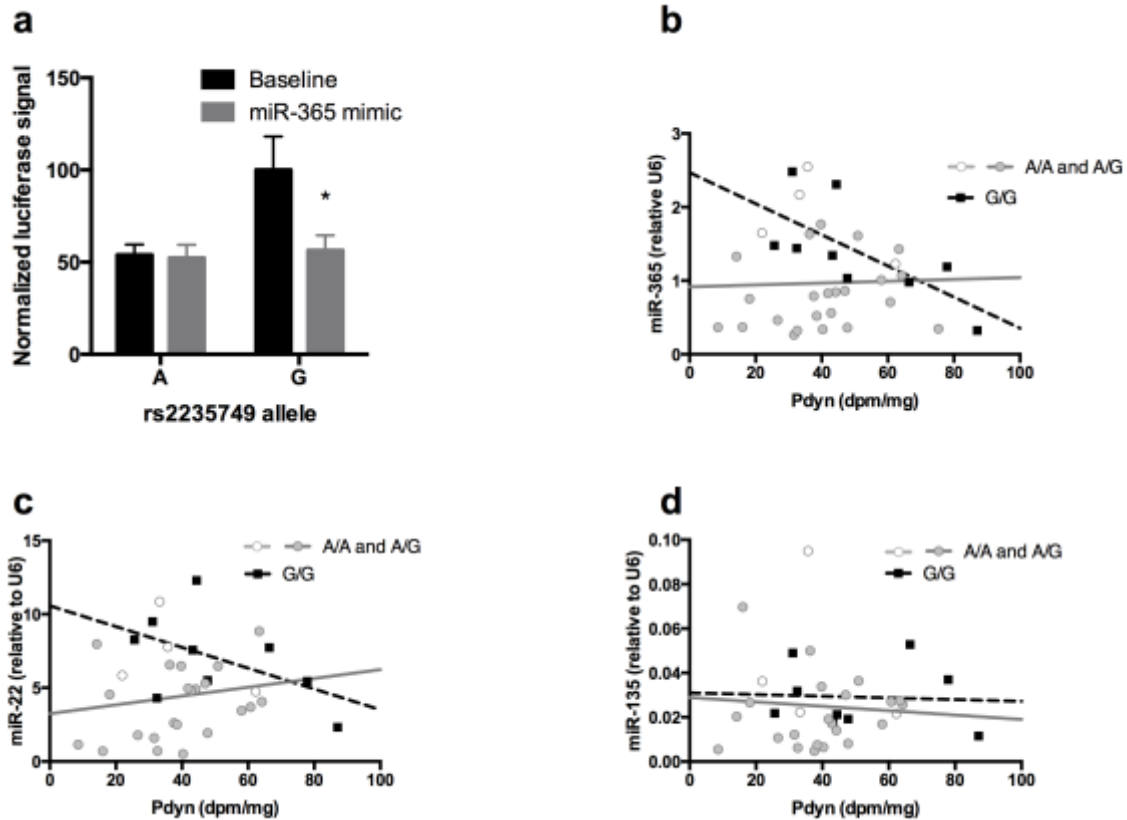


Figure 2: rs2235749 affects the binding of miR-365 to *PDYN*.

(a) Double luciferase assay showing *PDYN* mRNA levels in HEK 293 cells co-transfected with *PDYN* 3'UTR pFRL2 constructs corresponding to rs2235749G or rs2235749A alleles and miR-365 mimic or scrambled control vector (b-d) Correlations between *PDYN* mRNA and miR-365 (b), miR-22 (c) and miR-135 (d) levels in the post-mortem human striatum. Statistical analysis was carried out on pooled (A/A and A/G vs. G/G) genotypes. Error bars indicate SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

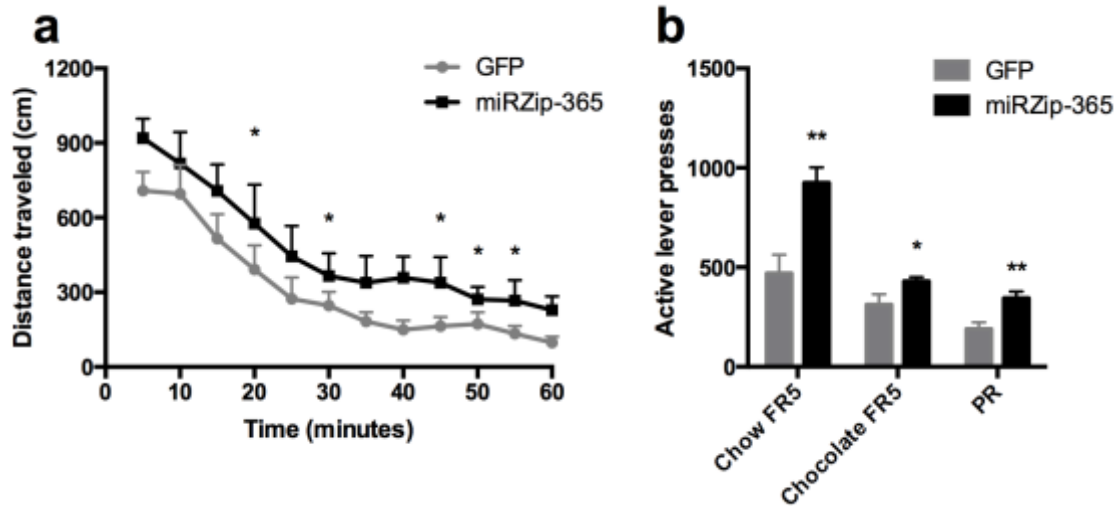
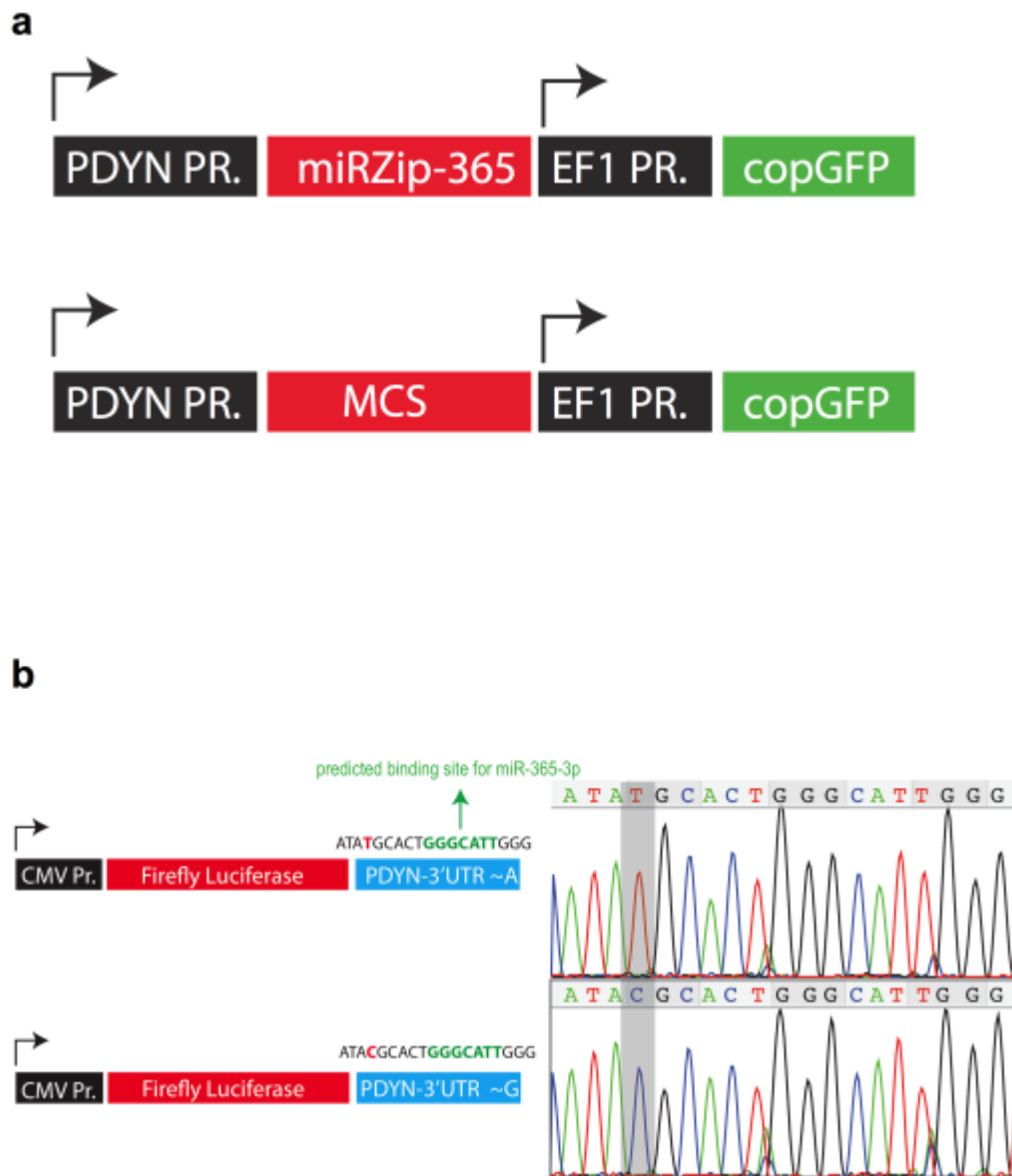


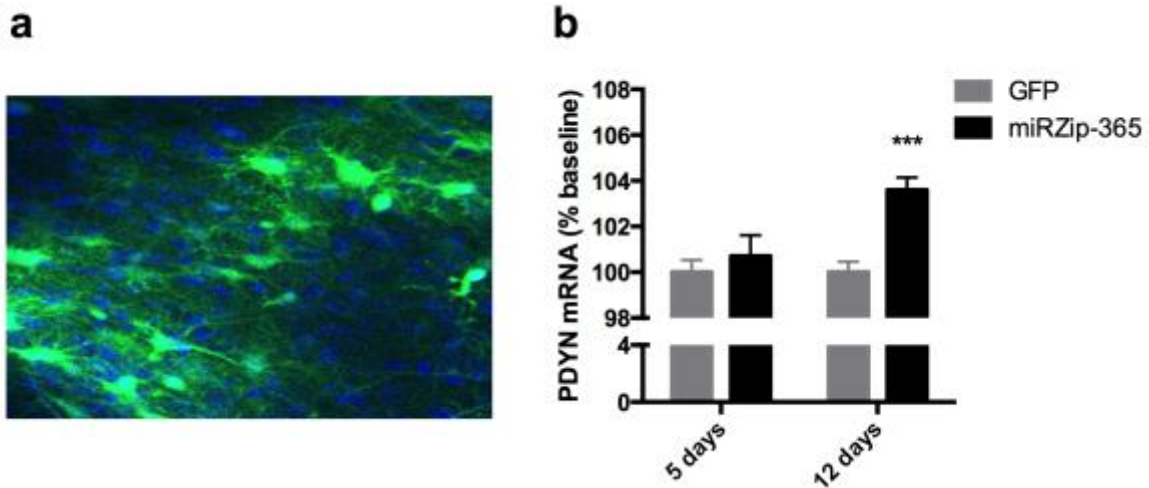
Figure 3: miR-365 inhibition in the nucleus accumbens shell impairs novelty seeking and positive reward learning behavior in rats.

(a) Novelty seeking behavior in adult male Long-Evans rats bilaterally injected with a lentiviral construct expressing miRZip-365 driven by the *Pdyn* promoter. (b) Food self-administration behavior in adult male Long-Evans rats bilaterally injected with a lentiviral construct expressing miRZip-365 driven by the *Pdyn* promoter. Error bars indicate SEM. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

SUPPLEMENTARY FIGURES

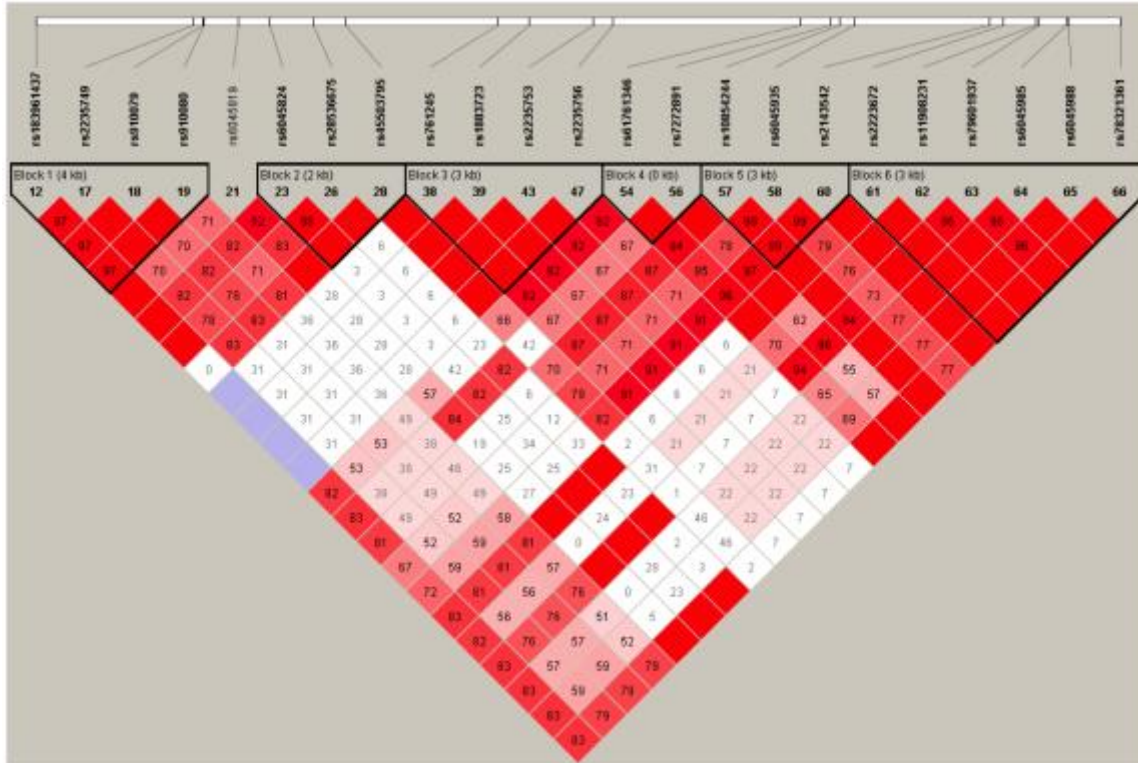
**Supplementary Figure 1: Construct maps.**

(a) Structure of the *PDYN*-miRZip-365-GFP expressing construct and the control vector with the empty multiple cloning site (MCS). The expression of miRZip-365 is driven by the human *PDYN* promoter (PR). (b) Structure of the *PDYN*-3'UTR-containing pFRL2 construct. The CMV promoter drives the expression of firefly luciferase and *PDYN* 3'UTR corresponding to either the G or the A allele of rs2235749. Since *PDYN* is described from the negative strand of DNA, the inserts corresponding to the G and A alleles harbors C or T nucleotides, respectively. The inserts were identical in every aspect other than rs2235749 as confirmed by Sanger sequencing. For the complete structure of pFRL2, see Malo *et al.* (2003).



Supplementary Figure 2:

For pilot experiments, adult male Long-Evans rats (n=4) were injected with the *PDYN*-driven miRZip-365 expressing lentiviral construct (one hemisphere) and the empty control vector (other hemisphere), targeting the nucleus accumbens shell (NAcSh). Animals were sacrificed at 5 and 12 days post-surgery. GFP expression was confirmed by confocal microscopy, and *PDYN* mRNA levels were measured using *in situ* hybridization histochemistry. (a) GFP expressing cells in the NAcSh. (b) 12 days following viral injections, miRZip-365 significantly increased *PDYN* mRNA levels in the NAcSh compared to controls.



Supplementary Figure 3: Linkage disequilibrium (LD) pattern in the *PDYN* gene.

rs2235749 is in strong LD with several 3'UTR SNPs including rs910080 and can be used as a good proxy for the 3'UTR. LD analysis was carried out using 1000 Genomes data representing a mixed population (CEU, IBS, MXL, CLM, PEL and PUR) with a minor allele frequency of at least 0.05. Numbers indicate D' values.