Temporally specific miRNA expression patterns in the dorsal and ventral striatum of addiction-prone rats

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Abstract

MicroRNA (miRNA) within the ventral and dorsal striatum have been shown to regulate addiction relevant behaviors. However, it is unclear how cocaine experience alone can alter the expression of addiction-relevant miRNAs within striatal subregions. Further, it is not known whether differential expression of miRNAs in the striatum contributes to individual differences in addiction vulnerability. We first examined the effect of cocaine self-administration on the expression of miR-101b, miR-137, miR-212 and miR-132 in nucleus accumbens core (NAcC) and shell (NAcS), as well as dorsomedial (DMS) and dorsolateral (DLS) striatum. We then examined the expression of these same miRNAs in striatal subregions of animals identified as being 'addiction-prone', either immediately following self-administration training or following extinction and relapse testing. Cocaine self-administration was associated with changes in miRNA expression in a regionally discrete manner within the striatum, with the most marked changes occurring in the NAcC. When we examined the miRNA profile of addiction-prone rats following self-administration, we observed increased levels of miR-212 in the DMS. After extinction and relapse testing, addiction-prone rats showed significant increases in the expression of miR-101b, miR-137, miR-212 and miR-132 in NAcSh, and miR-137 in DLS. This study identifies temporally specific changes in miRNA expression consistent with the engagement of distinct striatal subregions across the course of the addiction cycle. Increased dysregulation of miRNA expression in NAcSh and DLS at late stages of the

addiction cycle may underlie habitual drug seeking, and may therefore aid in the identification of targets designed to treat addiction.

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INTRODUCTION

Drug abuse is associated with cellular and molecular changes that impair the ability to elicit synaptic plasticity in specific brain regions, including the ventral and dorsal striatum (nucleus accumbens, NAC) (Corbit et al., 2012; Kasanetz et al., 2010; Mameli et al., 2009; Vanderschuren et al., 2005; Wang et al., 2015; Wolf, 2010). The NAC can be divided into two functionally distinct regions; the shell (NACsh) which regulates the rewarding properties of drugs of addiction, and the core (NACc) which plays a more important role in control of conditioned stimuli (Ikemoto, 2007). More recently, the dorsal striatum (DS) has been implicated in the addiction process (Balleine and O'Doherty, 2009). Like the ventral striatum, the dorsal striatum is composed of distinct subregions including the dorsomedial (DMS) and dorsolateral (DLS) striatum. The DMS is considered important for goal-directed learning, whereas the DLS mediates stimulus-response (habit) learning (Yin et al., 2004, 2005a). Recent evidence indicates that cellular and molecular adaptations in these striatal subregions following chronic drug is associated with impairments in the ability to evoke synaptic plasticity – changes that are linked with ongoing drug-seeking behaviours (Brown et al., 2010; Corbit et al., 2012; Quinn et al., 2015). Importantly, the precise regulatory mechanisms contributing to lasting molecular changes that promote addiction are largely unknown.

Like humans, not all animals exposed to drugs exhibit behaviors that resemble addiction. Models that incorporate the identification of 'addiction-prone' rats have

identified important differences in the synaptic and molecular profiles that are linked with addiction vulnerability (Brown et al., 2010; Deroche-Gamonet et al., 2004; Kasanetz et al., 2010; Quinn et al., 2015). These changes are often more subtle than what is observed between drug-exposed versus drug-naïve animals, but are arguably more relevant to human addiction (Brown et al., 2010). For example, Kasanetz et al., 2010 found that only addiction-prone animals displayed evidence of ongoing impairments in synaptic function (Kasanetz et al., 2010). Further, we showed that there was a widespread reduction in plasticity-associated genes, including dopamine receptor 1 (*Drd1*) and mechanistic target of Rapamycin (*mTOR*), in the nucleus accumbens and dorsal striatum of animals phenotyped as being addiction-prone (Brown et al., 2010). This pattern of suppression amongst synaptic plasticity associated genes suggests that these changes might be sustained by an epigenetic mechanism, such as microRNA (miRNA).

MiRNA are negative regulators of mRNA expression and have been implicated in addiction-relevant behaviors (Hollander et al., 2010; Im et al., 2010; Quinn et al., 2015). Specific miRNA have now been implicated in the addiction process. For example, miR-212, and to a lesser extent the closely related miR-132, are upregulated in the dorsal striatum of rats given extended access to cocaine. Further, knockdown of miR-212 in the dorsal striatum enhances whereas its overexpression curtails compulsive cocaine taking suggesting that it plays a protective role in controlling aberrant molecular

adaptations produced by cocaine (Hollander et al., 2010; Im et al., 2010). Presumably then addiction-prone rats would show suppressed miR-212 levels within the dorsal striatum relative to addiction-resistant rats. Consistent with this hypothesis we recently reported that expression levels of miR-212 were significantly suppressed in the DMS of addiction-prone rats (Quinn et al., 2015). As noted above, we also found that gene expression for *Drd1* and *mTOR* was suppressed in the striatum of addiction-prone rats. miR-101b is predicted to target *Drd1* and can regulate mTOR *in vitro* and its expression was altered in the DS of addiction-prone animals post relapse (Quinn et al., 2015). Currently, we have very limited information about temporal profile of these miRNA over the course of drug-taking. Therefore, the purpose of the present study was to examine the expression profiles of these specific miRNA during the early cocaine-taking phase in the DS. We also examined expression profiles in the ventral striatum at time points equivalent to the DS. miR-137 was included in our analyses as it has recently emerged as an important regulator of synaptic plasticity at the pre-synapse and has been implicated by gene pathway analyses as a relevant in the regulation of addiction-relevant genes.

METHODS AND MATERIALS

Animals and Ethics

Male Sprague-Dawley rats (Animal Resources Centre, WA, AUS) weighing 200-250g upon arrival were housed two per cage on a reverse 12-hour light/dark cycle (lights off

0700h) with *ad libitum* access to food and water. All procedures were carried out in strict accordance with protocols approved by the University of Newcastle Animal Care and Ethics Committee, New South Wales Animal Research Act and Regulations and the Australian Code of Practice for the care and use of animals for scientific purposes.

Intravenous Catheter Surgery

Rats were anaesthetized with isofluorane (1-3% with a flow rate of 2L/min) and a Silastic catheter was surgically implanted into the right jugular vein using aseptic techniques as described previously (Brown et al., 2010). Prior to surgery rats received a 0.3ml intramuscular injection of a broad-spectrum antibiotic (112.5mg/ml benzathine penicillin, 150mg/ml procaine penicillin; Norbrook Laboratories, UK) and a 0.2ml subcutaneous injection of a 50mg/ml solution of Caprofen (Norbrook Laboratories, UK). Following surgery catheters were flushed with 0.3ml of a 50mg/ml solution of Cephazolin (Mayne Pharma, Australia). Rats were allowed to recover for 7 days prior to commencement of cocaine self-administration training. Catheters were flushed daily with 0.2ml of 50U heparinized saline to ensure catheter patency.

Drugs

Cocaine hydrochloride (Johnson Matthey, Edinburgh, UK) was dissolved in sterile physiological saline (2.5mg/mL) (Brown et al., 2010).

Behavioral Testing Equipment

Behavioral procedures were conducted in standard operant conditioning chambers (Med Associates, VT, USA). Chambers were equipped with two retractable levers (6 cm above the floor), a white cue light above each lever, two speakers to deliver auditory stimuli, and a white house light at the top of the chamber wall opposite the levers. Auditory stimuli were produced by a white noise generator connected to a speaker that produced a 70dB white noise adjacent to the house light (Med Associates, VT, USA), and a tone source located in the top left corner (Sonalert with volume control, Med Associates, VT, USA), that was connected to a speaker that produced an intermittent tone (0.5 Hz at 70dB). A syringe pump (5 rpm motor, Med Associates, VT, USA) located on the outside of the cubicle delivered the IV cocaine. Behavioral testing equipment was controlled by a Windows-based PC, using MED-PC IV software (Med Associates, VT, USA).

Experiment 1 – Effect of cocaine on miRNA expression in the striatal subregions 24h after drug taking

To assess the effect of cocaine on miRNA expression, rats were implanted with jugular catheters and randomized to either cocaine (n=16) or yoked-saline (n=7) groups. Cocaine rats were trained to lever press for cocaine (0.25mg/0.1mL i.v.) on a fixed ratio (FR) 1 schedule for 2hr/day. Yoked-saline rats were placed in the operant chamber but lever presses had no scheduled consequence. Saline rats received non-contingent

infusions of saline when their yoked-cocaine counterpart received an infusion of cocaine. Cocaine rats continued on FR1 training until stable responding was established, which was defined as 20 infusions/session over 3 sessions, with <10% variability across those days. Rats then progressed to 3h/day sessions where cocaine availability was signalled by the presence of white noise (first 40 mins of each hour; referred to as the 'drug available' (DA) period), and cocaine non-availability was signalled by the illumination of a white house light (last 20 mins of each hour; referred to as the 'non-drug available' (NDA) period). After two FR1 cue sessions, rats progressed to an FR3 schedule for two sessions and then an FR5 schedule. Responding during the NDA period of FR5 testing was used as an index of rats' ability to refrain from drug seeking. After the final FR5 session, rats were tested on a progressive ratio (PR) schedule. PR sessions lasted for 5 hours or until rats ceased to press for 45 minutes, deemed the breakpoint. PR tests were carried out over 5 consecutive sessions and the breakpoint was used as an index of rats' of motivation for cocaine. Together, indices of rats' ability to refrain from drug seeking (responding during NDA period) and motivation for cocaine (PR break point) were used to determine rats' addiction propensity (see details below). Rats were sacrificed 24 hours after the final PR session. Brains were snap frozen and stored at -80°C for subsequent analysis of miRNA expression. For a timeline of Experiment 1, see Figure 1a.

Experiment 2: Striatal miRNA expression in addiction-prone rats

Because addiction is thought to result from an interaction between drug experience and individual vulnerability, we next sought to characterise the expression of striatal miRNA in addiction-prone rats at different time points across the addiction cycle. The first time point ('early time point') was immediately following self-administration training, and was selected because a similar time point was used in Experiment 1. The second time point ('late time point') followed extinction training and cue-induced reinstatement testing and was selected because relapse is a hallmark characteristic of addiction.

To identify addiction-prone rats, we used behavioral markers of addiction using methods that we and others have described previously. Briefly, rats were trained to self-administer cocaine on a FR1 and then FR3 schedule of responding before being tested for addiction-relevant behaviors as described for Experiment 1 – inability to refrain from drug-seeking during a signalled period of NDA on an FR5 schedule of responding, motivation to consume drug using repeated PR tests, and predicted (early time point; discussed below) or actual (late time point) responding during a cue-induced reinstatement test. Rats that scored in the top or bottom 40% of the distribution for reinstatement responding as well as the top or bottom 30% of the distribution for NDA and PR were phenotyped as addiction-prone or addiction-resilient, respectively. Behavioral data (Brown et al., 2010) and DS miRNA expression (Quinn et al., 2015) from the 'late time point' group has been published previously.

Prediction of reinstatement scores for 'early time point' animals

Because reinstatement behavior is a key criterion that we use to identify addiction-prone animals, we required an approach that would allow us to accurately predict relapse scores in these animals. To achieve this, we used a Bayesian model averaging (BMA) approach to identify early behavioural indices that can predict addiction-relapse vulnerability. BMA accounts for uncertainty in model selection in a principled framework, and allowed us to simultaneously test many candidate models that each contains a different combination of predictor variables. The influence or 'weight' of a candidate model is proportional to that model's ability to predict the outcome variable. The predicted outcome of a BMA analysis is therefore a weighted average of the predictions of the set of candidate models under consideration (Hoeting et al., 1999).

We developed this BMA model based on behavioral data from FR1, FR3, FR5 and PR sessions of a previously published cohort of rats (n=45) trained to self-administer cocaine, who were subsequently stratified as 'addiction-prone', 'addiction-resilient' or neither. The set of candidate predictor variables included the following: latency to first lever press in each drug available period, number of infusions earned across each session, mean interval between successive infusions, number of responses in the 20 second time out period that followed each infusion, the number of 'bursts' (5 infusions received in a 5 minute window), number of responses in the last 9 minutes of a session,

and the number of responses in the NDA period; and from the PR sessions the number of responses in the 20 second time out period that followed each infusion, and the breakpoint. These predictor variables were extracted separately from each session within each schedule (i.e., FR1, FR3, FR5, PR) and then averaged across sessions within a schedule to obtain a single value of the predictor for each schedule. Mean values were log-transformed for normality over rats where required. The outcome variable was the 'addiction-reinstatement' score described in this previous study (Brown et al., 2010). Due to technical issues, 3 (of 45) rats were missing data on the FR3 variables. For these rats we imputed the mean of the FR3 variables across all rats so we could obtain valid predictions (Figure 2a). The BMA model was then used to obtain a 'predicted reinstatement score' on rats (n=63) that were sacrificed after their final PR session (early time point group). These predicted reinstatement scores were then used to phenotype these rats as 'addiction-prone', 'addiction-resilient' or 'intermediate' using the criteria described above.

We used BMA over linear regression models to test how well the set of 23 variables predicted the relapse score, which involved comparison of the relative fit of $2^{23} \approx 8.3$ million models. We used methods described in the Bayesian Model Averaging library ('BMS' package) in the R programming environment (R Core Team, 2016; Zeugner and Feldkircher, 2015). We assumed a uniform prior over model size and birth/death Markov chain Monte Carlo (MCMC) sampling. We excluded the first 100,000 samples as burn in

and sampled 200,000 iterations from the stable posterior distribution. MCMC convergence was examined by comparing the frequency that the MCMC sampler drew from the best 2000 models with the marginal likelihood of the best 2000 models. The correlation between the two measures was $r=0.994$, indicating good convergence. Our primary outcome measure was the strength of the linear relationship between observed and predicted relapse scores.

Brain collection and miRNA analysis

Rats in Experiment 1 and rats in the 'early time point' group in Experiment 2 were sacrificed 24 h after their final PR session. Rats in the 'late time point' group in Experiment 2 were sacrificed 24 h after their cued reinstatement test, as per previous studies (Brown et al., 2010). Animals were decapitated, brains were snap frozen in isopentane and stored at -80°C for molecular analysis. We selected candidate miRNA previously implicated in drug taking, including miR-212 and the closely related miR-132 (Hollander et al., 2010; Im et al., 2010). MiR-137 was selected because of its involvement in pre and post-synaptic plasticity and neuronal development and maturation (Siegert et al., 2015). MiR-137 is also associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics, 2014), which has significant comorbidity with addiction (Buckley et al., 2009). miRNA with the potential to regulate *Drd1* and *mTOR*, were also chosen because of the association of these synaptic plasticity genes with addiction-relapse vulnerability (Brown et al., 2010). The

target prediction algorithms miRanda and TargetScan which identified miR-101b as a potential regulator of *Drd1* and *mTOR* expression. For further details on tissue dissection, qPCR and luciferase assay see Supplementary Material.

Statistical Analyses

Shapiro-Wilk test for normality was used to assess distribution. Unpaired two-tailed t-tests were performed for behavioral and qPCR data. Due to the prediction that miR-101b would negatively regulate *Drd1*, one-tailed t-tests were used for luciferase assay data. Mann-Whitney U tests conducted were required for data without normal distribution. An alpha value of 0.05 was adopted for all tests.

Results

Experiment 1

Cocaine self-administration decreased miRNA expression in the NAcC and DMS

To determine the effect of cocaine experience on striatal miRNA expression, we compared miRNA expression profiles of rats trained to self-administer cocaine versus yoked-saline controls. Cocaine trained rats showed robust self-administration behavior, whereas responding in the yoked-saline group was negligible (see Figure S1). Cocaine rats displayed significantly lower expression of miR-101b ($t_{20}=2.68$, $p=0.014$), miR-137 ($t_{20}=2.88$, $p=0.009$) and miR-132 ($t_{20}=2.75$, $p=0.012$) in the NAcC compared to saline controls (Figure 1b). No changes were observed in the NAcSh (Figure 1c). miR-212

expression approached significance in the NAcSh ($t_{19}=-2.01$, $p=0.059$) but no changes were observed in the NAcC of cocaine versus saline rats. In the dorsal striatum, cocaine rats displayed significantly lower miR-137 expression ($t_{20}=2.25$, $p=0.036$) in the DMS, but not DLS, of cocaine versus yoked-saline animals (Figure 1d, e). No changes in miR-101b, miR-212 or miR-132 were observed in either the DMS or DLS at this time point (Figure 1d, e).

Experiment 2

Early addiction-related behaviors robustly predicted future propensity to relapse

The relationship between observed and BMA-predicted relapse scores is shown in Figure 2a. The BMA model accounted for almost 50% of the variance in relapse scores. Importantly, the predicted relapse scores perfectly discriminated the animals classified as addiction-prone (squares) from those classified as resilient (Figure 2a; triangles; i.e., a horizontal line at the middle of the y-axis leads to perfect classification of the two target groups).

A summary of the BMA model is shown in Figure 2b. Overall, the strongest predictor variables were from sessions closer in time to the reinstatement session (i.e., PR, FR5) than earlier sessions (FR1). There was considerable uncertainty in the best set of predictors, with the largest posterior model probability reaching only 0.03. This highlights that there was considerable correlation among predictor variables, which can

cause instability problems in standard regression approaches. This demonstrates the utility of the BMA approach: If we had selected a single best-performing model, rather than averaging predictions over many models, we would have selected a single predictor – NDA responding in the FR5 session (i.e., leftmost column of Figure 2b). Predictions of this single-predictor model accounted for approximately half as much variance in relapse scores as the BMA model (25%).

To test whether the BMA model was over-fitting the data, we performed out-of-sample validation with an additional sample of rats ($n=6$) that served as vehicle controls in a previously published experiment (James et al., 2014) but otherwise experienced the same protocol as described above. The BMA model predicted the out-of-sample data well, explaining 55% of the variance in out-of-sample relapse scores. Although this relationship is somewhat unstable given the small sample size, it is of reasonable magnitude and in the expected direction, which suggests the BMA model has good generalization properties.

Using the BMA model we generated a ‘predicted reinstatement score’ (Table, see S2) for the rats in experiment 2. Using this predicted reinstatement score, as well as the scores from NDA and PR tests, we phenotyped rats as addiction-prone or addiction-resilient (Figure 2c-f). Addiction-prone rats showed significantly higher predicted reinstatement scores ($t_{14}=8.608$, $p<0.001$), NDA responding (Mann-Whitney $U=0$, $p=0.0002$) and PR breakpoints (Mann-Whitney $U=0$, $p=0.0002$) than resilient rats.

Importantly, no change was observed in cocaine consumption between the addiction-resilient (357mg/kg) and addiction-prone (449mg/kg) ($p>0.05$). These results indicate that the BMA model reliably classified rats as addiction-prone or addiction-resilient, despite the rats not having a reinstatement session.

Addiction-prone rats exhibit increased miR-212 in the DMS early in the addiction cycle

We compared the expression of candidate miRNA in striatal subregions of animals characterised as 'addiction-prone' to that of 'addiction-resilient' animals following self-administration training (Figure 3). At this time point, addiction-prone rats exhibited significantly elevated levels of miR-212 in the DMS (Mann-Whitney $U=11$, $p=0.028$; Figure 3c). No other differences in miRNA expression were observed in all other subregions at this time point ($p's>0.05$).

Addiction-prone rats exhibit increased miR-101b, miR-137, miR-212 and miR-132 in the NAcSh and increased miR-137 in the DLS late in the addiction cycle

We next examined the striatal expression of the same candidate miRNA in addiction-prone rats following extinction and reinstatement testing (Figure 4). In the NAcSh, miR-101b expression was significantly higher in addiction-prone versus resilient controls ($t_{5.323}=2.852$, $p=0.03$) (Figure 4b). A trend towards increased miR-101b expression was also observed in the NAcC ($t_{10}=2.181$, $p=0.054$). Addiction-prone animals exhibited significantly higher levels of miR-212 ($t_{10}=4.574$, $p=0.001$) and the closely related miR-

132 ($t_{10}=2.97$, $p=0.014$) in the NAcSh, with no change in the NAcC. Interestingly, the expression of schizophrenia risk gene miR-137 was significantly higher in the NAcSh ($t_{10}=2.535$, $p=0.03$) but not NAcC of addiction-prone animals.

We have previously shown that addiction-prone rats exhibit increased expression of a number of miRNA in the DMS, but not DLS, of addiction-prone rats following relapse (Quinn et al., 2015). However, we have not yet assessed the expression of miR-137 in the DS subregions post-relapse. Given that change in miR-137 observed following drug taking in the DMS, we assessed its expression in the DS subregions post-relapse in addiction-prone rats. We found increased miR-137 expression in the DLS ($t_6=2.38$, $p=0.05$) of addiction-prone rats, with no change observed in the DMS (Figure 4d, e).

miR-101b regulates Drd1 expression in vitro

To validate a potential functional interaction between miR-101b and *Drd1*, we used a luciferase reporter assay. We used antisense (AS) miRNA inhibitors to bind miR-101b in cells co-transfected with the *Drd1* binding site or miRNA recognition element (MRE) and a luciferase gene. Relative luciferase activity was increased by 17% when transfected with AS-101b compared to AS control ($t_5=2.05$, $p=0.048$) (Figure 5). These data suggest that inhibition of miR-101b leads to de-repression of the *Drd1* MRE, indicating that miR-101b has the potential to regulate its cognate recognition elements in *Drd1*.

DISCUSSION

We show that a history of cocaine self-administration changes the expression profile of a number of miRNAs in a regionally discrete manner within the striatum, with the most marked changes occurring in the NAcC. These findings align closely with previous demonstrations that the NAcC is highly sensitive to drug-evoked changes in synaptic plasticity. Because a major goal of addiction research is to understand the molecular mechanisms that underlie vulnerability to addiction, we next examined the striatal miRNA expression profiles of rats identified as exhibiting behaviors that resemble the essential diagnostic criteria for addiction (referred to as ‘addiction-prone’ rats). We show that early in the addiction cycle, addiction-prone rats show significantly increased levels of the addiction-related miRNA-212 in the dorsomedial striatum, a region implicated in driving initial goal-directed responding for drug. When we examined miRNA profiles at a later time point – following extinction and relapse testing – addiction prone rats showed changes in miRNA expression in regions associated with habitual responding for cocaine. Together, these findings indicate that levels of addiction-related miRNAs change in a spatially and temporally defined manner in addiction-prone animals, and that these changes closely track addiction stage.

Effect of cocaine exposure on striatal miRNA expression

Surprisingly, to our knowledge, a comprehensive characterisation of the effects of cocaine self-administration on miRNA expression in functionally segregated regions of the striatum has not previously been reported. To address this, we trained animals to

respond for cocaine over a period of ~4 weeks and examined the expression of candidate miRNAs shown to regulate drug-seeking behavior in ventral and dorsal striatal subregions. Compared to saline-yoked controls, cocaine-exposed animals showed a marked reduction in levels of miR-101b, miR-137 and miR-132 in the NAcC, with no such changes observed in the NAcSh. We also observed that cocaine exposure was associated with a reduction in miR-137 in the DMS, but had no effect on other miRNAs examined in either the DMS or DLS. Cocaine-induced changes in miRNA expression focused on NAcC are consistent with previous evidence that this region exhibits marked changes in synaptic plasticity following cocaine exposure (Conrad et al., 2008) and the suspected role of the NAcC in the conditioned reinforcing effects of abused drugs (Ito et al., 2004). Reductions in miR-101b, miR-137 and miR-132 in this NAcC may allow for an increase in the range of plasticity-associated gene expression in this region (discussed below), contributing to molecular and synaptic sequelae that reinforces cocaine seeking-behavior.

miRNA expression in addiction prone rats across the addiction cycle

There is increasing recognition that addiction cannot be explained by drug exposure itself, but rather through an interaction between drug experience and individual genetic vulnerability (Deroche-Gamonet et al., 2004; Everitt and Robbins, 2005; Nestler, 2001). Indeed, the inherent compensatory neuroadaptations in the brain are remarkably powerful at preventing the development of addiction as only 20% of drug users develop

what is defined as addicted (Anthony et al., 1994). We therefore used an established phenotyping approach to compare striatal miRNA expression profiles in addiction-prone versus addiction-resilient rats at two time points across the addiction cycle. We first examined miRNA profiles following approximately four weeks of self-administration training. We show that addiction-prone animals exhibit significantly greater levels of miR-212 in the dorsomedial striatum following 4 weeks of cocaine self-administration training. No differences were observed in miRNA expression in any other region examined. We next examined the striatal profile of miRNA expression in addiction-prone rats that were subjected to extinction training and cued-reinstatement testing. At this later time point, addiction-prone rats show significantly increased levels of all miRNAs examined in the NAcSh but not the NAcC. We also show addiction prone rats exhibit significantly increased expression of miR-137 in the DLS, but not in the DMS. This is in addition to our previously reported findings that these rats show increased levels of miR-101b, miR-132 and miR-212 and miR-431 in the DLS following extinction and reinstatement testing (Quinn et al., 2015). In contrast, an opposite pattern of miR-212 expression was seen in the DMS at this time point in addiction-prone rats (Quinn et al., 2015). With respect to the dorsal striatum, these results suggest that specific changes in miRNA expression in addiction prone rats may promote a shift from the DMS early in the addiction cycle, towards the DLS following further training and during extinction and relapse.

The distinct changes in miRNA expression across the addiction cycle are interesting given the divergent roles of striatal subregions in drug seeking. In the ventral striatum, studies generally support a transfer of information from the shell to the core – signals that may then gain access to the dorsal striatum through the core's descending projections to the midbrain dopamine cells (Haber et al., 2000). Data also indicates that synaptic changes occur in the NAc core and shell on different time scales (Jedynak et al., 2016; Kourrich et al., 2007; Kourrich and Thomas, 2009; Marie et al., 2012; McCutcheon et al., 2011; Rothwell et al., 2011). However, information flow from NAcC to NAcSh has also been predicted based on studies using protein synthesis inhibitors in the core - which prevents increased spine density formation in the NAcSh (Marie et al., 2012). Further, there is strong evidence that extended drug self-administration is associated with molecular and cellular changes that promote increased synaptic strength in NAcSh (Anderson et al., 2008; James et al., 2014; Mameli et al., 2009; Wolf and Ferrario, 2010). This may reflect the strengthening of associations between the hedonic properties of the drug and the environmental context, as NAcSh is important for the expression of context-driven drug-seeking (Bossert et al., 2007; James et al., 2014). Furthermore, with extended training NAcSh versus core neurons appear to respond to changes in reward outcome and modulate behaviour accordingly (Saddoris et al., 2011). Consistent with these findings, we show that late in the addiction cycle (i.e., post-extinction and relapse), all addiction-related miRNAs that we examined were

upregulated within the NAcSh, but not NAcC, in addiction-prone animals. Such differences were not observed immediately following self-administration training, which may reflect a gradual increase in the expression of these miRNAs in the NAcSh during extinction training, or an acute increase in response to reinstatement of drug seeking driven by drug-associated discriminative stimuli. Further studies are required to fully clarify the precise temporal pattern of these molecular changes.

Subregional differences in the dorsal striatum have also been described. DMS is thought to drive responding during early reward-seeking when behavior is outcome driven, whereas the DLS becomes increasingly engaged as reward-seeking transitions to a habitual, stimulus-driven behaviour (Yin et al., 2004; Yin et al., 2006; Yin et al., 2005b). The development of addiction is often described as a transition from casual drug taking to their compulsive and habitual pursuit, despite the negative associated consequences (Ostlund and Balleine, 2008). Indeed, when cocaine delivery is paired with an aversive conditioned stimulus, animals given limited access to cocaine suppress their behavior, whereas animals given extended access continue to respond (Vanderschuren and Everitt, 2004). This process may involve a gradual devolution of control from the DMS to DLS with prolonged drug use. We show here that an early time point, addiction-prone animals exhibited changes that were most pronounced in the DMS. This temporal profile points to an early engagement of DMS that may contribute

to the shift in control of behaviour to the DMS across the addiction cycle in rats identified as exhibiting key characteristics of addiction.

Previous studies have implicated the miR-132/miR-212 cluster in compulsive cocaine behaviour (Hollander et al., 2010; Im et al., 2010). miR-212 and miR-132 levels are elevated in the dorsal striatum in animals given extended access (6hr/day) to cocaine, a paradigm shown to promote compulsive cocaine seeking (Hollander et al., 2010; Im et al., 2010). Further, overexpression of miR-212 in DS reduced cocaine taking, whereas its knockdown promoted an increase in drug seeking behavior (Hollander et al., 2010). Consistent with these findings, addiction-prone rats that show high levels of compulsive responding during FR and PR training exhibit increased miR-212 expression in the DMS early in the addiction cycle. Interestingly, this pattern is reversed following extinction and relapse testing, with addiction-prone rats exhibiting decreased miR-212 within the DMS at this time point (Quinn et al., 2015). Although speculative, this may reflect an exhaustion of the neuroprotective effects of miR-212 in the DMS and a subsequent footprint of the changes that determine the devolution of control to the DLS over the addiction cycle in addiction-prone rats. Similarly, increases in miR-212 and miR-132 in the NAcSh of addiction prone rats may reflect shell activity associated with the presentation of contextual stimuli previously associated with drug availability. Further studies are required to understand if interruption of miR-212 and miR-132 signalling in either the DMS or NAcSh is protective against reinstatement of drug-seeking.

Similar to miR-212, a distinct expression profile of miR-137 was observed in striatal subregions across the addiction cycle. Variants in the MIR137 gene locus are associated with increased schizophrenia risk (Yin et al., 2014), a condition that is highly comorbid with addiction (Buckley et al., 2009). Further, recent evidence implicates miR-137 as an important regulator of presynaptic plasticity. For example, Siegert et al. (2015) showed that miR-137 over-expression significantly impaired the induction of LTP in the hippocampus (Siegert et al., 2015). Disrupted hippocampal-dependent contextual fear learning was also observed in mice over-expressing miR-137, providing a behavioral surrogate for impaired LTP (Siegert et al., 2015). Moreover, miR-137 regulates gene expression in the presynapse including synaptotagmin-1 (*Syt1*), N-ethylmaleimide sensitive fusion protein (*Nsf*) and complexin-1 (*Cplx1*), proteins important for vesicle trafficking and neurotransmitter release (Siegert et al., 2015). Our finding that cocaine exposure decreased miR-137 in the NAcC and DMS aligns closely with evidence that cocaine exposure substantially enhanced presynaptic drive to medium spiny neurons in the DMS (Corbit et al., 2014). Further, increased DLS miR-137 in addiction-prone rats following relapse testing may reflect altered pre-synaptic plasticity within this region, resulting in inflexible, habitual behavior.

Finally, we were interested in the expression of miR-101b, as this miRNA is a known regulator of *mTOR*, a plasticity-associated kinase that regulates drug motivated behavior (James et al., 2014; James et al., 2016; Neasta et al., 2010; Quinn et al.,

2015). Our observation that self-administration of cocaine decreased miR-101b in the NAcC aligns closely with previous findings from our laboratory and others' that cocaine and alcohol increase mTOR levels in the NAc (James et al., 2014; Neasta et al., 2010). Similarly, increased levels of miR-101b in the NAcSh in addiction-prone rats following relapse supports our previous observation that these rats show deficits in mTOR in this ventral striatum (Brown et al., 2010). We also identified miRNA-101b as a potential regulator of *Drd1*, a relationship that we confirm here via luciferase assay. NAcC dopamine release has been shown to be important in the acquisition of conditioned stimuli, and infusions of *Drd1* antagonists have been shown to disrupt memory consolidation for conditioned stimuli (Di Ciano et al., 2001). The decrease in miR-101b that we observed as a result of cocaine experience may result in a subsequent increase in *Drd1* expression, which may facilitate the acquisition of cocaine-stimuli associations. In contrast, our finding that addiction-prone rats display increased miR-101b expression in the NAc following relapse testing is highly consistent with previous rat and primate studies that report reduced *Drd1* expression in the striatum following extended cocaine use (Nader et al., 2002). Therapies that target miRNA-101b may therefore be useful for addressing pathological changes in both striatal mTOR and *Drd1* levels in individuals at high risk of relapse.

Conclusion

In summary, we show that cocaine exposure results in significant suppression of addiction-relevant miRNA expression within the NAcC and DMS. These changes may reflect compensatory responses to the drug itself that permit increased expression of plasticity-associated genes responsible for modulating drug-evoked plasticity. We also show that in addiction-prone rats, striatal miRNA expression levels change in a spatially and temporally defined manner according to addiction stage. Specifically, as the addiction cycle progresses, the expression of miR-101b, miR-137, miR-212 and miR-132 tend to increase in the NAcSh and DLS of addiction-prone rats. We suggest that these changes may produce deficits in plasticity within these regions, in turn promoting habitual drug seeking. Alternatively, these changes may reflect neuroadaptations in response to extinction training. It will be important that future studies seek to clarify these processes. In addition, further understanding of the mechanisms by which these miRNA influence synaptic plasticity may lead to the identification of therapeutic targets to aid in recovery from drug addiction.

Authors contribution

RKQ, MHJ, and CVD were responsible for the study concept and design. RKQ and ALB contributed to the acquisition of animal data. GEH and AH performed the BMA analysis. RKQ performed qPCR and luciferase assay. DWS and MJC assisted with data analysis and interpretation of findings. RKQ, MHJ and CVD drafted the manuscript. GEH, DWS,

and MJC provided critical revision of the manuscript for important intellectual content.
All authors critically reviewed content and approved final version for publication.

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Figure 1

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Figure 2

Figure 5 This article is protected

Figure Legends

Figure 1: Changes in miRNA expression in striatal subregions with

cocaine use. To assess changes during the drug-taking phase of the addiction cycle (A) rats were first implanted with an i.v. catheter and trained to self-administer cocaine (0.25 mg/0.1ml i.v.) on an FR1 schedule of responding which progressed to an FR3 and finally an FR5 schedule of responding. Rats were tested for NDA responding for 10 sessions, followed by 5 consecutive sessions of progressive ratio (PR) testing. Rats were sacrificed twenty-four hours following final PR session. MiRNA expression is altered in the NACc (B) and DMS (D) but not NACsh (C) or DLS (E) of cocaine (n=16) versus saline rats (n=7). * $p < 0.05$; ** $p < 0.01$. Data presented $\pm$ SEM.

Figure 2: Bayesian Model Averaging analysis identifies addiction-prone

rats. (A) The y-axis shows the 23 predictor variables where the vertical space occupied by each predictor represents the posterior inclusion probability (PIP) for the variable, so a larger space represents a better predictor. The x-axis shows the cumulative model probability, where the leftmost model has the largest weight sorted to lower weights on the right. Coloured squares code which predictors (y-axis position) were included in each model (x-axis position). Blue and red fill respectively represent positive and negative regression coefficients in a model. For simplicity, only the 10 highest PIP predictors (y-axis) and the 200 highest ranked models (x-axis) are shown. (B) Observed versus predicted relapse scores (x- and y-axis, respectively). Rats phenotyped as vulnerable and resilient are plotted with square and triangle symbols, respectively. Remaining rats are plotted with crosses. Using this

BMA model we identified animals that showed addiction-relapse vulnerability. Animals classified as addiction-relapse vulnerable showed greater active lever pressing in non-drug available periods (C) and higher progressive ratio breakpoint (D) and significantly higher predicted relapse scores (F). No changes in cocaine consumption were observed between groups (E).

*** $p < 0.001$. Data presented $\pm$ SEM. $n = 8/\text{group}$

Figure 3: Changes in miRNA expression in addiction vulnerable versus resilient rats during drug taking. Animals phenotyped as addiction vulnerable using the BMA model displayed altered miR-212 expression the DMS (C) compared to addiction resilient controls. * $p < 0.05$ Data presented $\pm$ SEM. $n = 8/\text{group}$

Figure 4: Changes in miRNA expression in addiction vulnerable versus resilient rats following reinstatement. (A) To phenotype animals into addiction vulnerable or resilient groups, animals were first implanted with an i.v. catheter and trained to self-administer cocaine (0.25 mg/0.1 ml i.v.) on an FR1 schedule of responding which progressed to an FR3 and finally an FR5 schedule of responding. To assess changes following relapse rats were tested for NDA responding for 5 days. Following this animals were tested for motivation to consume drug using a progressive ratio test, followed by a final FR5 cocaine session. Drug-seeking behaviour was then extinguished by exposing animals to the operant chamber. Drug was absent and lever pressing did not result in a drug reward. Once responding returned to baseline levels, animals were re-exposed to cues associated with drug availability in a reinstatement test and sacrificed 24 hours later. For detailed description of

behavioural training and phenotyping see Brown et al. 2010. For both groups, animals that scored in the top third of the distribution for each behavioural test were deemed to be addiction vulnerable, while those in the bottom third of the distribution were identified as addiction resilient. Following reinstatement, rats phenotyped as addiction-relapse vulnerable show changes in miRNA expression in the NACsh (C) compared to addiction resilient controls, with no change in the NACc (B). miR-137 expression was altered in the DLS (E), but not DMS (D) of addiction vulnerable rats following reinstatement.

Figure 5: miR-101b regulates *Drd1* expression in vitro. AS-101b increased luciferase activity when co-transfected in HEK-293 cells with *Drd1* MRE luciferase construct, suggesting miR-101b has the ability to regulate *Drd1* expression in vitro. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ Data presented +/- SEM. n=6/group

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