Brain Region and Cell Type Specific Approaches to Study Drug Abuse

Dissertation

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By

Bartholomew J Naughton IV, B.S.

Graduate Program in Neuroscience

The Ohio State University

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Dissertation Committee:

Howard Gu, Advisor, PhD

Rene Anand, PhD

John Oberdick, PhD

Wolfgang Sadee, PhD

Susan Cole, PhD

Abstract

Drug dependence is a persistent problem throughout the world. Once addicted to a drug, many users have difficulty quitting use, even when they desire to stop using. This substance dependence is both psychological as well as neurophysiological. In 2008 the U.S. Department of Health classified 22 million Americans as having a significant degree of drug dependence. Additionally, the U.S. Office of National Drug Control Policy stated that the health care costs in 2004 for substance abuse was estimated to be comparable to cancer. There is therefore undoubtedly both an individual and societal need for therapeutic interventions in drug dependence.

While a great deal is known about the molecular action of these addictive substances, very little is understood about the underlying neurocircuitry of addiction. The process of addiction is a learned behavior and is not solely driven by homeostatic adaptations to the drug itself. Psychoactive drugs overrun the natural reward circuitry of the brain, forcing maladaptive learning of drug associated rewards which then become overvalued in comparison to natural rewards. This dissertation approaches the study of drug addiction using two separate techniques: one regionally specific to the nucleus accumbens, and one cell type specific to dopaminergic and GABAergic neurotransmitter systems of the central nervous system.

All drugs of abuse increase striatal dopamine release despite their widely varied mechanisms of action. The ventral striatum primarily consists of the Nucleus Accumbens (NAc), which has long been thought of as the origin of addiction-like behaviors. As such, the NAc has

been proposed to be one of the primary reward centers of the brain. Utilizing viral delivery of short-hairpin RNA (shRNA) into the NAc, knockdown of specific subtypes of receptors can be achieved. In this manner, behavioral testing can investigate changes in rodent addiction-like behavior following region specific knock down of dopamine receptor isoforms.

The widespread dopaminergic connections leaving the ventral tegmental area (VTA) modulate the glutamatergic and GABAergic connections that instigate reward valuation and associative memory formation. An integrated view of these circuits is needed in order to better understand the progressive neurophysiological changes that are occurring in addiction. Bac transgenic techniques can be used to generate mouse lines with inducible Cre expression specifically within dopaminergic or GABAergic neuronal cell types. Utilizing these mouse lines, both neurotransmitter systems can be characterized in a manner never before possible.

In conclusion, this dissertation shows that techniques can now be employed to begin studying the neurocircuitry of addiction with regional or cell type specificity. The method for rapid screening of shRNA that is presented here will expedite research using transcriptional regulation. Our results on D2L knockdown of the dopamine receptor within the nucleus accumbens are an informative first pass at attempting to differentiate these near indistinguishable splice variants. The experimental mouse models generated by our bac recombineering will advance research into the role that these specific neurotransmitter systems are playing in addiction. Further understanding the brain regions and neurotransmitter systems associated with drug addiction can lead to potential pharmacotherapeutics.

Dedication

To BLT

Thank you, always, for keeping my head up.

Acknowledgements

I would like to thank my advisor, Dr. Howard Gu, for being a wise and patient mentor. Your calm poise and guidance has shown me the appropriate way to approach research, as well as life. You took me into your lab as lab-less lost soul, and molded me into an excellent scientist. The direction you gave me was a constant reminder that it is not only the end, but the journey itself which teaches us the greatest lessons. You taught me to embrace science with the energetic mind of a child, and only asked that I did so with conscientious caution. Thank you for the opportunity to be your student and for taking the time to be my greatest teacher.

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I am thankful to faculty members, students, and staff in the Pharmacology department and within the Neuroscience Graduate Studies program for their assistance and generosity. Special thanks to Sherry Ring for helping me with my computer software issues.

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Thanks to Stephanie and Larry Gilbert, Bart, Cassie, Kitt and Donna Naughton, Dan, Danny and Mark McGown. You have always been there for me. In memory of Doris Naughton, you told me I could and I did, I only wish you could have seen it.

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Vita

2004 ……...Undergraduate Summer Intern Johns Hopkins Applied Physics Lab
2005 …….. B.S., Molecular Genetics, Ohio State University
2005-2008 ……. Graduate Research Assistant, AC DeVries Lab, Ohio State University
2008-2011 ……. PhD Candidate, HH Gu Lab, Ohio State University

Publications

BJ Naughton, DD Han, HH Gu. "Fluorescence based evaluation of shRNA efficacy Analytical Biochemistry" *Analytical Biochemistry 2011*

B Martin, BJ Naughton, K Thirtamara-Rajamani, D Yoon, D Han, AC DeVries, HH Gu. "Dopamine transporter inhibition is necessary for cocaine-induced increases in dendritic spine density in the nucleus accumbens" *Synapse* 2010

Fields of Study

Major Field: Neuroscience

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Chapter 1: Fluorescence based screening of shRNA as a method of estimating *in vivo* efficacy

N.B. Portions of this chapter were published: B.J. Naughton, Dawn D. Han and Howard H. Gu: Fluorescence based evaluation of shRNA efficacy. *Analytical Biochemistry* 2011

1.1 Abstract

RNA interference is a cellular mechanism regulating levels of mRNAs. It has been widely exploited to knockdown specific protein targets. The selected interfering RNA sequence greatly influences its ability to knockdown the target. Here we present a method for constructing multiple testing plasmids which express small hairpin RNAs (shRNA) targeting different regions of an mRNA. A simple fluorescence test in cultured cells allows convenient evaluation of mRNA knockdown by many different shRNAs on 96-well plates. We show that software predicted shRNAs have varying efficacies and only 2 of the 7 tested shRNAs significantly knocked down their targets. Using this *in vitro* screening method, one can choose a shRNA sequence that is more likely to work effectively *in vivo*. This technique was used to find the most effective viral genome prior to packaging into adeno associated viral particles (AAV) and beginning *in vivo* and behavioral testing of D2 dopamine receptor knockdown. Ultimately, we were able to find shRNA sequences which were able to non-specifically knock down both isoforms of the D2 receptor (shRNA D2L/S) and additional sequences capable of specifically knocking down the D2L isoform (shRNA D2L) by targeting exon 6, which is only included in this isoform. Despite

computer predictions however, no sequence tested was found to effectively knock down the D2S isoform (shRNA D2S). The only unique portion of D2S was the junction between exon 5 and exon 7, the rest of this mRNA was identical to D2L. Therefore targeting D2S without affecting D2L was very difficult, and in our experiments did not prove possible.

1.2 Background

RNA interference is a biological process which allows for the controlled degradation of specific mRNA targets. Researchers can directly manipulate this endogenous activity in order to silence gene expression. This is directed by sequence specificity of small-interfering RNA (siRNA) to an mRNA target. In this manner, RNA interference has allowed researchers to investigate the functions of specific proteins and their roles in various disease models. Small hairpin RNA (shRNA) is similar to siRNA, but can be expressed continuously in a plasmid or a viral vector. The hairpin secondary structure, when designed properly, can increase the effectiveness of the shRNA at knocking down its target (Cheng and Chang 2007). Bioinformatic studies have produced rules for selecting the sequences of siRNA. Computer programs that utilize these rules are available for predicting the sequences that are likely to be effective. However, some of the predicted sequences are much more effective than others and validation is necessary. Frequently used methods to confirm effective RNA knockdown include qPCR, flow cytometry, western blot or immunhistochemical techniques, which can be tedious and time consuming (Martinez, Naguibneva et al. 2002; Paddison, Caudy et al. 2002; Paddison, Silva et al. 2004; Kamio, Hirai et al.). More convenient, rapid methods of evaluating shRNA efficacy would greatly expedite interfering RNA research.

The design of shRNA usually starts with using computer programs to predict the sequence of siRNA that will likely be able to knockdown a specific target. There are also a number of commercial sources available where shRNAs can be purchased. However, novel shRNAs from these sources are made based on software predictions and have not been validated. We have developed a system for easily making a large number of shRNA constructs and screening for those with the highest efficiencies of knocking down targets expressed in cultured cells. We used this method to design shRNA against the individual splice variants of the D2 dopamine receptor.

The D2 Dopamine receptor can be translated into two different splice variants, a long isoform (D2L) which contains the 29 base pair exon 6, or the short isoform (D2S) which does not (Moyer, Wang et al. 2011). These receptors are proposed to have very different functions and localizations (Guiramand, Montmayeur et al. 1995; Boundy, Lu et al. 1996). However, they cannot be differentiated by immunhistochemical or pharmacological methods, and the transgenic knockout animals in which both isoforms (D2-/- mice) or specifically the long isoform (D2L-/- mice) were removed have been difficult to interpret (Wang, Xu et al. 2000; Holmes, Lachowicz et al. 2004). Our ultimate goal was to design one viral vector that delivered shRNA that non-specifically knocked down both isoforms of the D2 receptor (shRNA D2L/S) and to also generate two additional vectors capable of specifically knocking down either the D2L isoform (shRNA D2L) or the D2S isoform (shRNA D2S). To bypass having to screen for specificity and efficacy in vivo, we developed a method in which green fluorescence protein (GFP) expression could be actively correlated with knockdown of the target isoform. By doing so, we were able to screen for sequences that were specific to one isoform over the other.

1.3 Materials and methods

1.3.1 Construction of the GFP-cDNA reporter constructs

In order to screen for knockdown efficacy using GFP as a reporter, we designed reporter plasmids which would be co-transfected along with our shRNA plasmids. A cDNA fragment of a target mRNA (D2L or D2S), containing the majority of the cDNA sequence, was fused with green fluorescence protein (GFP) cDNA to form a single transcript. Our GFP reporter vector contained a CMV promoter followed by a nonstable GFP (GFPns) cDNA and a BGH polyA signal. We modified pcDNA3 (Invitrogen, Carlsbad, CA) by inserting D2L or D2S cDNA into the NotI and XbaI sites. We then inserted GFPns via BamHI. The end result of these modifications was pCMV driven expression of GFPns followed immediately by cloned D2L or D2S cDNA, all within a single transcript. The plasmid expressed a single transcript with GFPns being translated into protein and the targeted cDNA lying just after the polyA signal. This cDNA was located after the 'stop"signal, and was not translated. Therefore, this targeted cDNA acted as the 3' untranslated region (3'UTR) (Figure 1.1). Since the degradation of the target (D2L or D2S) by the shRNA complex would also degrade the GFPns mRNA, this results in a direct reduction in GFPns expression, which can be actively visualized across several days. Importantly, GFPns was

Figure 1.1

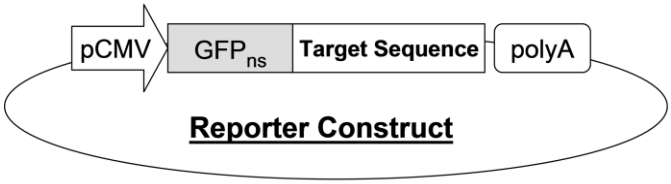

Figure 1.1: Reporter construct design Reporter construct design. A CMV promoter drives expression of a single transcript containing the GFPns coding sequence followed by a piece of the target cDNA sequence and a polyA stop signal. The cDNA sequence acts as a 3'UTR and only GFPns protein is synthesized.

used, this form of GFP has a shorter half life and thus reflects the mRNA knockdown more readily (Andersen, Sternberg et al. 1998). This allowed more active visualization of knockdown, since GFP or enhanced GFP (eGFP) would build up in the cells, and changes in protein levels may not have been evident even after hindering translation.

1.3.1 Construction of the shRNA expressing construct

Our shRNA expressing construct was derived from pTRIPZ shRNAmir vectors (Open Biosystems, Huntsville, Al)(Paddison, Caudy et al. 2002; Paddison, Caudy et al. 2002). First, a modified pTRIPZ vector was created that could be readily digested, and rapidly ligated with shRNA as a small double stranded DNA insert. In order to accomplish this, pTRIPZ was first digested with BspQ1 (SapI) and NcoI, followed by klenow fill in and self ligation. This removed the only BspQ1 restriction enzyme site that existed within the original plasmid. Norepinephrine transporter cDNA was amplified from mouse (mNET) using bspF (TTCTCGAGGTATATTGCTGTTGACAGTGAGCGTGAAGAGCCACAGTGTGGAAGATCTGCC) and bspR (TTGAATTCCGAGGCAGTAGGCATGAAGAGCGGCTTGAAGTTGATGATG) primers. The resulting gel purified amplicon was then cut with EcoRI and XhoI, followed by gel purification of the 1.3Kb fragment. Following digestion of the modified pTRIPZ by EcoRI and XhoI, it was treated with cip enzyme, and the 7.8Kb band was gel purified. The modified pTRIPZ was then ligated to the mNET fragment. This effectively inserted mNET, and the BspQI restriction enzyme sites that it contained, directly in between the 5'mir30 and 3'mir30 regions of pTRIPZ (these elements are described briefly below). mNET was inserted into this construct simply to allow for easy separation via gel purification, following digestion with BspQ1. This is the portion of the viral

genome that is designed to express shRNA. Once digested with BspQ1 and gel purified, the double digested vector (7.8kb) then has two incompatible 5' overhang ends and can be easily isolated from any single digested vector, thus eliminating self-ligation background in later procedures.

Plasmids expressing shRNA were constructed by inserting annealed oligonucleotides with desired sequences into the BspQI restriction site of our shRNA expressing vector. Our shRNA was designed by using freely available siRNA design software, siRNA wizard (v3.1, in vivogen). This was applied to the shRNA backbone provided by the pTRIPZ vector. To do this, we designed a single set of primers containing this full sequence, and then did a simple annealing reaction (95˚C to 50˚C in 3 minutes by 0.3˚C increments) in order to make a small double stranded insert, with BspQ1 restriction fragment overhang ends. Example:

5'- gcg**CTGGGAGTTTCCCAGTGAACAG***TAGTGAAGCCACAGATGTA***CTGTTCACTGGGAAACTCCCAT** -3'
3'- **GACCCTCAAAGGGTCACTTGTC***ATCACTTCGGTGTCTACAT***GACAAGTGACCCTTTGAGGGTA**acg -5'

This short insert contains the target specifying sequences (underlined, which can be replaced with other siRNA sequences) and the microRNA-30 loop (in italics; which would remain the same regardless of the siRNA sequence used). Appendix A contains all of the primers used to generate shRNA which were used for the purpose of knocking down both isoforms of the D2 receptor (shRNA D2L/S), knocking down the D2L isoform specifically (shRNA D2L) or the D2S isoform specifically (shRNA D2S). We used the shRNA designing software in order to find potential shRNA sequences which would recognize the regions of D2 mRNA homologous between both isoforms of the receptor (shRNA D2L/S). Additionally, shRNA sequences were designed to have homology to exon 6, the exon which is only included in the D2L isoform (shRNA D2L). Finally, shRNA sequences were designed to target the junction between exon 5 and exon 7 of the mRNA, which would only be present in the D2S isoform of the receptor (shRNA D2S). FOr each

purpose approximately 7 sequences were chosen, and therefore 7 seperate shRNA expressing constructs were cloned and tested.

Figure 1.2

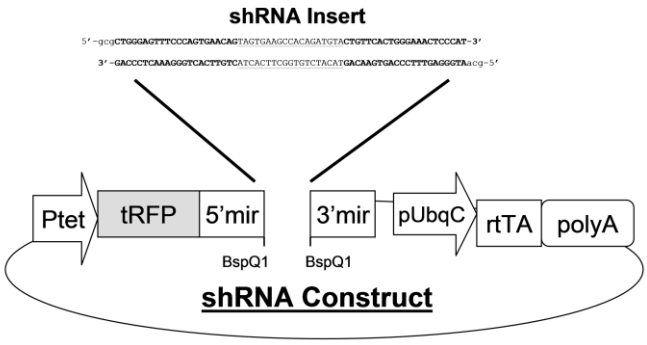

Figure 1.1: shRNA construct design Design of the shRNA expressing construct. A tetracycline-inducible promoter (TetO) drives the expression of the RFP-shRNA transcript with the shRNA acting as the 3'UTR. Different double stranded shRNA sequences with compatible ends, made by annealing pairs of oligonucleotides, can be readily ligated into the two BspQ1 restriction sites between the 5'mir30 and 3'mir30 regions. The Ubiquitin C driven reverse transactivator (rtTA) is also in the plasmid, which is necessary for inducible expression.

Figure 1.2 shows the major components of the plasmid. A tetracycline-inducible promoter (TetO) was used to drive the expression of turboRFP (tRFP) (Merzlyak, Goedhart et al. 2007) with a shRNA structure as its 3'UTR. The expression level of tRFP allows one to track the expression levels of the shRNA. The shRNA structure contains the 5' and 3' flanking sequences (5'mir, 3'mir) and the loop sequences of microRNA-30 because these sequences have been shown to significantly increases the Drosha and Dicer processing of the expressed hairpins and thus the effect of RNA interference (Paddison, Silva et al. 2004; Silva, Li et al. 2005). These elements are important for the proper formation of shRNA secondary structure, which in turn decides its stability and incorporation into the Dicer mRNA digestion protein complex. The double stranded stem portion of the shRNA defines target specificity. Our modified pTripz vector also contains the 5' and 3' long terminal repeats (LTR) of the adeno associated virus (AAV). In the presence of a plasmid containing viral packaging genes, the DNA between these LTR's could be packaged into virus particles, without the need for further subcloning.

The intensity of green fluorescence expressed by the reporter plasmid allows tracking of the target mRNA knockdown. Once the degradation complex that contains the shRNA sequence

recognizes the target cDNA, the entire transcript is degraded, including the GFP portion of the mRNA (Valencia-Sanchez, Liu et al. 2006). Therefore, the extent of green fluorescence reduction indicates the effectiveness of the shRNA at knocking down its mRNA target.

1.3.2 Transfection of Chinese Hamster Ovary (CHO) cells

Chinese Hamster Ovary (CHO) cells (Invitrogen, Carlsbad, CA) were used for all in vitro studies. Lipofectamine (Inivtrogen, CA) was used for all transfections, and was done according to the manufacturer's protocol. Each shRNA containing plasmid was transfected in a 1:1 ratio with the chosen reporter construct. All transfections were done in triplicate. The target plasmid and a shRNA plasmid were cotransfected into cultured cells in 2 sets of wells of a dish and doxycycline is added to one of the 2 sets to induce RFP-shRNA expression. The viral genome vectors express both shRNA and RFP upon induction with doxycycline. Cells in the uninduced wells exhibited strong green fluorescence but no red fluorescence, while cells in the induced wells will show strong red fluorescence and reduced green fluorescence as compared to the uninduced control wells, if the shRNA is efficacious.

1.3.3 Relative quantification of shRNA efficacy

Two days after transfection/induction, GFP pictures were taken of GFP expression using a fluorescent microscope. Using ImageJ (National Institute of Health, MD) analysis, one can quantitatively represent this reduction in fluorescence. Non-induced controls

were pooled for statistical purposes. One-way ANOVA with Tukey post-hoc analysis (SPSS 17.0, Somers, NY) was used to investigate significance of shRNA efficacy in reducing GFP-reporter expression as compared to uninduced wells GFP expression ($p < 0.05$ considered significant).

1.4 Results

We tested 7 or 8 different shRNA sequences for their ability to knockdown either both isoforms (D2L and D2S) or to specifically knock down one isoform (D2L or D2S) of the D2 receptor. Figure 1.3 shows a test of shRNA efficacy using representative pictures of wells transfected with D2L and D2S reporter constructs. These wells were also transfected with shRNA expressing constructs designed to either knockdown D2L and D2S, or to specifically knockdown D2L only. RFP expression is only visible in wells with doxycycline induction (lower panels), and GFP expression is substantially lower as the D2L specific shRNA degrades the GFP-D2L fusion mRNA transcript, compared to those wells without induction (Figure 1.3D versus 1.3A). This effect was isoform specific, as GFP expression from a GFP-D2S construct was not affected by the same shRNA construct. Figure 1.3F is the merged image of GFP and RFP images, showing that most RFP expressing cells had no or low GFP expression, indicating efficient D2L knockdown. Also shown in Figure 1.3F, a few cells expressed both GFP and RFP (yellow cells) indicating insufficient knockdown in these cells, which could be due to more reporter plasmid than the shRNA plasmid being transfected into these cells. The results are consistent with the fact that shRNA knockdown usually does not reach 100%. However, the difference between an effective shRNA and an ineffective one is readily observable in our fluorescence assays in multiple wells for each condition.

Figure 1.3

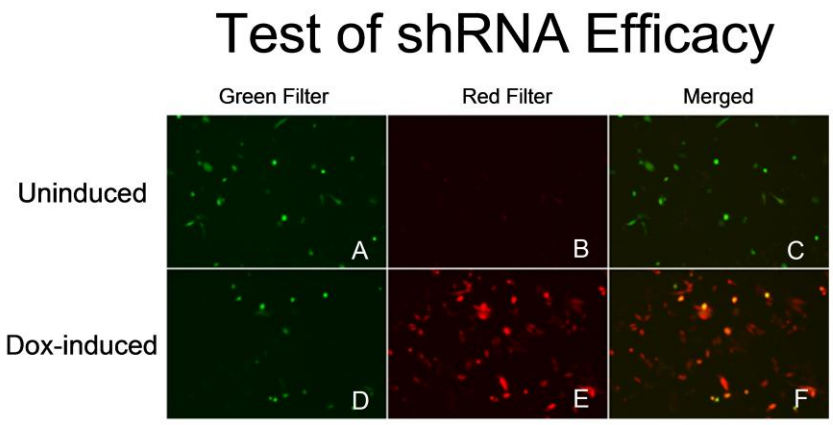

Figure 1.3: Test of shRNA efficacy Knockdown of the specific mRNA targets by shRNA expression. CHO cells were cotransfected with a reporter construct expressing GFPns-D2L fusion transcript and tRFP-shRNA constructs targeting D2L. Panels A-C shows the same field of cells in a well without doxycycline induction viewing through a green filter, a red filter, or a merged view of the two. Panels D-F are similar but showing cells in a well with doxycycline induction. The presence of doxycycline induced strong tRFP-shRNA expression (Panel E versus B) paralleled by substantially lower GFP-D2L expression (Panel D versus A).

Figure 1.4 represents single trials of testing for several shRNA sequences. Each sequence was tested in triplicate, and was repeated several times in 2-3 separate plates. Figure 1.4A shows our *in vitro* test using shRNA designed to knock down both isoforms of the D2 dopamine receptor. The sequence of "shRNA-D2L/S-g" was found to be the most reliably efficacious, as seen in several trials and was retested, as seen in Figure 1.5. Figure 1.4B shows our *in vitro* test using shRNA designed to knock down the D2L dopamine receptor without affecting expression of D2S. The sequence of "shRNA-D2L-d" was found to be the most reliably efficacious, as seen in several trials and was also retested, as seen in Figure 1.5. Figure 1.4C shows our *in vitro* test using shRNA designed to knock down the D2S dopamine receptor without affecting expression of D2L. None of the 7 tested shRNA sequences were efficacious in selectively knocking down D2S reporter expression. However, "shRNA-D2S-c" showed the most promise, and was retested, as seen in Figure 1.5

Figure 1.4

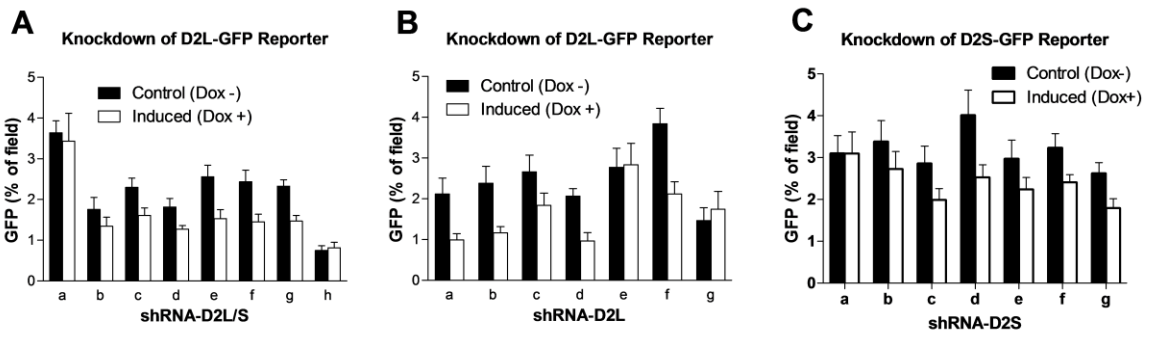

Figure 1.4: *In vitro* screening of shRNA *In vitro* screen of all shRNA constructs for efficacy of GFP reporter knockdown. (A) Eight different shRNA sequences all designed to knockdown D2L and D2S were initially screened for efficacy of D2L reporter knockdown. Further testing revealed that shRNA-D2L/S-g was most efficacious at knocking down D2L, as well as D2S. (B) Seven different shRNA sequences all designed to specifically knockdown D2L were screened for efficacy of D2L reporter knockdown. Further testing revealed that shRNA-D2L-d was most efficacious at knocking down D2L, without affecting D2s reporter expression. (C) Seven different shRNA sequences all designed to knockdown D2S were screened for efficacy of D2S reporter knockdown. Further testing could not find any sequences which reliably knocked down D2S without affecting D2L reporter expression. Statistical significance could not be achieved since only 3 wells were transfected in order to test the efficacy of each shRNA sequence. Reliably effective shRNA sequences were tested using a larger number of wells in Figure 1.5.

Figure 1.5 shows a summary of the most effictive shRNA in knocking down either both isoforms (D2L and D2S) or specifically knocking down one isoform (D2L or D2S). Unlike Figure 1.4, all shRNA were tested in 6 separate wells for each condition, in order to allow for statistical significance of GFP reduction to be achieved. Reporter only wells did not differ and were pooled for statistical purposes. As shown in figure 1.5, the 7 shRNAs made according to software predictions had different efficacies in knocking down D2L mRNA and only 2 out of the 7 shRNAs knocked down their target significantly on average (ANOVA with a Tukey Pot-hoc analysis. *$p<0.05$. SPSS 18.0, IBM, NY). This result strongly suggests that validating shRNA is necessary. If one were to simply pick a computer generated RNAi sequence without validation, the chance of getting an effective knockdown is less than 30%.

1.5 Discussion

Figure 1.5

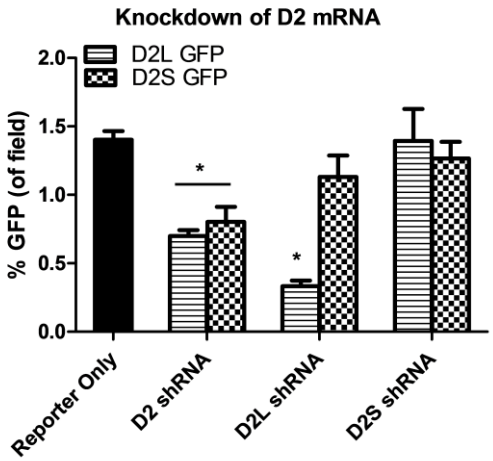

Figure 1.5: Comparison of final shRNA sequences Final i*n vitro* screen of all shRNA constructs previously found to be effective at knocking down their target(s). Each shRNA sequence is compared to it's ability to either knockdown the D2L GFP or D2S GFP reporter constructs. Significant differences were defined as $p < 0.05$)

Previous work has transfected cells under two different conditions (with or without an shRNA expressing plasmid) which will cause alterations in transfection efficiency (Kamio, Hirai et al.). By using an inducible shRNA construct, transfection conditions can be kept constant, with induction used to activate shRNA expression. Our method also allows shRNA expression to directly correlate to RFP expression, allowing us to indirectly track shRNA expression. Additionally, our method only requires the use of a fluorescent microscope and ImageJ freeware, and does not necessitate the use of flow cytometry or qPCR. This allows our technique to be more generally used by the scientific community, as well as towards cost effective high throughput screening of shRNA. What we (and Kamio et al) have shown are that certain computer predicted sequences are more effective than others in triggering the RNA interference mechanism in cultured cells. While showing efficacy of a shRNA sequence in vitro does not necessitate efficacy in vivo, it is assumed that such sequences are more likely to be effective in doing so. This provides a method for acquiring information that allows researchers to make more educated guesses pertaining to which sequences they would then like to test in vivo. This ultimately reduces the cost and time spent doing in vivo studies.

We were able to use this method to successfully find a shRNA sequence which could efficiently degrade D2L and D2S mRNA, as well as a sequence which was able to selectively degrade the D2L mRNA sequence. Unfortunately, we were unable to find a sequence which specifically degraded D2S mRNA. Since D2S is identical to D2L, except for the fact that it does not contain exon 6, shRNA needed to target the junction between exon 5 and exon 6 within the mRNA in order to be selective. siRNA wizard software was only able to suggest one sequence for this purpose. Additionally, we tried to come up with 6 other sequences using our own knowledge of shRNA design. In the end, all 7 sequences proved to be ineffective at degrading D2S, without affecting D2L expression. Since the D2L selective sequence had the greatest specificity, we ultimately used this sequence for our *in vivo* studies.

We present here a simple method for evaluating multiple shRNA sequences in knocking down their mRNA targets in cultured cells using fluorescence as indicators. It is relatively rapid and easy compared to histochemical or qPCR confirmation. We also show an inexpensive way of making many different shRNA constructs by annealing pairs of oligonucleotides and ligating directly into our shRNA vector. Finally, the ability to induce shRNA expression in a portion of identically transfected cells allows more controlled comparison of the cells with and without knockdown. Taken together, we describe here a convenient, inexpensive, rapid and thus high-throughput suitable method for simultaneously evaluating the efficacies of multiple different shRNAs at knocking down specific mRNA targets. In conclusion, our method resulted in finding an efficacious shRNA sequence against both isoforms of the D2 dopamine receptor (D2L and D2S) as well as one specifically against the D2L isoform.

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Chapter 2: Isoform specific knockdown of the D2L dopamine receptor

2.1 Abstract

Dopamine signaling in the nucleus accumbens is critical in mediating the effects of cocaine. There are two splice variants of dopamine D2 receptors, D2L and D2S, which are believed to have different functional roles. Here we show that knocking down D2L selectively using viral mediated shRNA led to a slight but significant decrease in basal locomotor activity with no significant change in cocaine induced stimulation of locomotion. The knockdown appears to produce a trend of reduced conditioned place preference to cocaine but the difference was not statistically significant. Our results demonstrated that splice variants of D2 receptors can be selectively manipulated in specific brain regions, allowing functional studies of each D2 receptor isoform within the context of addiction neurocircuitry.

2.2 Background

Drug dependence is a persistent problem throughout the world. In 2008 22 million Americans were classified as having a significant degree of drug dependence (2008 National Survey on Drug use and Health). Additionally, the health care cost in 2002 for substance abuse was estimated to be comparable to cancer (Economic Costs of Drug Abuse 2004). All drugs of

abuse increase striatal dopamine release despite their widely varied mechanisms of action (Volkow, Wang et al. 2006). After repeated exposure to drugs of abuse, a loss of D2 dopamine receptors (D2Rs) within the striatum occurs. Moreover, D2R availability in this brain region has been predictive of susceptibility to addiction (Dalley and Everitt 2009; Thompson, Martini et al. 2010). The ventral striatum primarily consists of the nucleus accumbens (NAc), an area which has been considered the origin of addiction behaviors. D2R expression within this region is relatively high (Centonze, Grande et al. 2003). Aberrant D2R signaling within the NAc has been continuously implicated in the initiation of drug addiction (Giordano, Satpute et al. 2006).

Drugs of abuse cause a significant increase in synaptic dopamine (DA) to occur within the NAc via increased mesocorticolimbic DA activity (Feltenstein and See 2008). DA has also long been studied as a modulator of glutamatergic and GABAergic signaling within the central nervous system (CNS). The sources of DA within the CNS originate in the midbrain, from the ventral tegmental area (VTA) and the substantia nigra (SN). The mesolimbic circuit, where the VTA projects to the NAc, amygdala, and hippocampus, has largely been attributed to modulation of memory formation. However, these widespread dopaminergic connections are not suited for encoding memory information. The individual glutamatergic and GABAergic circuits within the CNS are responsible for the more direct acquisition and retrieval of memories. Instead, DA projections coordinate the responses of these circuits to salient stimuli, including rewards. Evidence of this is seen when ablating the VTA or the NAc, which severely attenuate cocaine and herion self-administration (Feltenstein and See 2008). The mesocortical pathway connects the dopamine neurons of the VTA with the glutamatergic prefrontal, orbitofrontal and cingulate cortices. The mesocortical pathway has been implicated in schizophrenia and addiction, although its specific role in each has been difficult to ascertain. Ultimately, the VTA and its extension the SN are aptly positioned to modulate the glutamatergic and GABAergic connections that allow associative memory formation and reward valuation to occur. These mesocorticolimbic

connections would then be best suited to synchronize information across various brain regions in order to alter the motivational state of the entire organism (Hyman, Malenka et al. 2006). It has thus been postulated that it is this neurocircuitry that is responsible for the reinforcing effects of drugs of abuse, as well as craving and relapse following chronic use (Feltenstein and See 2008). Taken together, addiction can be viewed as a rapid learning process that is largely dependant on DA signaling, however the details of this processes have yet to be fully elucidated.

The striatum is one of the largest single structures within the brain, and is believed to function by integrating information in order to organize an appropriate motor response. Perhaps the most important aspect of the striatum toward this end, are its wide spread connections to the limbic circuitry of the brain. These connections allow the ventral striatum to rapidly influence memory formation. The NAc has long been proposed as the origin of addiction-like behaviors (Hyman, Malenka et al. 2006). This area is one of the primary reward centers of the brain, the activation of which can lead to euphoria and the hedonic consequences of substances of abuse (Hyman, Malenka et al. 2006). It is the VTA-NAc connection that is initially activated during the early stages of drug abuse, leading to the profound increase in DA within this region. Dopamine release in the ventral striatum allows the hedonic value of a goal to be attached to the motivation to initiate behaviors that allow the goal to be achieved (Hyman, Malenka et al. 2006). Dopamine release within the NAc occurs after administration of nearly every addictive drug tested (Volkow, Wang et al. 2006). To summarize this more simply, the NAc is responsible for the primary reinforcing effects of drugs of abuse (Feltenstein and See 2008). Taken together, the normal associative learning mechanisms that lead to reward behavior are dominated by the intense pharmacologically induced DA release caused by drugs of abuse (Hyman, Malenka et al. 2006).

The signaling modulated by this dopamine release has been closely linked to aberrant learning, particularly in forming associations to drug-related cues (Dalley and Everitt 2009). This aberrant learning process has been proposed to be regulated, in part, by each of the dopamine

receptors (D2Rs). There are 5 different dopamine receptors, all of which are G-protein coupled and have been categorized into two separate subfamilies. The D1-like receptors include D1 and D5 and are characterized by stimulation of adenylyl cyclase upon activation. The second subfamily is the D2-like receptors which include D2, D3 and D4 and are collectively characterized by inhibition of adenylyl cyclase upon activation. D2-like receptors have long been proposed as primary regulators of drug induced reward (Thanos, Michaelides et al. 2008; Johnson and Kenny 2010). D2-like receptor availability has been predictive of susceptibility to addiction and drug-induced loss of D2Rs within the striatum occurs after repeated drug administration. (Dalley and Everitt 2009; Thompson, Martini et al. 2010). This reduction in D2-like receptor expression persists for weeks following cessation of drug administration in animals (Dalley and Everitt 2009). In this way, aberrant D2R signaling within the striatum has been strongly linked to addiction.

The D2R gene can be translated into two different splice variants, a long isoform (D2L) which contains the 29 amino acid sequence derived from exon 6, or the short isoform (D2S) which does not (Moyer, Wang et al. 2011). It has become generally accepted that the D2S isoform of the receptor exists presynaptically, while D2L is predominately postsynaptic in localization and function. To this end, the D2S receptor has been strongly implicated as an autoreceptor with actions toward inhibiting dopamine release within the striatum (Centonze, Gubellini et al. 2004). However, there have been some reports that D2L may also exist presynaptically, but its role there is less understood (Centonze, Grande et al. 2003). Notably, both isoforms regulate GABA-mediated inhibition of striatal interneurons. Localization of these receptors is difficult to asses, since most techniques cannot differentiate the two. Moreover, D2S receptors are expressed at much lower levels within the striatum, with ~80% of striatal D2 receptors being of the D2L isoform (Wang, Xu et al. 2000; Smith, Fetsko et al. 2002). Beyond localization, the individual D2 receptor isoforms appear to also differ in their signaling

mechanisms. Both isoforms of the D2 receptor lead to the inhibition of adenylyl cyclase activity. This inhibition reduces the quantity of cyclic adenosine monophosphate (cAMP) produced by adenylyl cyclase. This reduction of intracellular cAMP in turn leads to reduced activity of phosphokinase A (PKA). However, the D2S receptor has been observed to be more efficient at this inhibition *in vitro* (Boundy, Lu et al. 1996). This difference suggests that intracellular signaling mechanisms may also differ between the two isoforms. To this end, D2L and D2S receptors do appear to bind different G proteins (Guiramand, Montmayeur et al. 1995; Boundy, Lu et al. 1996; Centonze, Gubellini et al. 2004). Perhaps relating to these differences in G protein coupling, D2L receptors are more resistant to desensitization by phosphokinase C (PKC) (Morris, Van et al. 2007). Taken together, it appears as though D2L and D2S receptors, while similar, may differ quite significantly in both localization and function (Guiramand, Montmayeur et al. 1995; Boundy, Lu et al. 1996). This idea has been furthered by genome wide association studies which correlated drug abuse with genomic variability. The widely studied Taq1A allelic variant of the D2 receptor gene has been implicated in increased risk to substance abuse in humans (Munafo, Clark et al. 2004; Munafo, Matheson et al. 2007). Most individuals with Taq1A also have several intronic single nucleotide polymorphisms (SNPs), which have been associated with alternative splicing of the D2 receptor (Moyer, Wang et al. 2011). It is therefore possible that alterations in expression of D2L and D2S might be responsible for the increased risk of addiction associated with these polymorphisms. In mice sensitized to amphetamine for example, D2L but not D2S mRNA expression increased in the dorsal, but not ventral striatum (Giordano, Satpute et al. 2006). This is contrary to the reduction in D2R expression after repeated drug administration, as mentioned above. However, those agonist-based studies were largely unable to differentiate between the D2, D3 and D4 receptors. Taken together, there seems to be enough evidence at present to suggest a complex, region specific role for each D2R isoform in the context of addiction.

The majority of what is currently known about D2R was acquired from studying agonists, antagonists and transgenic mice (Beaulieu and Gainetdinov 2011). In some animal studies, D2 partial agonist treatment has been able to attenuate cocaine craving and relapse (Chen, Chen et al. 2009). At the time of writing however, no agonist or antagonist could fully differentiate between D2, D3 and D4 receptors, let alone the individual isoforms of D2R. Work with transgenic mice has been much more informative in helping understand the differences between the D2L and D2S splice variants. In mice lacking both isoforms of the D2 receptor (D2R -/- mice), reduced tyrosine hydroxylase positive neurons and lower levels of neurotrophins are found within the striatum (Holmes, Lachowicz et al. 2004). This indicates that without D2R, dopaminergic neurons are reduced in number, furthering the importance of these receptor isoforms in dopamine signaling. In D2R -/- mice, dopamine in the substantia nigra or ventral tegmental area is no longer able to inhibit the release of dopamine into the striatum, as mediated by these two nuclei (Holmes, Lachowicz et al. 2004). Amphetamine induced dopamine release is also disrupted, further suggesting that one or both of the D2R receptor isoforms is the primary autoreceptor on dopaminergic neurons (Holmes, Lachowicz et al. 2004). Work with transgenic animals in which the long isoform (D2L-/- mice) was removed have been difficult to interpret since these mice exhibited overexpression of D2S mRNA (Wang, Xu et al. 2000). Since D2L-/- mice still exhibit dopamine induced reduction of subsequent dopamine release, these mice are considered to have intact autoreceptor activity. Combining the studies using both transgenic animals, this is one of the most convincing arguments for D2S as the primary dopamine autoreceptor. Furthermore, this same argument implies that D2L receptors might act predominately as postsynaptic receptors.

Behavioral differences have also been found in these transgenic animals. D2 knockout mice, which do not express either variant of the D2 receptor, have severely reduced baseline locomotor activity (Chausmer, Elmer et al. 2002). D2L-/- mice also have exhibited equally reduced basal locomotor activity, as well as reductions in addiction-like behavior to some, but not

all, substances of abuse (Smith, Fetsko et al. 2002). This work has strongly implicated D2L as the primary isoform involved in basal locomotor activity and has further suggested its involvement in the initiation of addiction. These knockout mouse models have been informative, but improved methods will be necessary if conclusions specific to an individual isoform are to be made. Developmental compensation is likely occurring since dopamine receptors are involved in the formation of the central nervous system (Missale, Nash et al. 1998). Beyond this, the increased expression of D2S in D2L-/- mice greatly hinders decisive interpretation of these animals both behaviorally and molecularly.

Improved methods are necessary in order to make conclusions pertaining to the specific roles that the individual isoforms of the D2 dopamine receptor are playing in addiction. To this end, we generated a viral delivery system which administered shRNA that specifically targeted the long isoform of the receptor, while leaving expression of the short isoform unchanged. In the previous section, we discussed how we screened for efficient shRNA *in vitro*. During those screening processes we were only able to find shRNA sequences that effectively knocked down D2L mRNA without affecting D2S mRNA. A virus expressing this sequence was applied specifically to the nucleus accumbens, allowing us to investigate not only this particular isoform, but also its involvement within this region of the brain. In this manner, we were able to test addiction-like behaviors in animals which had D2L knockdown within the nucleus accumbens.

2.3 Materials and Methods

2.3.1 Animals

Male C57/bl6 mice were acquired through Jackson Labs (8-10 wks, Bar Harbor, ME) and were treated in accordance with the Institutional Laboratory Animal Care and Use Committee, the Ohio State University. They were group housed and kept on a 12-h day and 12-h night cycle and provided *ad libitum* access to food (Harlan Teklad 8640 Rodent Diet, Madison, WI.).

2.3.2 Viral Vector Preparation

Several shRNA against D2L (shRNA-D2L) were designed by siRNA wizard (3.1, Invivogen, San Diago, CA) and evaluated in vitro (Naughton 2011). The most effective shRNA (CTGGGAGTTTCCCAGTGAACA) was cloned into an AAV vector as described previously. Replication deficient AAV1 serotype viral vectors were prepared at a concentration of 1.5×10^{14} vgu/µl (During, Young et al. 2003). A virus expressing shRNA against GFP (shRNA-GFP) was used as a negative control for all experiments.

2.3.3 Surgical Procedures

Mice were anesthetized with ketamine (100 mg/kg), xylazine (30 mg/kg) and stereotaxicaly injected with the AAV1 viral vector (1.0×10^{12} vgu/µl). Unilateral injection: Animals were injected with virus randomly into the right or left striatum (coordinates in mm: 0.75

anterior, 1.75 lateral; 4.25 and 3.00 ventral to Bregma) at a ventral (0.5 µl volume) and a dorsal (0.75 µl volume) injection site. Bilateral NAc injection: NAc was injected (coordinates in mm: 1.3 anterior, 1.3 lateral; 4.4 ventral to Bregma) at a 10° angle from the midsagittal plane. Animals received 0.5 µl per injection site. Bilateral whole striatum injection: Animals were injected with virus (coordinates in mm: 0.75 anterior, 1.75 lateral; 4.25 and 3.00 ventral to Bregma) at a ventral (0.5 µl volume) and a dorsal (0.75 µl volume) injection site. All injections were at a rate of 0.1 µl per minute.

2.3.4 qPCR Analysis

All animals received unilateral injection of either shRNA-D2L or shRNA-GFP virus. After a 3 week recovery animals were sacked via cervical dislocation, followed immediately by rapid brain removal. A 1 mm section was cut surrounding the injection site using a mouse brain matrix. One hole-punch was taken from each hemisphere (injected and uninjected), and striatal mRNA was extracted according to the protocol of the RNeasy Lipid Tissue Minikit (Qiagen, Valencia, Ca.). Reverse transcriptase reactions were performed by the iscript cDNA synthesis kit (Thermo Scientific, Waltham, Ma). Mastermix for duplex qPCR was optimized from Maxima Hotstart Taq Polymerase (Thermo Scientific, Waltham, Ma). cDNA was analyzed with a duplex qPCR reaction, using β-actin as a within sample control. All reactions were run in triplicate with probes conjugated to either Fam or Hex (Sigma, St. Louis, MO) using a Mastercycler ep Realplex^2 (Eppendorf, westbury, NY). Standard curves were established for each reaction. The primer and probe sequences, as well as their efficiency and R^2 values are included as Appendix B. Standard curves were applied to each sample's cycle threshold, which was then normalized to β-actin.

2.3.5 Drugs

Cocaine was provided by the National Institute on Drug Abuse drug supply program. Cocaine was dissolved in 0.9% saline and injected interperitoneally in a volume of 0.1 ml/10 g of body weight. Sulpiride and Quinpirole were purchased from Sigma (St. Louis, MO) and were also injected in a volume of 0.1ml/10g of body weight.

2.3.6 Conditioned Place Preference Behavior

We used an unbiased conditioned place preference (CPP) paradigm after a 2 week recovery following virus injection. During the preconditioning day, mice freely explored a 3-chamber box and were monitored using video tracking software (ANYmaze, Stoelting Company, Wood Dale, IL). The acrylic CPP boxes consisted of a smaller middle chamber (12.5 x 7.5 cm) and two larger conditioning chambers (12.5 x17.5 cm). One larger chamber had black solid stripes as a visual cue with mesh flooring as a tactile cue, and the other larger chamber had thin wavy stripes with porous flooring. Based on pretest results, the drug-paired chambers were assigned so that the shRNA-D2L and shRNA-GFP groups were both counterbalanced and unbiased toward the environmental cues.

During conditioning, all 3 chambers contained either the saline or cocaine paired cue set. Mice were again allowed to freely explore all 3 chambers for 30 minutes while locomotor activity was recorded. Animals were injected with saline in their assigned saline-paired cue set on days 1, 3, 5, 7, and 9, and injected with cocaine (10mg/kg) in the other cue set on days 2, 4, 6, 8, and 10. During postconditioning, animals were placed into the CPP boxes with a setup identical to the preconditioning day, and time spent in each chamber was recorded over 30 minutes. Mice in the

control group received saline on all conditioning days and had each viral injection group represented (shRNA-GFP and shRNA-D2L). Saline groups did not differ and were pooled.

2.3.7 Open-field Testing of Locomotor Activity

After a two week recovery period from bilateral injection of virus into the whole striatum, mice were baseline tested in open field chambers (acrylic; 25 x 25cm) using video tracking software (ANYmaze, Stoelting Company, Wood Dale, IL.). Animals were habituated to the chambers for 30 minutes prior to every testing session. Each animal then received cocaine (5mg/kg), and was again placed into the open field chambers, this time for a 1.5 hr recording session. This occurred every day for 5 consecutive days. After a 5 day drug-free period, animals were again tested (5mg/kg cocaine). The following day, animals began an escalating drug treatment paradigm. The day after their last 5mg/kg injection, animals underwent testing with 10, 20 and 40mg/kg cocaine over 3 consecutive days.

Animals unilaterally injected with either shRNA-D2L or a virus that was designed to knock down both isoforms of the D2 receptor (shRNA-D2B) also underwent locomotor behavioral testing in the open-field test. Two weeks after injection, animals began a testing paradigm similar to what has been described above. On consecutive day's animals were injected (i.p.) with saline, cocaine (10mg/kg), Sulpiride (10mg/kg), and Quinpirole (0.1mg/kg). Rotation frequency contralateral to the viral injection site as well as total locomotor activity was recorded.

2.3.8 Immunohistochemistry for the D2 Receptor

Immediately after the CPP post-test, animals were given a lethal dose of averdin and sacrificed via transcardial perfusion with 4% paraformaldahyde. Brains were rapidly extracted and post fixed for 4 hours. Brain slices were taken of the striatum surrounding the viral injection site via microtome, and were cut to a thickness of 40um. Serial sections were then stained for D2 receptor expression by Keerthi Thirtamara-Rajamani using anti-D2 primary antibody (1:500; Millipore, Billerica, Ma), goat anti-rabbit secondary antibody (1:600; Sigma, St. Louis, Mo.) and rabbit anti-goat Pap conjugated antibody (1:300; Jackson Labs, bar Harbor, Maine). Pictures were taken of the ventral striatum at 20x magnification of regions that also visibly expressed RFP. Three pictures were taken from three different sections and/or hemispheres for each animal. D2 expression was quantitatively estimated using ImageJ software (NIH, Bethesda, MD). The percent area of the visual field that contained D2 receptor staining was pooled for the three pictures taken for each animal.

2.3.9 Statistics

Statistics were performed using SPSS (SPSS 18.0, Chicago, IL.). Two-way analysis of variance (ANOVA) was used to assess overall effects of shRNA-D2L x shRNA-GFP on CPP score and total locomotor activity. One-way ANOVA was used to determine whether there was a significant difference across each treatment group. Post-hoc Tukey tests were performed after one-way ANOVA to look for differences between cocaine treated shRNA groups and the pooled saline group. Locomotor differences across time between shRNA groups were assessed via

multivariate ANOVA. All qPCR data sets were analyzed via independent-sample two-tailed T tests.

2.4 Results

2.4.1 Striatal expression changes after D2L knockdown

To confirm D2L knockdown, we unilaterally injected shRNA-D2L (n=8) into the striatum and compared it to shRNA-GFP (n=8) control injections. mRNA expression after virus injection was presented as a ratio compared to that of the uninjected hemisphere. Expression profiles within the non-injected hemispheres of both groups of mice did not differ, and were pooled. Injection of shRNA-D2L into the striatum led to a significant reduction (~50%) in D2L mRNA expression, as compared to both the uninjected and shRNA-GFP treated hemispheres (Figure 2.1A). D2S mRNA expression was not affected (Figure 2.1B). Immunohistochemical staining for the D2 receptor (Figure 2.2B) showed an insignificant trend towards a ~50% reduction in D2 protein after sHRNA-D2L injection into the NAc (Figure 2.2C). This may have been influenced by a variety of factors, the most important being that the primary antibody for D2 receptors cannot differentiate between the individual isoforms. Both isoforms of the D2 receptor are expressed at approximately equal levels within the NAc (Centonze, Gubellini et al. 2004). Therefore, since this manipulation is knocking down only D2L, without altering D2S, it is possible that the effect on protein knockdown is largely being masked.

Figure 2.1

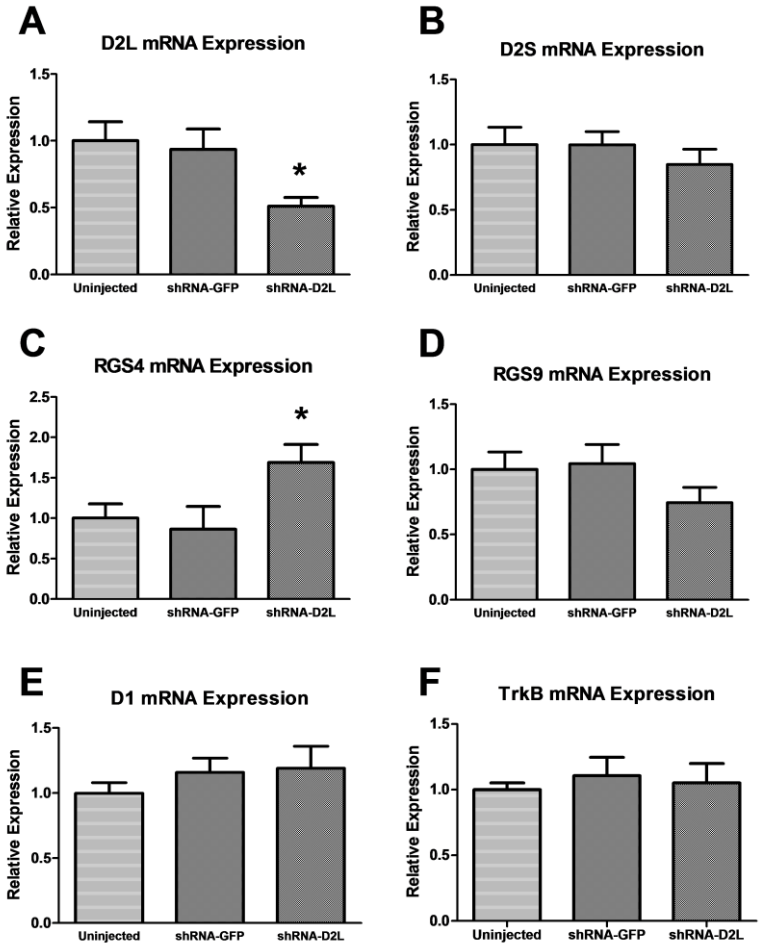

Figure 2.1: qPCR results after shRNA-D2L treatment Relative mRNA expression in animals unilaterally injected with either the shRNA-D2L or the shRNA-GFP control virus. Expression of the D2L receptor (A), D2S receptor (B), RGS4 (C), and RGS9 (D) within the striatum. The quantity of mRNA is presented as a fraction of the uninjected hemisphere. A significant reduction of D2L mRNA occurred as compared to both the uninjected hemisphere and the control virus injected mice (A). A significant increase of RGS4 mRNA was seen, as compared to both controls (C). shRNA-D2L had no effect on the mRNA levels of D2S (B), RGS9 (D), D1 receptor (E) or TrkB (F).

Figure 2.2

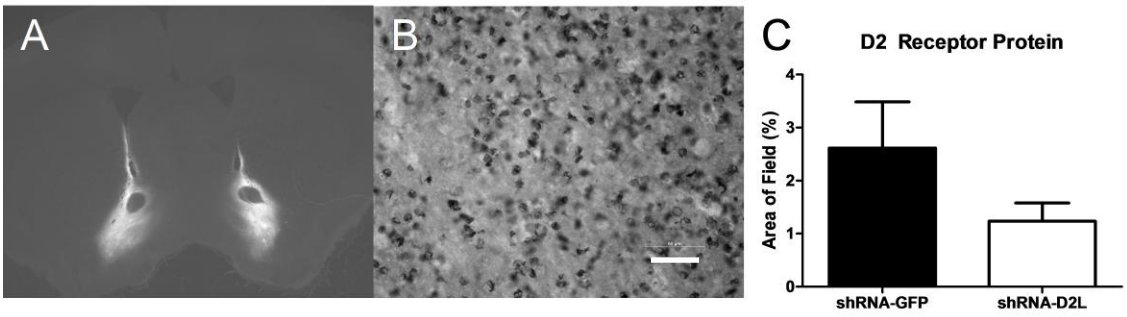

Figure 2.2: Viral and D2R histochemistry A) A representative figure showing RFP expression from AAV1 infected cells. A coronal section of the mouse striatum (40um thick, +1.0mm anterior of Bregma) is being visualized at 1x with fluorescence microscope. RFP expression is confined to the NAc with little expression occurring in the dorsal striatum. B) A representative figure of D2R immunohistochemical staining (indicated by black color) as done by Keerthi Thirtamara-Rajamani. All pictures were taken at 20x magnification and the scale bar represents 50 um. C) D2 receptor protein quantified by ImageJ analysis of immunohistochemically stained tissue. The Y axis shows the percentage of the NAc picture which contained D2 receptor staining (Black) for each viral group. A trend towards decreased D2 protein was seen ($p = .151$).

In addition to the changes seen in D2L mRNA expression, regulators of G protein signaling were also affected. RGS4 and RGS9 are known negative regulators of intracellular D2 receptor signaling. After D2L knockdown, RGS4 mRNA (Figure 2.1C) significantly increased by approximately 60%. RGS9, D1 receptor and TrkB mRNA did not change (Figure 2.1D, E and F).

2.4.2 Conditioned place preference behavior after D2L knockdown

To test the role of D2L in cocaine reward, we knocked down D2L with bilateral injections of shRNA-D2L into the NAc. RFP is co-expressed with shRNA from the viral genome following viral infection. RFP expression was examined for all animals after behavioral testing and those with patterns different from that shown in Figure 2.2A were removed from the study (<10%).

Figure 2.3

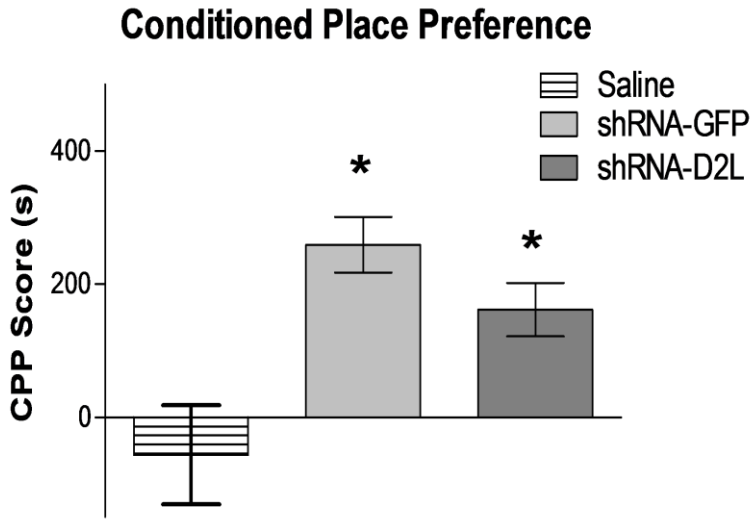

Figure 2.3: Conditioned place preference to cocaine Conditoned place preferance (CPP) testing in mice with bilateral NAc injection of either the shRNA-D2L or the shRNA-GFP control AAV virus. CPP score was calculated as the time spent in the durg paired chamber during the post –test subtracted by the amount of time spent during the pre-test. A sub group of animals were injected with only saline throughout the paradigm. This saline-only group consisted of animals representing both viral injection groups. No differences between these saline animals were seen, and they were therefore pooled. Both the shRNA-GFP and the shRNA-D2L groups showed a significant (* $p < 0.05$) preference for the cocaine (10mg/kg) paired chamber, as compared to animals only injected with saline. A trend toward reduced preference for cocaine was found in the shRNA-D2L group as compared to shRNA-GFP.

Figure 2.3 shows that cocaine induced significant CPP in both shRNA-D2L injected mice (n=15) and shRNA-GFP injected mice (n=20) compared to saline conditioned control mice (6 shRNA-D2L mice and 5 shRNA-GFP mice, which were pooled). There is a trend towards reduction of CPP score after D2L knockdown compared to the shRNA-GFP group, but the difference was not significant (p=0.16).

2.4.3 Locomotor differences after D2L knockdown in the nucleus accumbens

Locomotor activity was measured during the pretest and conditioning periods of the CPP procedure. Significantly less total baseline locomotor activity was seen in shRNA-D2L animals

as compared to the shRNA-GFP group during the CPP pretest (Figure 2.4A). When looked at in 5 min bins (Figure 2.4B), the baseline locomotor activity difference between the two groups was most prominent after 20 and 25 minutes. At these two time points, animals with D2L knockdown within the NAc showed significantly decreased basal locomotor activity. However, no significant difference in locomotor activity was seen between viral groups after the first saline, or cocaine injection (Figure 2.4C). The remainder of the conditioning days also did not show a difference in activity across viral groups (Figure 2.5), although significant sensitization to the locomotor stimulating effects of cocaine was evident.

Figure 2.4

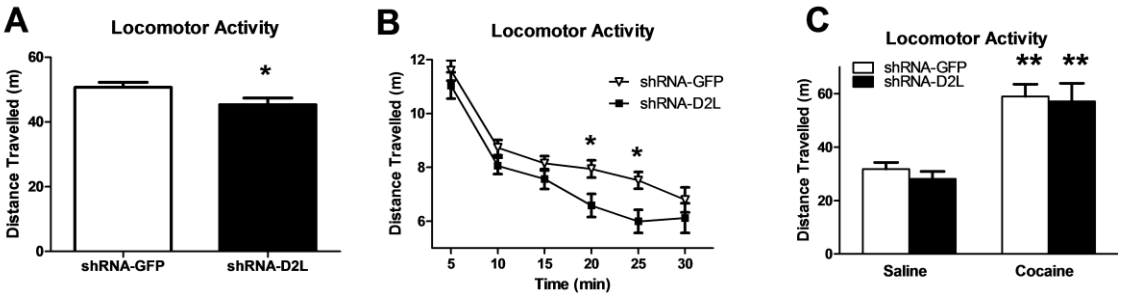

Figure 2.4: Baseline locomotor activity after NAc injection Behavioral tests in mice with bilateral NAc injection of either the shRNA-D2L or the shRNA-GFP control AAV virus. A) Total locomotor activity during the 30 minute CPP pretest. Animals showed a significant (* $p < 0.05$) reduction in baseline locomotor activity after shRNA-D2L knockdown in the NAc, as compared to shRNA-GFP injected controls. B) Time course of basal locomotor activity in 5 min intervals. While the distance traveled did not differ between groups during the first 15 minutes, a significant (* $p < 0.05$) difference occurred between the two groups after 20 and 25 minutes. C) Total locomotor activity after the first saline or cocaine injection. Cocaine significantly increased locomotor activity (** $p < 0.01$) in both groups but there was no difference between viral treatments.

Figure 2.5

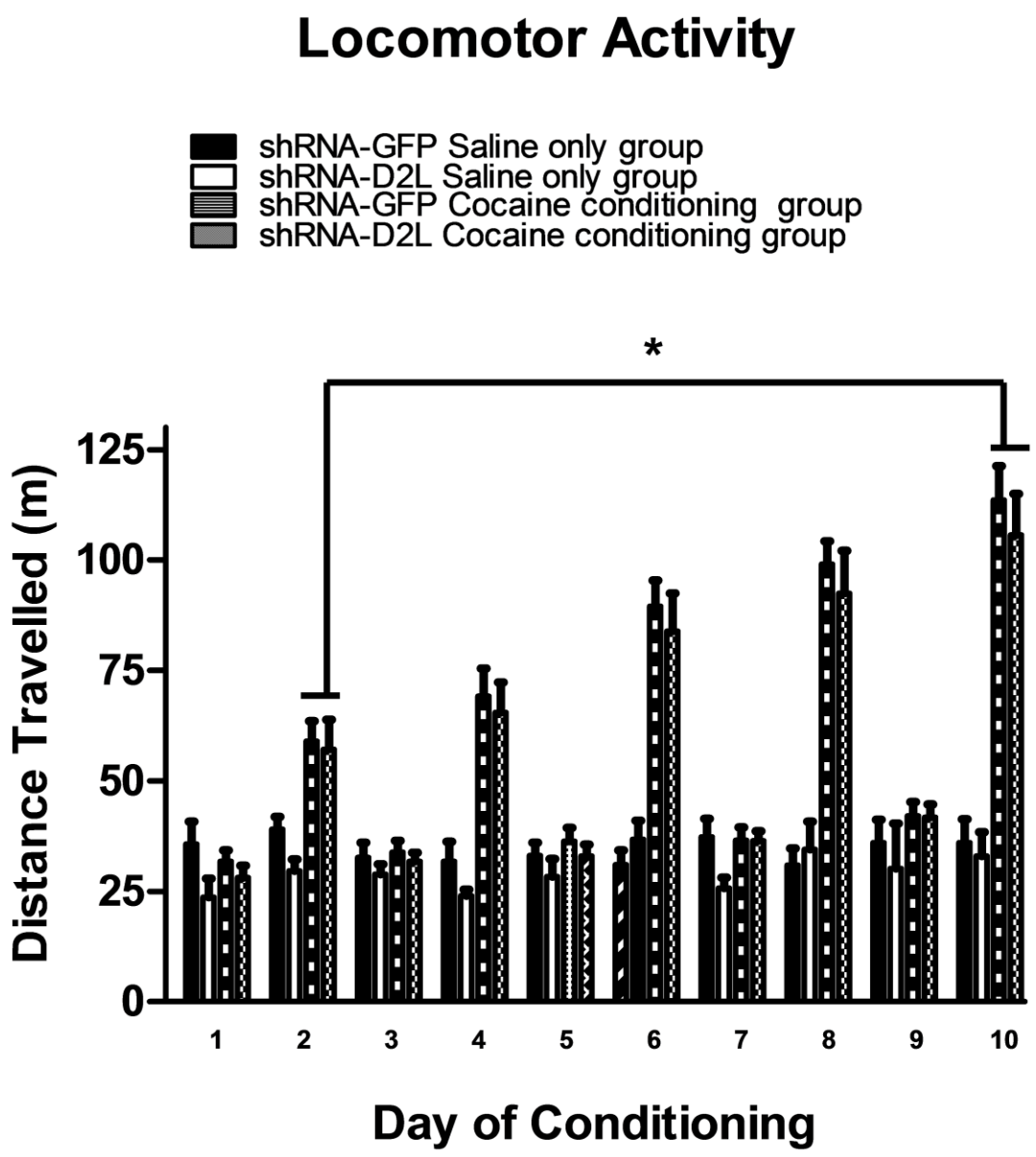

Figure 2.5: Locomotor activity during CPP conditioning Locomotor activity with saline or cocaine during 30 min of CPP conditioning after bilateral nucleus accumbens (NAc) injection of either the AAV-shRNA-D2L or the AAV-shRNA-GFP control virus. Animals within the cocaine conditioning group received Saline (i.p.) on odd days, and cocaine (10mg/kg, i.p.) on even days. Animals in the saline only group acted as negative controls and received saline injections on all days. Both the shRNA-GFP and the shRNA-D2L groups showed significant (* $p < 0.05$) sensitization to the locomotor stimulating effects of cocaine. This is evident when comparing locomotion on Day 10 after cocaine (with Day 2 after cocaine injection (10mg/kg). No significant differences were seen between viral groups on any of the 5 cocaine administration days.

2.4.3 Locomotor differences after D2L knockdown in the whole striatum

Animals which were bilaterally injected with either shRNA-D2L (n=3) or shRNA-GFP (n=4) into the entire striatum were behaviorally tested in the open-field locomotor activity test. Figure 2.6 shows locomotor activity during each of the 5 consecutive times that these animals were injected with cocaine (5mg/kg, i.p.), as presented in 15 min time bins (Figure 2.6A-E). This occurred daily for 5 days consecutively. Each test began with a 30 minute habituation period, followed immediately by cocaine injection and 1 hour of continued monitoring. Animals with shRNA-D2L consistently showed a trend toward reduced locomotor activity, as seen both before (basal activity) and after cocaine injection (stimulation). Following this repeated drug administration, Figure 2.6F shows the locomotor activity induced by a 5mg/kg dose of cocaine, administered after the animals were kept drug free for a 5 day period. Only shRNA-GFP animals appeared to show sensitization to the stimulating properties of cocaine, as compared to shRNA-D2L animals, following this drug-free period (Figure 2.6F versus Figure 2.6A). Starting 24 hours after the last 5 mg/kg injection of cocaine, these same animals immediately underwent locomotor testing using escalating doses of cocaine (10, 20 and 40 m/kg) (Figure 2.7). Open-field testing was performed as mentioned previously, but each consecutive day of testing used a greater dose of cocaine. Even after escalating doses of cocaine were administered, shRNA-D2L continued to show a trend towards reduced locomotion.

Since our results indicated the involvement of D2 receptors in locomotor activity, we conducted an additional pilot study using unilateral injection into the dorsal and ventral striatum with either shRNA-D2L (N=6) or a virus containing shRNA designed to knockdown both isoforms of the D2 receptor (shRNA-D2B, N=7). Open-field locomotor testing was conducted on these animals. The coordinates of injection were identical to the previous

Figure 2.6

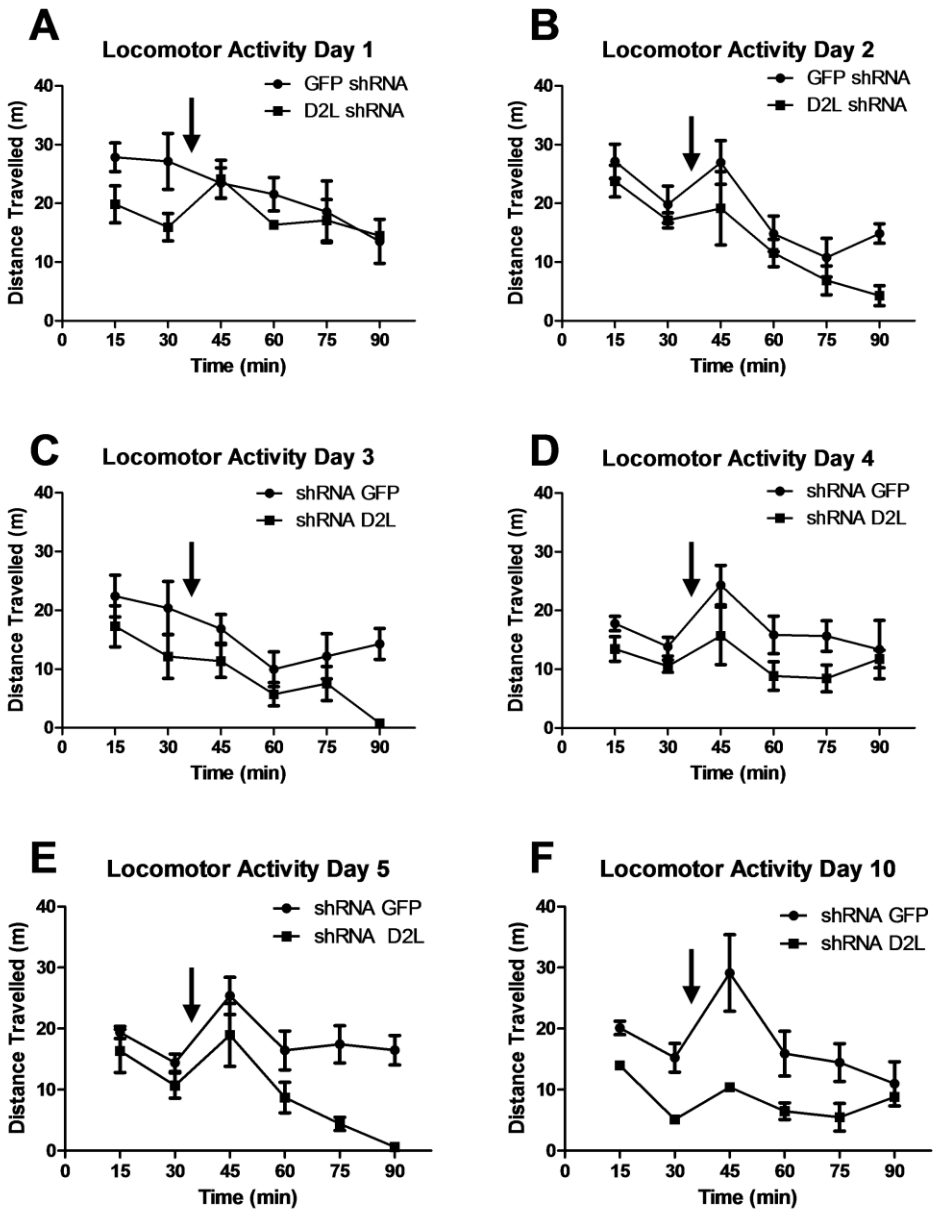

Figure 2.6: Locomotor activity after whole striatum injection Locomotor activity of animals bilaterally injected with either shRNA-GFP or shRNA-D2L into both the dorsal and ventral (whole) striatum. Testing occurred within open-field chambers after 5 days of repeated cocaine (5mg/kg) injections (A, B, C, D and E). Each test included a 30 minute habituation session, followed immediately by injection of cocaine (indicated by the black arrow) for a one hour recording session. Data is presented in 15 min time bins over the 1.5 hour total testing period. After a 5 day drug-free period (F), animals were again injected with cocaine (5mg/kg) and tested as before. shRNA-D2L animals consistently showed a trend toward a slight reduction in locomotor activity. This was seen on every day of testing, and occurred both before (baseline) and after cocaine injection.

Figure 2.7

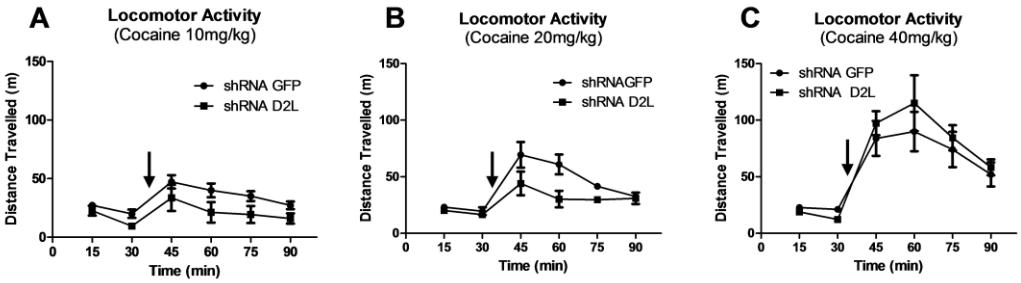

Figure 2.7: Escalating cocaine dose effects on locomotor activity Locomotor activity of animals bilaterally injected with either shRNA-GFP or shRNA-D2L into both the dorsal and ventral (whole) striatum. Starting 24 hrs after the last time they received 5mg/kg (Figure 2.6), animals were injected with escalating doses of cocaine each day, 10mg/kg (A), 20 mg/kg (B) and 40 mg/kg (C). Each test included a 30 minute habituation session, followed immediately by injection of cocaine (indicated by the black arrow) for a one hour long recording session. Data is presented in 15 min time bins over the 1.5 hour total testing period. shRNA-D2L animals again consistently showed a trend toward a slight reduction in locomotor activity. This was seen at all three doses, and generally occurred both before (baseline) and after cocaine injection.

Figure 2.8

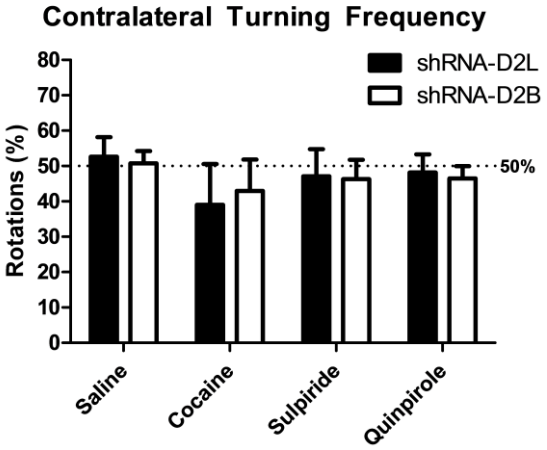

Figure 2.8: Rotational behavior of unilaterally injected animals Rotational behavior after unilateral injection into both the dorsal and ventral (whole) striatum of either shRNA-D2L or a virus designed to knockdown both isoforms of the D2 receptor (shRNA-D2B). Data is presented as rotational frequency (%) contralateral to the viral injected hemisphere over the 1 hour testing period. Saline injection acted as baseline testing, and animals spent half the time turning in both directions as would be expected. After administering the indirect D2 receptor agonist Cocaine (10mg/kg), a slight trend toward a reduction in contralateral turning frequency was seen. No differences in rotational behavior were seen after administering the D2 specific antagonist Sulpiride (10mg/kg) or the agonist Quinpirole (0.1mg/kg). Taken together, it appears as though a hemispheric imbalance of D2 mRNA does not influence rotational behavior.

locomotor study. Since these injections were unilateral, and since our previous data suggested D2Ls involvement in locomotion, it was predicted that these animals would exhibit significantly more rotations as occurring contralateral to the virally injected hemisphere. These rotations would indicate an imbalance in dopamine signaling across hemispheres. However, this unilateral manipulation did not significantly influence the percentage of contralateral rotations in these animals. Even when treated with various D2 receptor agonists and antagonists, no differences in rotations or overall locomotor activity were seen between viral groups (Figure 2.8). Taken together, it appears as though a hemispheric imbalance of D2 mRNA does not influence rotational behavior.

2.5 Discussion

We present here the first published report of nucleus accumbens specific knockdown of the D2L receptor. We were able to confirm appropriate bilateral injection of the virus by the expression of RFP in infected cells. This region of the brain is heavily involved in addiction-like behavior. Our results indicate that viral-mediated delivery of shRNA was able to significantly knockdown D2L mRNA expression by approximately 50%, without altering the expression of D2S. This effect persisted for at least three weeks, likely longer, so the animals undergoing behavioral testing had reduced D2L expression within the NAc throughout the paradigm. D2 dopamine receptor activation leads to inhibition of adenylyl cyclase, causing accumulation of intracellular cAMP, which in turn activates phosphokinase A (De Mei, Ramos et al. 2009). This is one of several intracellular signaling systems attributed to D2 receptor activation, and is perhaps the most understood. D1 receptor activation leads to the opposite intracellular signaling response, stimulating adenylyl cyclase activity (Beaulieu and Gainetdinov 2011). Therefore, alterations in D1 dopamine receptor mRNA levels might have been indicative of compensation to

the knockdown of D2L mRNA, however no such changes were found. The tyrosine kinase B (TrkB) receptor activates upon binding to its endogenous ligand, brain-derived neurotrophic factor (BDNF) and participates in LTP formation and learning (Minichiello, Korte et al. 1999). The involvement of adenylyl cyclase in learning behavior signified that alterations in TrkB mRNA expression might have been indicative of alterations in LTP or of compensation, however no expression changes were found when analyzing TrkB mRNA (Kheirbek, Britt et al. 2009). Since D2S is an alternate splice variant of the D2R gene, expression changes in D2S mRNA could have been indicative of compensation to D2L knockdown, however none were seen in our study. It is worth noting that the primary problem with previous attempts at investigating D2L receptors, namely D2L knockout mice, were difficult to interpret due to an increase in D2S mRNA expression within the brain (Wang, Xu et al. 2000). In that study, conclusions were hard to make, since the alterations in behavior may have been caused by D2S, and not D2L. While we cannot say what, if any, compensatory changes in mRNA expression occurred after D2L knockdown, we did analyze several likely molecules, and found no changes.

The most important deciding factor in the choice of intracellular signaling pathways that are induced by dopamine receptor activation is the cellular milieu of proteins such as β-arrestin, GRK, Regulator of G-protein signaling (RGS) and G proteins (Beaulieu and Gainetdinov 2011). RGS can inhibit receptor signaling by accelerating intrinsic GTPase activity of Gα proteins (Beaulieu and Gainetdinov 2011). D2 dopamine receptors are Gα protein coupled receptors that signal by transitioning the GDP bound form of inactive G proteins to the active, GTP bound form. In this manner, RGS proteins halt G protein signaling, including those mediated by D2 receptor activation. Importantly, expression of the five most common RGS proteins within the striatum are specifically regulated by dopamine D1 and D2 receptors (Taymans, Leysen et al. 2003; Beaulieu and Gainetdinov 2011). This feedback loop allows for rapid and controlled G protein signaling. Acute cocaine, amphetamine and morphine treatment increases RGS4 mRNA levels

within the striatum (Bishop, Cullinan et al. 2002). Of these RGS proteins, RGS4 and RGS9-2 appear to most notably regulate D2 dopamine signaling (Taymans, Leysen et al. 2003; Celver, Sharma et al. 2010; Beaulieu and Gainetdinov 2011). RGS4 protein exerts its GTPase activity predominately on Gαi/o, which is the same G protein subtype that couples with D2 receptors (Taymans, Leysen et al. 2003). This RGS mediated regulation of $G\alpha_{i/o}$ protein prevents D2 receptor activation from inhibiting adenylyl cyclase function. RGS9-2 appears to act predominately on Gβ5 proteins, and influences the agonist dependant internalization of D2 receptors (Celver, Sharma et al. 2010). Similarly to RGS4, RGS9-2 appears to be associated with D2 receptor signaling, but its specific interaction with D2L receptors has not been characterized (Maple, Perna et al. 2007; Celver, Sharma et al. 2010).

Our results showed a significant up regulation of RGS4 mRNA in animals which had D2L expression knockdown. This is the first study to show a direct effect of D2L expression changes on RGS4 receptor expression. This is a perplexing result since D2 receptor activation has been shown to increase RGS4 expression, and D2 receptor antagonism has been shown to decrease RGS4 expression (Taymans, Leysen et al. 2003; Schwendt and McGinty 2007). However, previous work was not able to differentiate between D2L, D2S, D3 or D4 receptors. The signaling of these other D2-like receptors is widely varied, since it includes both pre- and post-synaptic receptor signaling (Beaulieu and Gainetdinov 2011). The stimulation of these other D2-like receptors may explain these different results. Therefore, this seemingly contradictory effect may be outlining a novel role of D2L receptors in RGS4 expression, different from other receptors within the D2-like subtype. The potential importance of these results lie in the fact that RGS4 also regulates the signaling of group I metabotropic glutamate receptors (mGluRI), μ-opioid receptors, $M_{1\text{-}4}$ muscarinic receptors, and 5-hydroxytryptamine (serotonin) receptors (Schwendt and McGinty 2007). It is possible that D2L signaling is able to regulate other G-protein coupled receptors by altering the expression of RGS4.

D2R has been implicated in both animal and human studies of addiction. While many groups attribute the hedonic and locomotor affects of cocaine to D1 dopamine receptor activation, a growing body of evidence has suggested the involvement of D2R (Centonze, Grande et al. 2003; Giordano, Satpute et al. 2006). Our results indicate a trend toward reduced addiction-like behavior in animals with knockdown of D2L in the NAc. However, this was not found to be significantly different from control virus injected animals ($p = 0.16$). Our results may suggest that D1 or D2S is playing a greater role in this process (Takahashi, Matsui et al. 2010; Moyer, Wang et al. 2011). However, since we are only able to knockdown D2L expression by approximately 50%, we cannot definitively rule out D2Ls involvement in addiction-like behavior.

Our results indicate a mild, but significant affect on basal locomotor activity after D2L knockdown. This may reflect expedited habituation to the novel environment, or it may imply a reduction in exploratory behavior. D2L knockout mice showed reductions in locomotor activity that were greater then what was seen in our study, but they also had subsequent over expression of D2S (Wang, Xu et al. 2000; Holmes, Lachowicz et al. 2004). Our manipulation did not significantly change D2S expression, and so we are able to attribute these mild but significant locomotor changes more specifically to reduced D2L mRNA expression within the nucleus accumbens. It is noteworthy that we were able to show a trend towards reduced activity in open-field testing, both before and after cocaine injections. shRNA-D2L seemed to be primarily reducing basal locomotor activity, and did not appear to influence the locomotor stimulating properties of cocaine despite testing a variety of doses. This inhibition occurred not only when we injected shRNA-D2L into the striatum (dorsal and ventral), but also when we injected it specifically into the NAc. This indicates that D2L knockdown within the NAc may be sufficient to reduce basal locomotor activity; however a more thorough manipulation is necessary before that conclusion can be made. Manipulations that affect D2L mRNA expression greater than what we were able to achieve in this study would likely be necessary.

Taken together, we present here nucleus accumbens specific knockdown of D2L dopamine receptors. This reduction in D2L slightly, but significantly, reduced locomotor activity, and increased RGS4 mRNA expression. This is the first body of evidence implicating the D2L splice variant in regulation of RGS4 expression. Future studies focusing on the interaction between different isoforms of the D2 receptor and RGS4 may elucidate novel regulatory pathways involved in addiction (Schwendt, Gold et al. 2006).

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Chapter 3: Inducible and cell type specific expression of Cre recombinase in novel BAC transgenic mouse lines

3.1 Abstract

Cell type specific expression of exogenous genes will allow more detailed investigation of addiction related neurocircuitry by precisely manipulating particular neurotransmitter systems. GABAergic or dopaminergic specific expression of Cre recombinase opens many avenues for investigation of the central nervous system. Several previously established loxp mouse lines could be bred with these mice. By adding an inducible component, developmental compensation can be largely avoided. We present here our method for developing and genotyping Bac transgenic mice with inducible, cell type specific expression of Cre. There is a necessity for proper and thorough genotyping of these animals, as the size of the Bac fragment inserted into the mouse genome may dictate the specificity of Cre expression. By using these novel genotyping methods, we can establish these GABAergic and dopaminergic specific mouse lines with greater certainty.

3.2 Background

Cell type specific expression of exogenous or endogenous genes has obvious utility for a wide array of biological disciplines. Historically, researchers have tried to clone promoters out of the mouse genome and then incorporate it into a plasmid vector, driving a select exogenous or endogenous gene (Igarashi 2004; Rao and Monks 2009). Following this *in vitro* cloning, researchers would then randomly insert the vector into the genome of a transgenic animal. The goal of such projects was to use the cell type specific regulation of the promoter to drive expression of the gene of interest. However, utilizing the promoter alone is often unable to achieve cell type specificity (Chen, Kelz et al. 1998). It became readily apparent that regulatory elements lying up or down stream of the promoter also play a significant role in the specific expression of genes. Very large plasmids, referred to as bacterial artificial chromosomes (Bacs), have already been used to establish genomic libraries. These Bacs can incorporate >200,000 base pairs (bp) of genomic DNA, and therefore, can easily include not only the gene of interest but also the regulatory elements lying nearby. Until recently, work with these large constructs has been limited. Conventional cloning reactions with Bacs are near impossible, since a single occurrence of a restriction enzyme site is incredibly rare. This means that any given restriction enzyme would dice the 200k bp construct into many small, unusable fragments.

Several techniques have been developed to allow efficient cloning of Bac constructs, with the most successful being the recombination-mediated genetic engineering (recombineering) devised by Soren Warming *et al* (Warming, Costantino et al. 2005). This group modified the DH10B strain of Escherichia coli (E coli) to contain temperature-sensitive expression of recombination-inducing genes (exo, bet and gam). This allows for recombination to be 'turned on' by heat shocking the bacteria at 42˚C. In this manner, double stranded DNA containing 5'

and 3' ends, which are homologous to sequences within the Bac, can be inserted site specifically at relatively high frequencies via recombination. DH10B and the strain of recombineering E coli produced by Warming *et al* (SW102) are missing the galk gene, which is one of 4 genes necessary for the bacterium to utilize galactose as a sole carbon source. This allows positive or negative selection for successful recombination. SW102 therefore acts as an excellent tool for proficient modification of large genomic fragments.

This method takes advantage of the inability of SW102 bacteria to processes galactose, and ultimately expedites screening for successful recombination. First, a Bac is chosen based on the sequence of genomic DNA that it contains. Second, this Bac must be electroporated into the SW102 strain of E coli. Under the appropriate conditions, even large constructs such as Bacs can be easily electroporated into bacteria. Third, the galk gene needs to be inserted into the Bac so that it may act as a negative selection marker for subsequent recombination. The galk cassette is amplified using primers that contain 5' and 3' ends with sequence homology to the desired insertion site within the Bac. This double-stranded DNA fragment is electroporated into the Bac-containing SW102 bacteria, which is immediately heat-shocked to induce recombination. The bacteria are then grown on minimal media plates which are selective for recombination since they contain galactose as the only available carbon source. Without the presence of the Galk cassette, the bacteria will not be able to grow. Finally, the exogenous gene of interest is amplified using primers that again contain sequence homology to the desired insertion site within the Bac. This fragment is then electroporated into the galk/Bac-containing bacteria, and recombination is again induced via heat shock. They are then grown on a different set of minimal media plates, this time with the purpose of negative selection for galk. These plates contain both glucose and 2-deoxy-galaxactose (DOG) as their only carbon sources. A complete galactose operon (ie with galk) processes DOG into a toxin, preventing further bacterial growth. This then selects for proper insertion of the exogenous gene, since it should have overwritten the galk fragment that was

originally inserted. Taken together, this multi-step process screens for sequence specific insertion of any desired exogenous gene into a Bac.

As was already mentioned in the previous chapter, the striatum is an important brain area in the initiation of addiction behavior (Volkow, Wang et al. 2006). Dopaminergic projections from the VTA and substantia nigra release dopamine within the striatum, greatly altering neuronal signaling and assisting in the formation of addiction (See, Elliott et al. 2007; Berridge and Kringelbach 2008; Everitt, Belin et al. 2008; Beaulieu and Gainetdinov 2011). The striatum itself primarily consists of γ-aminobutyric acid (GABA) releasing neurons. These neurons are morphologically defined as medium, spiny neurons (MSNs) and are believed to be the principal interneurons of the striatum (Surmeier, Ding et al. 2007; Lovinger 2010). To date, much of the research on these two neurotransmitter systems has been acquired via systemic administration of agonists/antagonists, or via ablation studies. While both sets of work have proven to be invaluable in ascertaining the basic function of these systems, they each lack the subtlety necessary to answer more specific questions. Precise manipulations will be necessary if dopaminergic and GABAergic signaling within addiction is to be investigated in greater detail.

In order to use Bac recombineering to manipulate only DA or GABA releasing neurons, we first needed to find genes which were exclusively expressed within these neuronal populations. Dopaminergic neurons are difficult to target, since vesicular monoamine transporters and the aromatic L-amino acid decarboxylase synthesis enzyme are shared between DA, epinephrine and norepinephrine. The dopamine transporter (DAT) however, is expressed almost exclusively in dopaminergic neurons and glia. This makes the DAT promoter and its associated regulatory elements excellent for the purposes of dopaminergic-specific expression. For GABAergic specific targeting, it is important to note that the vesicular GABA transporter also transports glycine and the extracellular GABA transporters are very highly expressed in glia. However, the synthesis enzyme L-glutamic acid decarboxylase (GAD), which is responsible for converting

glutamate into GABA, is expressed exclusively in GABA releasing neurons. GAD exists in two different isoforms (GAD65 and GAD67) expressed from two separate genes (gad1 and gad2). Both isoforms show a great deal of overlap in their expression patterns but GAD67 has been previously implicated in addiction (Lindefors 1993; Souza, Toniazo et al. 2009). In summary, by modifying a Bac that contains the DAT gene we can achieve dopaminergic specific expression and by similarly modifying a Bac that contains the GAD67 gene we can achieve GABAergic specific expression. By generating mouse lines using these two Bacs, we would effectively be able to investigate the most common neuronal bodies of the striatum (GABAergic MSNs) or a specific population of neurons that are afferent to the striatum (dopaminergic neurons).

3.3 Materials and Methods

3.3.1 Choosing a Bac

A Bac containing the DAT gene (RP23-408F13) and a Bac containing the GAD67 gene (RP24-395F14) were purchased form the Children's Hospital Oakland Research Institute (Bac-Pac Resources, chori.org). Bac sequences were confirmed by diagnostic digestion using restriction enzymes. The predictable nature of this digestion pattern makes it indicative of the Bacs genetic architecture. The DAT Bac was overnight digested with HindIII or BglII and the GAD Bac was digested with PstI or EcoRI, each producing the expected digestion patterns (an example of this can be found with Figure 3.2).

3.3.2 Creation of prtTA.m2.2 and TetO-Cre constructs/inserts

rtTA M2.2 was removed from pCL-tet-on (Wang and Tsien 2006) and inserted into the Bluescript KS plasmid (Fermentas, Glen Burnie, MD) followed by a SV40 poly A signal. In order to generate the rtTA DAT-Bac insert, this expression cassette was amplified with PCR using primers with 5' homology to the ATG start of the DAT promoter and 3' homology to the middle of the first exon of the DAT gene. Primers were then used to amplify approximately 1kb upstream and downstream of DAT. These three fragments were gel purified and then combined in a reaction that also contained polymerase and dNTP's. This led to the elongation of a single, large insert which had 1kb homology to the DAT gene on both the 5' and 3' ends. To generate rtTA GAD-Bac inserts, a similar PCR amplification of the rtTA cassette was used. All primer sequences are list in Appendix C and D. Cre was removed from pMC-Cre (Gorski and Jones 1999) and inserted into Bluescript followed by a BGH-polyA and the WPRE regulatory element. The CMV promoter was replaced with the tet inducible TetO promoter (pTripz, Open Biosystems, Huntsville, AL). In order to generate TetO-Cre Bac inserts, this expression cassette was amplified using primers with homology to either Lpcat1 (for the DAT-Bac) or for the hypothetical gene (hpg, for the GAD67-Bac) which was also included within the Bac (Appendix C and D).

3.3.3 Insertion of rtTA and Cre in the Bacs

Once purchased, Bacs were inserted into SW102 recombination bacteria for storage and modification. Bacs were purified using standard alkaline-lysis preparations and were then electroporated (BioRad GenePulser Xcell, 900V, 25uF, 200Ω) into SW102 bacteria (grown to an

optic density (OD600) of .55). Selection for Bac insertion was done on Lysogeny Broth (LB) plates containing chloramphenicol (12.5mg/ml). Bacteria were stored in 15% glycerol at -20˚C.

To begin Bac modification, the galk cassette was electroporated into Bac-containing SW102 bacteria. Galk was amplified from pGalk (Warming, Costantino et al. 2005) using PCR (Herculase II, Agilent, Santa Clara, CA). Primers were designed to have sequence homology to the ATG start of either the DAT (Appendix C) or GAD67 (Appendix D) gene. Bac-containing SW102 bacteria were first grown to an optic density of .55. Double stranded galk-inserts were electroporated into heat-shocked SW102, followed immediately by a 4 hour recovery in LB media without the presence of a selection marker. Bacteria were washed with a salt buffer and plated onto galactose (1%) minimal media plates containing chloramphenicol (12.5 mg/ml) and grown at 30˚C for 3 days. Robust colonies from these plates were then streak-plated onto McConkey agar minimal media plates containing galactose (1%) and chloramphenicol (12.5 mg/ml), and grown over night. Bright red colonies were then screened using PCR for galk (Appendix C and D). Restriction enzyme digestion of the Bac was also used to confirm. This predictable change in fragment size confirmed galk insertion into the desired area of the Bac (example: Figure 3.2).

To insert rtTam2.2 directly after the ATG start of either DAT or GAD67 genes, Bac/galk-containing SW102 bacteria were heat shocked and electroporated with rtTA inserts containing appropriate homology ends. After a 4 hour recovery in nutrient rich LB media, removal of galk was selected for by plating bacteria on minimal media plates containing DOG (1%), glucose (1%) and chloramphenicol (12.5 mg/ml) which were grown at 30˚C for 3 days. Colonies were screened with PCR for the presence of rtTA (Appendix E). Restriction enzyme digestion was again used to confirm insertion into the desired area of the Bac. Following positive results in both conformational screens, Bacs were sequenced across the insertion site for confirmation of

integrity as well as for location within the Bac (i.e. directly after the ATG start of the respective promoter).

Insertion of the TetO Cre cassette occurred exactly as described above for both the DAT (Appendix C) and GAD67 (Appendix D) Bacs. Colonies were screened with PCR for the presence of TetO-Cre (Appendix G). Restriction enzyme digestion was again used to confirm insertion into the desired area of the Bac. The digestion pattern was heavily scrutinized to ensure that both the rtTam2.2 and TetO-Cre insertions altered the Bac predictably. Following positive results in both conformational screens, Bacs were sequenced across both of the insertion sites. Bacteria were stored in 15% glycerol at -20˚C.

3.3.4 DAT-Bac mouse generation and genotyping

DAT-Bac transgenic mice were produced by the University of Michigan Transgenic Core. Tail DNA from founder pups was genotyped by PCR for the presence of rtTA, Cre and the chloramphenicol resistance gene (cmr), as performed by Dr. Shengcai Wei (primer sequences listed in Appendix G). All BAC transgenic founders had genotypes positive for all 3 Bac markers. Potential positive founder mice were then analyzed using qPCR (Eppendorf Mastercycler Realplex^2, Hauppauge, NY). SYBR green mastermix (Applied Biosystems, Carlsbad, CA) and primers specific to rtTA, Cre and cmr were used to more closely analyze Bac insertion within founder mice (Appendix H). β-actin was used as a control for genomic quality/concentration. Additionally, regions of Bac DNA lying just before (Region A), and just after the DAT gene (Region B) were used to estimate the size of the inserted sequence. A third region located just

after where Cre was inserted (Region C) was also used for this purpose. All three of these regions share homology with the genome, and can only be analyzed via qPCR.

3.4 Results

3.4.1 GAD67-Bac synthesis and confirmation

The Gad67-Bac (see Figure 3.1) was modified in two primary ways. First, rtTA m2.2 was inserted directly after the ATG start of the GAD67 gene. Since GAD67 is only expressed within GABAergic neurons, this will presumably lead to GABA neuron-specific expression of rtTA. The rtTA gene was followed by the simian vacuolating virus 40 polyadenylation signal (SV40pA). Second, TetO-Cre was inserted at a second location within the Bac.

Figure 3.1

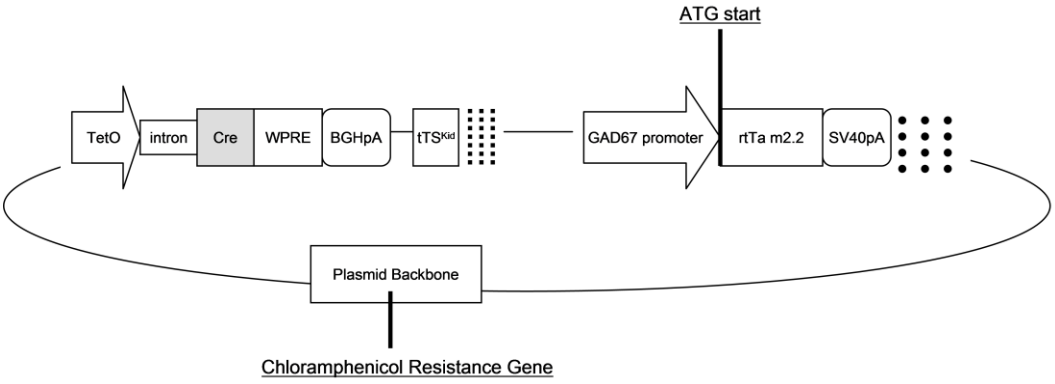


Figure 3.1: Map of the modified GAD67 Bac A map of the modified GAD67 Bac (RP24-395F14) following insertion of TetO-Cre and rtTa. The plasmid backbone containing the chloramphenicol resistance gene is pictured on the bottom of the Bac. The dashed lines after the tTS^{kid} of the Cre cassette indicate the remaining 9 exons of the hypothetical gene (LOC66748). The Dotted lines after the SV40 polyadenylation signal (SV40pA) of the rtTA cassette indicate the remaining 4 exons of the GAD67 (gad1) gene.

While gad1 was the only full gene present within this Bac, a second hypothetical gene was also partially present. The promoter for this gene (hypothetical protein LOC66748) was not included within this Bac sequence; however some exons were upstream of gad1. TetO-Cre was inserted over the first included exon (exon 4) of this second, partially present gene. This will hopefully avoid disrupting the regulatory elements of the gad1 gene, and may even make TetO-Cre more available for inducible expression (Giraldo and Montoliu 2001). Additionally, an intron was placed just before TetO-Cre and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) just after, in order to allow more efficient translation and to stabilize the mRNA, respectively (Klein, Ruttkowski et al. 2006; Rao and Monks 2009). tTS^{Kid} produces tTS protein which binds and represses TetO in the absence of doxycycline (Lamartina, Silvi et al. 2003). When doxycycline is present, tTS cannot bind TetO but rtTA can, leading to gene transcription. Thus, the inclusion of tTS^{Kid} further protects from gene expression occurring outside of induction (so called 'leaky' expression). Modifications to the Bac were confirmed by sequencing as well as by overnight digestion with pstI, SmaI or KpnI (Fgiure 3.2). Sequencing was important since all inserts were created via PCR with Herculase II self correcting polymerase. While the risk of polymerase error is low, it is still possible that the inserts were not amplified accurately. Digestions were performed after every recombinant insertion. This confirmed that no large scale insertions or deletions were happening as a consequence of the induced recombination. If this did happen, then the digestion pattern would deviate from what was expected. A table of all expected band lengths (in bp) for each recombineering step can be found in Appendix E.

Figure 3.2

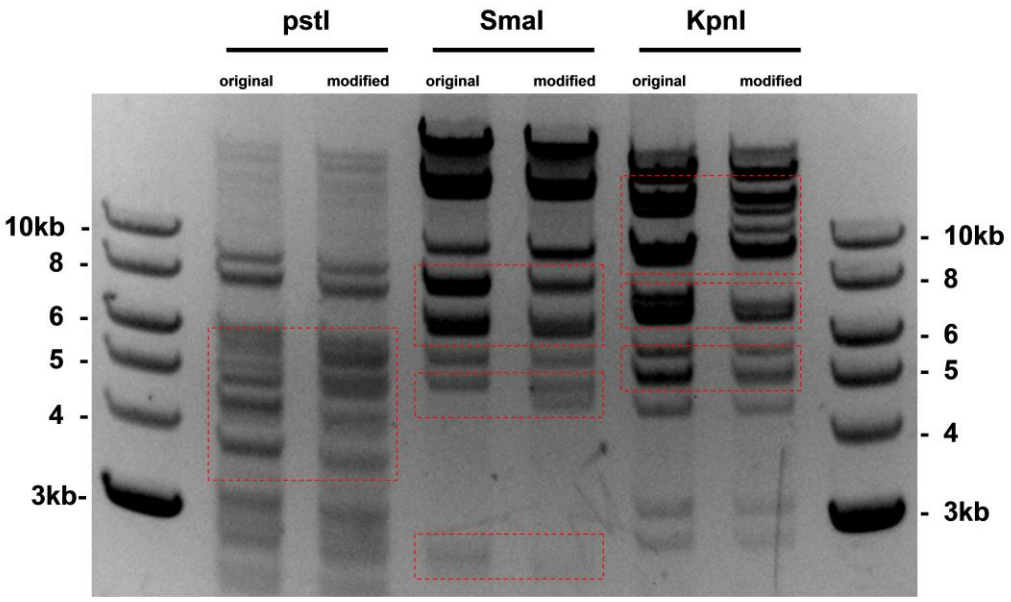


Figure 3.2: Diagnostic digestion of GAD67 Bac Confirmation of Bac integrity by restriction enzyme digestion. Digestion fragment size of the original GAD67 Bac is compared to the modified Bac which contains rtTA and TetO-Cre. Expected changes in overall band size after overnight digestion with either pstI, SmaI or KpnI between the two constructs are highlighted. Beyond confirming that recombinant insertion was successful, this procedure also helps asses whether or not large scale insertions/deletions occurred within the Bac. By producing the predictable digestion pattern, it is very unlikely that any gross changes of the Bac sequence took place. The results were also confirmed by sequencing the inserts and their surrounding Bac sequence.

3.4.2 DAT-Bac synthesis and confirmation

The DAT-Bac (Figure 3.3) was also modified via the insertion of rtTA and TetO-Cre. rtTA was inserted directly after the ATG start codon of the DAT promoter. This should allow cell type specific expression of rtTA in all cells that express DAT, which would be all dopaminergic neurons of the CNS. The organization of the rtTA cassette was identical to what was inserted into the GAD67 Bac, as both inserts were PCR amplified from the same rtTA-vector. The TetO-Cre cassette also had identical organization to what was inserted into the GAD67 Bac, as it too was amplified from the same TetO-Cre-vector. The DAT-Bac contained a second gene, Lpcat1. In order to prevent over expression of this gene, the TetO-Cre insert was designed to

replace the promoter and first exon of Lpcat1, therefore silencing this gene. Taken together, the DAT-Bac should lead to transgenic mice similar to GAD67-Bac mice, but with inducible, dopaminergic specific expression of Cre.

Figure 3.3

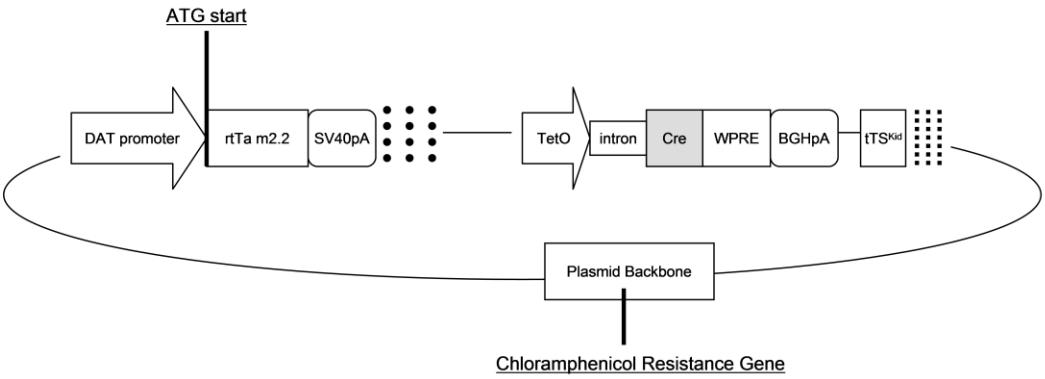


Figure 3.3: Map of the modified DAT Bac A map of the modified DAT Bac (RP23-408F13) following insertion of rtTA and TetO-Cre. The plasmid backbone containing the chloramphenicol resistance gene is pictured on the bottom of the Bac. The Dotted lines after the SV40 polyadenylation signal (SV40pA) of the rtTA cassette indicate the remaining 14 exons of the DAT gene (slc6a3). The dashed lines after the tTS^{kid} of the Cre cassette indicate the remaining 13 exons of the lysophosphatidylcholine acyltransferase 1 (Lpcat1) gene.

Correct modification of the DAT-Bac was also confirmed by using overnight digestion with restriction enzymes combined with sequencing the inserts and the surround Bac sequences. It was during this initial confirmation by digestion that a mistake was found. When the DAT-Bac was first designed both the rtTA and Cre inserts were followed by SV40pA signals. Because of this sequence homology, unintended recombination occurred. Figure 3.4 shows an altered Bgl2 digestion pattern, in which it is apparent that the insertion of the TetO-Cre-SV40pA led to a very significant deletion of the Bac. As can be seen when comparing the expected fragment sizes, the region of the DAT-Bac lying between the two SV40pA sequences (~70,000 bp) was lost (Appendix F).

Figure 3.4

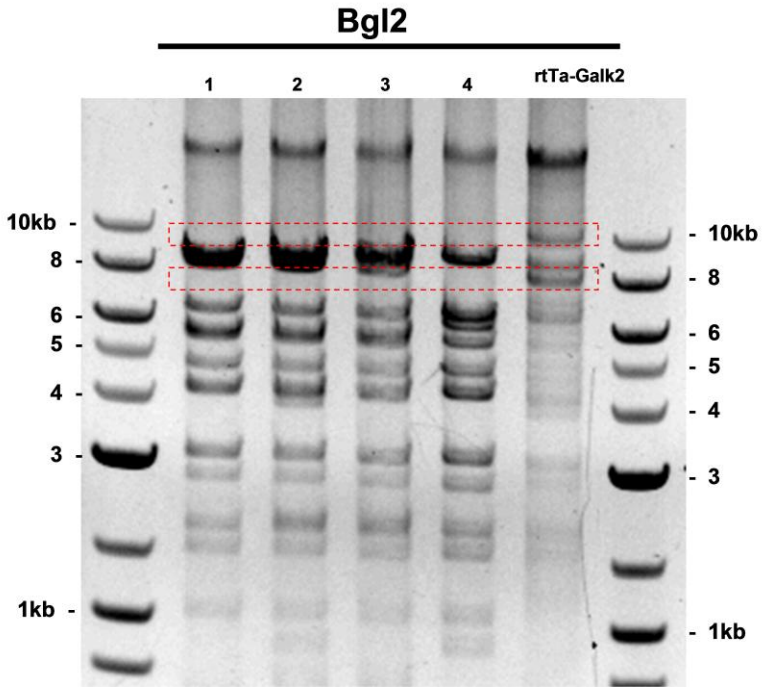


Figure 3.4: Confirmation of unwanted recombination in DAT Bac A digestion pattern of the modified DAT Bac (RP23-408F13) following insertion of rtTA-SV40pA and TetO-Cre-SV40pA. When compared to the DAT-Bac which had rtTA inserted after the DAT promoter and galk inserted over the Lpcat1 gene (rtTA-GALK2), unwanted recombination had obviously occurred during TetO-Cre insertion (clones 1, 2, 3 and 4). It was later found that since rtTa and TetO-Cre each had a sV40pA, recombination was occurring between these sequences, removing ~70,000 bp of Bac. Evidence of this was seen in the loss of the 10kb and 7.9kb bands (see Appendix F). Ultimately, a new TetO-Cre vector was cloned, this time with a BGHpA in order to alleviate this problem.

In order for this digestion pattern to be seen, two separate recombination events would have needed to occur. First, TetO-Cre-sV40pA would have to be inserted into the DAT-Bac. A second recombination event must then have occurred, which removed the ~70,000 bp between the two SV40polyA signals of rtTA and TetO-Cre. This shows not only a potential pitfall when using this technology but also outlines its ability to efficiently induce recombination between any homologous sequences within the Bac. The importance of using restriction enzymes to confirm that no large scale insertions/deletions occurred during the recombineering process is also very important when using this method. Luckily, this mistake was caught early enough that it could be

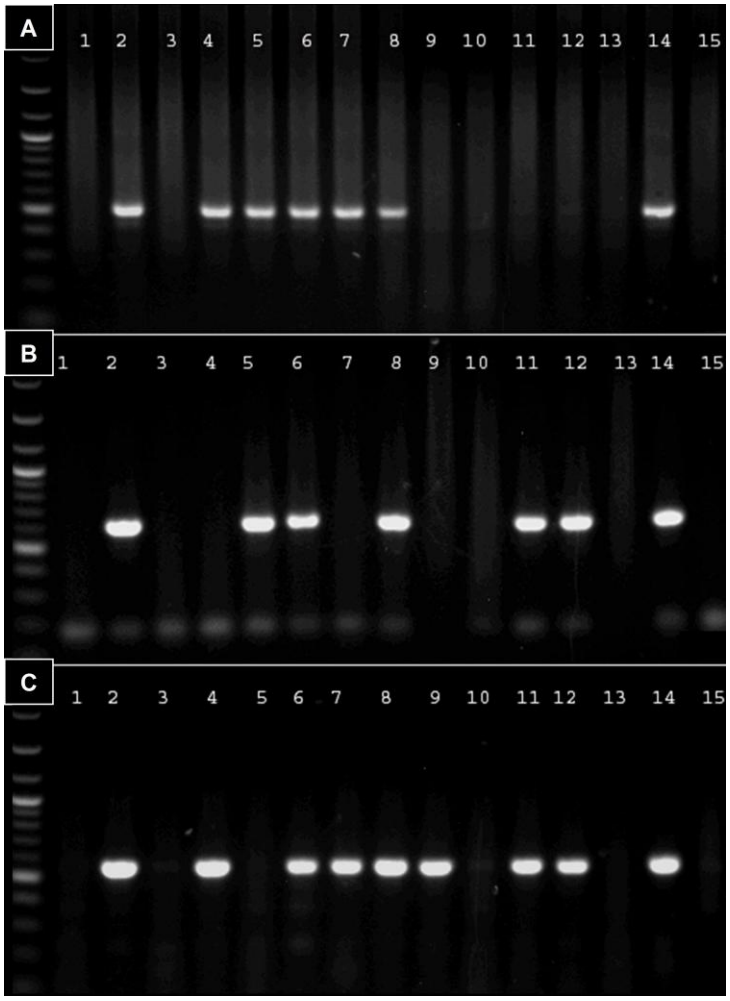
readily fixed by simply cloning a BGHpA instead of the SV40pA after the TetO-Cre expression cassette.

3.4.3 DAT-Bac mouse confirmation with conventional genotyping

Potential Dat-Bac mice were screened for genomic Bac insertion using conventional PCR-based genotyping techniques (primer sequences are listed in Appendix G) as well as via qPCR (primer sequences are listed in Appendix H). Tails from animals generated by fertilized egg injection of the DAT-Bac (F0 generation) were screened for the presence of the 3 primary exogenous markers: rtTA, Cre and cmr. An example of the conventional and qPCR methods of genotyping are presented in Figure 3.5. Of the original 215 pups born following injection of the modified DAT-Bac, 18 animals had at least one Bac marker present. Of these 18 animals, 13 contained partial fragments of the Bac sequence. Some of them had only cmr (n=2), rtTa (n=2) or Cre (n=2), others contained fragments with rtTa+cmr (n=2), Cre+cmr (n=2), or rtTa+Cre (n=3). Only 5 pups had all 3 Bac markers inserted into their genomes, and therefore presumably had the entire DAT-Bac sequence inserted.

Our results indicate that it is almost 3 times more likely for only a fragment of the Bac to be inserted into the genome, as compared to the chance of having the entire Bac inserted. This is a serious concern when generating these mouse lines since it is vital that the inserted Bac sequence contain all regulatory elements in order for cell type specific expression to be complete. If only a third of all Bac inserted animals actually contain the entire Bac sequence, then genotyping using multiple exogenous markers is certainly a worthwhile practice.

Figure 3.5



D	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
CMR	37.1	25.3	36.1	26.8	26.5	28.6	27.2	35.4	>40	37.4	>40	>40	37	25.2	>40
CRE	38	26.4	36.8	36	28.1	28.6	37	35.5	38.9	36.9	28.8	28.4	>40	26.1	36.9
rtTa	36.3	24.5	34.7	26.5	35.1	28.4	26.8	33.4	29.6	34.1	27.7	26.7	35.5	25	35.4

Figure 3.5: Genotyping of potential DAT Bac founder (F0) mice Genotyping of potential founder mice following DAT-Bac genomic insertion. Genomic DNA was collected from mouse Tails. Figure 3.4A-C was created by Dr. Shengcai Wei via conventional genotyping techniques. Animals 1-13 are potential founder mice (generation F0). Animal 14 is a positive control, known to have all 3 markers inserted into its genome. Animal 15 is a negative control (wild type mouse). Animals were genotyped for the presence of cmr (A), CRE (B) and rtTa (C). Figure 3.4D is a table of cycle thresholds (CTs) acquired via the qPCR method of genotyping. Results indicating the presence of the gene are highlighted in gray. Animals #3 and #10 in Figure 3.4C had a faint band which might have indicated the presence of rtTA. However, when using qPCR to screen, it was evident that these animals do not posses this marker (Figure 3.4D, animal #3 and #10).

The chloramphenical resistance gene (cmr) is included in the vector backbone of most Bac libraries, and makes for an excellent second marker for genotyping. While one cannot say for certain that the Bac has been inserted in entirety, genotyping for cmr still greatly increases the likelihood of having the entire Bac present. For example, our data shows that if we were to only screen our animals for the presence of rtTA then we would have found 12 animals with this marker. However, of these 12 pups, only 5 of them actually contained all 3 exogenous markers. In our study, genotyping with only one marker was more likely to identify animals with only a portion of the DAT-Bac sequence. Alternatively, if we were to genotype for the presence of both rtTa and cmr, we then would have identified 7 animals, 5 of which contained all three markers. Since not all groups can screen for 3 separate exogenous markers, screening for these two alone still helps insure that a larger fragment was inserted.

Animals positive for all 3 markers were bred with wild type (WT) mice. Only pups containing all three insertion markers were considered to be part of the positive, first generation (F1) DAT-Bac line. Of the 40 pups produced from this breeding (Figure 3.5, animal #2 bred to wild type), 19 of them tested positive for all 3 exogenous markers. 2 of them appeared to contain only a piece of the Bac, indicating that the founder mouse was may have been chimeric in nature (Figure 3.6). This is a strange occurrence, especially given the extremely low frequency of transmission. Approximately 5% of the F1 generation received only rtTA, when the expected would have been closer to 50%. It is difficult to say precisely why this was the case. Overall, it can be concluded that some form of chimeric distribution had likely occurred in our original F0 founder line. These 2 animals were investigated in more detail using qPCR (Table 3.1).

Figure 3.6

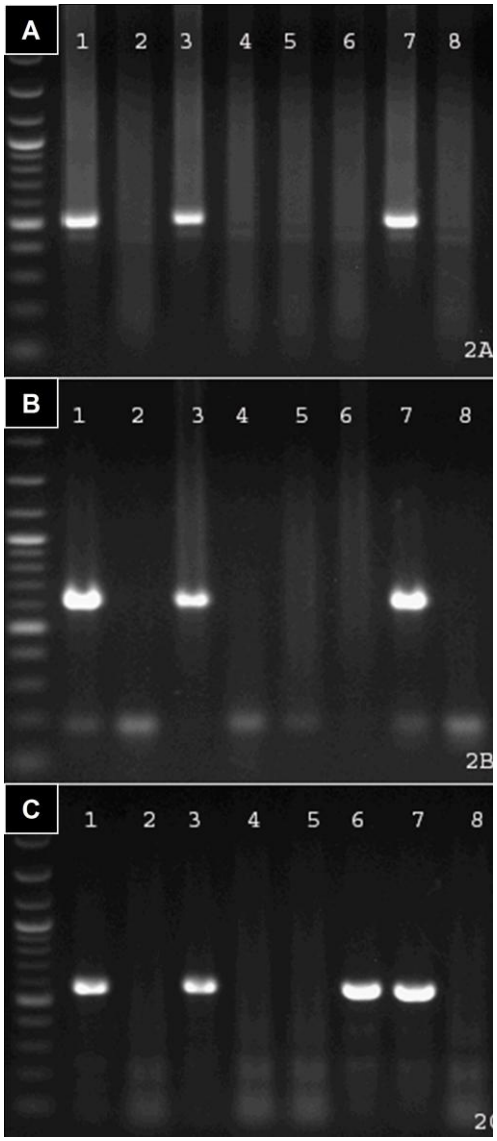


Figure 3.6: Genotyping of first generation (F1) of DAT Bac mice Genotyping of the first generation (F1) of DAT-Bac mice. Data was collected by Dr. Shengcai Wei via conventional genotyping techniques. The mice included in this gel are representative of the 13 total mice produced by this founder mouse. Animals 1-6 are F1 generation mice with potential transmission of the inserted DAT-Bac. Animal #7 is a positive control, the father of animals #1-6, and is also animal # 2 from Figure 3.4. Animal #8 is a negative control (wild type mouse). Animals were genotyped for the presence of the cmr (A), CRE (B) and rtTa (C) genes. These results indicate that only animals #1 and #3 received the entire DAT-Bac from the founder mouse. Mouse # 6 only received rtTa, indicating that the founder mouse might have been chimeric for Bac insertion.

3.4.4 DAT-Bac mouse qPCR confirmation of Bac insert size

Animals were also tested using qPCR, which amplified not only the three genotyping markers but also the regions of homologous DNA surrounding them. This method was used to assess the size of the Bac inserted into the mouse genome. Figure 3.7 shows a basic layout of the DAT-Bac and which regions of the Bac were amplified with qPCR.

Figure 3.7

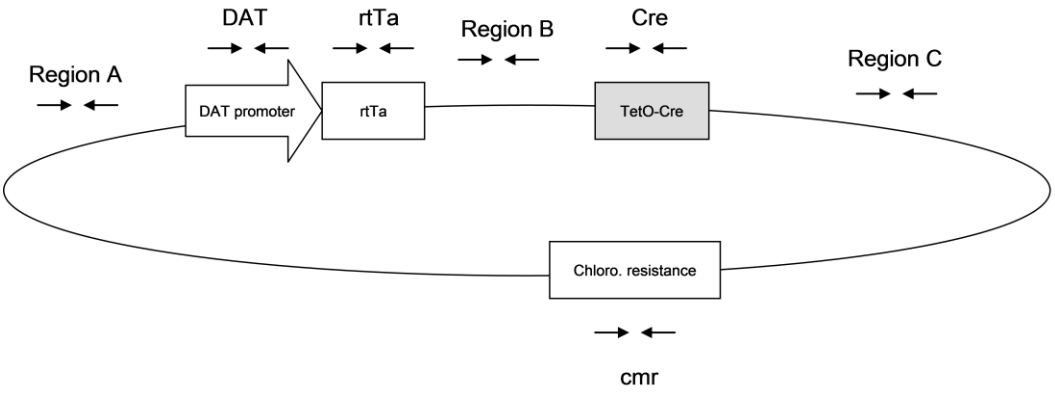


Figure 3.7: Map of DAT Bac qPCR primer sequence homology Basic layout of the DAT-Bac outlining the regions amplified by qPCR in order to test the insertion length in our DAT-Bac inserted mice. qPCR primers were designed over the 3 markers tested by conventional genotyping (rtTA, Cre and cmr) but some were also designed for regions homologous to the mouse genome (Region A, B, C and DAT). These homologous regions could not be looked at via conventional genotyping since they are not specific to Bac insertion. This allowed us to identify mice that had only received part of the Bac, which may have otherwise been considered positive for entire Bac insertion if genotyping was performed solely with conventional-PCR.

While Figure 3.7 shows an outline of the DAT-Bac, it is important to note that this Bac is inserted into the genomes of these animals. It is presented in the above manner for clarity purposes only. We cannot definitively say in which orientation the Bac was inserted into the mouse genome, but by using qPCR we can further validate that the Bac was inserted in near-entirety. It is important that we investigate the size of the inserted fragments, since the location of the regulatory elements that allow cell type specific expression is unknown. Table 3.1 shows a representation of the qPCR data used for this purpose.

Table 3.1

	A	DAT	rtTa	B	Cre	C	cmr
WT	-0.3	1.05	10.9	1.11	13.7	-0.4	10.2
Founder	-0.82	-1.65	-0.26	-0.35	2.64	-1.43	0.69
F1-1	-3	-2.63	-1.08	-1.57	0.45	-3.3	-1.51
F1-2	-0.41	0.5	9.6	0.49	14.1	-0.76	10.6
F1-3	-1.48	-0.77	0.27	-1.08	1.8	-1.44	0.31
F1-4	-0.68	-0.04	9.93	-0.32	14.5	-0.53	10.3
F1-5	-0.16	0	11	0.23	15.5	-0.66	12.6
F1-6	0.51	-1.05	1.48	1.11	14.2	-0.87	14.4
F1-28	-0.48	-1.37	1.24	0.52	23.9	-0.84	15.5

Table 3.1: qPCR genotyping of DAT-Bac mice qPCR of tail DNA acquired from a wild type (WT), founder, or first generation (F1) of Bac-inserted mice. Mice F1-1 through F1-6 are identical to animals #1-6 in Figure 3.6 above. Data are presented as delta-CT values, with all cycle thresholds (CT) being subtracted by the CT for genomic β-actin. Figure 3.6 shows a basic layout of the DAT-Bac, highlighting which areas are being amplified by these qPCR reactions. The mice included in this chart are representative of the 13 total mice produced by this founder mouse. Genomic regions found to be more frequent then occurring in the WT or Bac-negative (F1-2/4/5) mice are highlighted in grey. Some mice only received a partial Bac (F1-6 and F1-28). Therefore, the founder mouse appeared to be chimeric. Several mice had the entire Bac sequence inserted into their genome (F1-1 and F1-3). qPCR was able to show definitively that close to the entire DAT-Bac sequence was included in these 2 animals.

The first generation of mice (F1) born from the original founder (F0) either contained the entire Bac sequence, or only a fragment containing the DAT promoter and rtTA. This fragment was suspected when doing conventional genotyping as it tested positive for only one marker (rtTA) however qPCR was able to validate the size of this fragment in greater detail. The perplexing qPCR results acquired from the original founder mouse also imply its chimeric nature. While this mouse was shown to be able to transmit the Bac genotype via germline transmission, DNA collected from the tail is difficult to interpret. After forming the F1 generation however, the interpretation of this mouse line becomes much clearer.

3.5 Discussion

We designed transgenic mice with tet-inducible expression of Cre-recombinase in order to generate tools that can be used in conjunction with a wider array of cell type specific studies. While this has been done successfully in other tissue types, without using Bac recombineering, our method greatly increases the likelihood of getting cell type specific expression, and is also inducible (Rao and Monks 2009). Recombineering was used to insert rtTA directly after the ATG start of either the DAT or GAD67 genes. rtTA activates gene expression of the tet-promoter only when it interacts with a tetracycline derivative, such as doxycycline. Without both rtTA and doxycycline, Cre expression does not occur. Therefore, Cre can only be induced in dopaminergic or GABAergic cells respectively. Systemic treatments of pharmaceuticals, as well as knock-in/knockout transgenic studies all have a drastic effect on the entire central nervous system (CNS). In the case of conventional transgenic animals, this could include alterations in the development of the CNS. It is therefore very important that tools be designed which allow investigation of the CNS while avoiding these common potential confounds. The mouse lines described herein will act as powerful tools for investigation of the dopaminergic or GABAergic neurocircuitry.

Current methods which have been used to investigate the neurocircuitry of addiction lack the specificity needed to distinguish the individual neurotransmitter systems. At the heart of what is currently known about addiction is the knowledge that all drugs of abuse cause a significant increase in synaptic dopamine (DA) to occur within the NAc (Feltenstein and See 2008). DA indirectly modulates the output of the striatum via compelling control of interneuronal signaling, thereby modulating reward (Centonze, Grande et al. 2003). Dopamine signaling is also heavily involved in learning, with DA receptors located all throughout the limbic system. The limbic

circuitry of the CNS consists of the amygdala, hippocampus and thalamus and is involved in the formation of long-term memories. It is through these pre-existing neural circuits that associations to the rewarding properties of drugs are engrained over time (Hyman, Malenka et al. 2006). These normally helpful associative mechanisms likely exist to allow rapid learning of cues to natural rewards, such as food or opportunities for mating. Psychoactive drugs lead to excessive DA release, forcing maladaptive learning of drug associated rewards which then become overvalued in comparison to natural rewards (Hyman, Malenka et al. 2006). In many ways, addiction can be viewed as a rapid learning process that is largely dependant on DA signaling with the NAc (Hyman, Malenka et al. 2006; Everitt, Belin et al. 2008).

Drug addiction can perhaps be more readily understood when considered as uncontrolled learning of associative cues. The mesolimbic circuit, where the ventral tegmental area (VTA) projects to the NAc and hippocampus, has largely been attributed to modulation of memory formation. The VTA and the substantia nigra (SN) are the only sources of DA within the CNS, and both lie in the midbrain. However, the widespread dopaminergic connections leaving the VTA are not suited for directly encoding memory information, which is determined by the glutamatergic and GABAergic systems of the CNS. These connections would instead be best suited to synchronizing information across various brain regions in order to alter the motivational state of the organism (Hyman, Malenka et al. 2006). Evidence of this is seen when ablating the VTA or the NAc, both of which severely attenuates self-administration of cocaine or heroin (Feltenstein and See 2008). It has thus been postulated that activation of the dopaminergic, mesolimbic pathway is responsible for the reinforcing effects of drugs of abuse, as well as craving and relapse following chronic use (Feltenstein and See 2008). Thus, DA release in the NAc appears to allow the hedonic value of a goal to be attached to the environmental cues associated with the drug (Hyman, Malenka et al. 2006). Taken together, the normal associative learning mechanisms that lead to reward behavior are greatly modulated by dopaminergic

signaling. While DA itself may not be influencing memory formation directly, it indirectly reinforces drug related memories and behaviors, making them much more persistent.

While theories pertaining to the role of dopaminergic signaling are backed by a great deal of evidence, ultimately, very few results have been concrete. More precise manipulation of the dopaminergic system is necessary to further understand the complexities of this neuromodulatory circuit. Inducible, cell type specific expression of Cre recombinase opens many doors in this area. DAT-Bac animals could be bred to mice containing loxp-interrupted genes, apt for induced expression. This would lead to DA specific expression of proteins or shRNA (which would in turn reduce protein expression indirectly). The most obvious targets for such an amazing tool would be the receptors of the primary neurotransmitter systems (DA, glutamate and GABA). Mice with DA specific over expression or knockdown of any of these three neurotransmitter receptor types would greatly accelerate our knowledge in learning and addiction. Not only does dopaminergic enervation regulate the release of other neurotransmitters, there are also many feedback loops (especially in terms of glutamate and GABA) to the VTA (Briand, Vassoler et al. 2010). This reciprocal neurocircuitry is poorly understood, and discoveries in this area may increase our understanding of many CNS disorders, with one obvious example being Parkinson's disease (Fuentes, Petersson et al. 2010). Receptors expressed presynaptically on dopaminergic terminals have also been difficult to study, and could be elucidated using similar means (de Jesus Aceves, Rueda-Orozco et al. 2011). Additionally, AAV mediated delivery of loxp-interrupted genes could also accomplish these goals, all while adding an additional layer of specificity (Witten, Lin et al. 2010). AAV particles infect the cell bodies, as well as the projections afferent to the injection site (Franich, Fitzsimons et al. 2008). By using viral mediated delivery to a specific brain region (such as the striatum), genetic targets could be increased/decreased in expression within the subpopulation of dopaminergic neurons projecting to that area. This would

allow manipulation of afferent DA projections, while avoiding most of the potential confounds currently plaguing this type of research.

Taking this concept even further, the particulars of dopaminergic neuron activation or suppression could also be rigorously tested in DAT-Bac animals. Optogenetic control could achieve inducible, DA specific stimulation or suppression of neuronal firing. By breeding DAT-Bac mice with animals containing loxp-interrupted genes for light sensitive channels, dopaminergic firing could be directly controlled during behavioral testing for drug addiction. Since the light-activated cationic channels (channelrhopdsins) stimulate neuronal depolarization and the light-activated chloride-pumps (halorhodopsins) inhibit neuronal depolarization, a wide array of experiments could be conducted on dopaminergic neurons during different stages of addiction or learning (Lobo, Covington et al. 2010; Witten, Lin et al. 2010). These experiments would allow for stimulation or suppression of dopaminergic activity by placing a light filament in certain brain regions of these cross-bred animals. In so doing, region specific activation/suppression of DA terminals could be achieved. Outlined here are only a few of the near infinite experimental manipulations that these DAT-Bac mice could be used for.

The GAD67-Bac mice will also be extremely useful in the study of addiction and learning, as they will allow manipulation of yet another important neurotransmitter system of the CNS. The striatum is one of the largest single structures within the brain, and is believed to function by integrating information. Perhaps the most important aspect of the striatum toward this end, are its wide spread connections to the limbic circuitry of the brain. These connections allow the NAc to rapidly influence memory formation. The ventral striatum primarily consists of the Nucleus Accumbens (NAc) which is responsible for the primary reinforcing effects of drugs of abuse (Feltenstein and See 2008). The dorsal striatum is also involved, although its role becomes more evident in the later stages of addiction (Robbins, Ersche et al. 2008). Both areas of the striatum predominantly consist of GABAergic medium-spiny interneurons (MSNs). MSNs

not only integrate modulation by DA afferents, but also coordinate memory formation as regulated by glutamatergic projections from the hippocampus, amygdala and prefrontal cortex (PFC). This integration is very important since the formation of drug-paired memories is not only mediated by the hippocampus but also by the amygdala, and its connections to the limbic circuit (Robbins, Ersche et al. 2008). Additionally, the PFC assigns value to the rewarding properties of psychoactive drugs (Hyman, Malenka et al. 2006). Thus, GABAergic interneurons likely regulate the integration of afferent signaling mediated by striatum.

The integration mediated by these striatal cells is vital to learning, memory and addiction, but it is poorly understood from a circuitry perspective. Current methods used to manipulate this system are not specific to the GABAergic cells of the striatum. The striatum also contains larger cholinergic interneurons, which are believed to serve a different function (Beaulieu and Gainetdinov 2011). Additionally, pharmaceutical interventions injected directly into the striatum also influence the glutamatergic and dopaminergic afferents entering this brain region. More precise manipulation of the GABAergic system is therefore necessary to further understand the amalgamation of afferent signaling as occurring in this brain region. Since GAD67 positive medium-spiny neurons are by far the most common interneuron of the striatum, our GAD67-Bac mice will be a valuable tool. By breeding our GAD67-Bac animals to mice with loxp interrupted receptor or shRNA expression, the integration process mediated by the striatum could be investigated. Since the striatum receives and integrates a multitude of neurotransmitter signals, the GABAergic neurons residing in this area possess a large array of postsynaptic receptors. By selectively overexpressing or knocking down these receptors, the striatal GABAergic responses to each individual afferent neurotransmitter could be studied. This would greatly assist in shedding light on the aberrant striatal signaling that occurs following chronic drug use. Investigation of this brain area using these animals could also elucidate mechanisms underlying many disorders related to aberrant striatal signaling, such as Parkinson's disease, Huntington's disease and

schizophrenia (Tang, Chen et al. 2007; Ward, Kellendonk et al. 2009; la Fougere, Popperl et al. 2010).

The subregions of the striatum influence learning, memory and addiction in different ways (Lovinger 2010). AAV particles containing loxp-interrupted gene expression could be used to separately investigate striatal subregions, such as the NAc versus the dorsal striatum. Previous studies have been confounded by the inability to separate afferent signaling from interneuronal signaling. By utilizing AAV, the MSNs of the striatum could be investigated without directly affecting the glutamtergic or dopaminergic afferents of these striatal subregions. Alternatively, optogenetic control could also be used to activate or suppress neuronal firing of GABAergic neurons located within subcompartments of the striatum, as has already been done with ChAT-Bac mice expressing Cre (Witten, Lin et al. 2010). ChAT is primarily expressed by larger, cholinergic interneurons, which also reside within the striatum. These large interneurons are seperate and distinct from the GABAergic MSNs that predominately comprise the striatum. Research utilizing optogenetic control of ChAT neurons within the NAc found some very interesting aspects of ChAT neuronal control in a model of addiction-like behavior. It would be incredibly informative to begin this research anew using similarly bred GAD67-Bac animals. Utilizing optogenetic control to induce a state of aberrant striatal signaling could also greatly increase our knowledge of striatal disorders, such as Parkinson's disease. The experimental possibilities discussed above are only the beginning of a long list of potential uses for these amazing transgenic tools.

The learned process of addiction is neither wholly dependant on striatal signaling nor is it fully reliant on DA release by the VTA. Instead, an integrated view of these circuits is needed in order to better understand the progressive neurophysiological changes that lead to addiction. Armed with DAT-Bac and GAD67-Bac animals, these changes can be investigated in manner specific to the individual neurotransmitter systems. By focusing progressive research efforts on

the GABAergic neurons of the striatum and/or their DA afferents, the neurocircuitry of addiction can be investigated in a way never before possible. As other groups continue to generate novel animals containing loxp-interrupted or flanked genes, these Bac mice will continue to play a vital role in the study of addiction. Similarly, as viral mediated delivery systems evolve to include their own level of increased cell type specificity, use in our Bac animals will continue to be vital to this research. In conclusion, our transgenic animals will act as tools for a multitude of studies, the limitations of which will only be confined by the imagination of the investigator.

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Appendix A – Table of oligonucleotide sequences used to generate shRNA

Target	Label	Primer	Sequence
D2L & D2S	a	Forward	gcgCTGTCATGATGTGCACAGCAAGTAGTGAAGCCACAGATGTACTTGCTGTGCACATCATGACAT
	a	Reverse	gcaATGTCATGATGTGCACAGCAAGTACATCTGTGGCTTCACTACTTGCTGTGCACATCATGACAG
	b	Forward	gcgCACCCTGACAGTCCTGCCAAACTAGTGAAGCCACAGATGTAGTTTGGCAGGACTGTCAGGGTT
	b	Reverse	gcaAACCCTGACAGTCCTGCCAAACTACATCTGTGGCTTCACTAGTTTGGCAGGACTGTCAGGGTG
	c	Forward	gcgAACCGTTATCATGAAGTCTAATTAGTGAAGCCACAGATGTAATTAGACTTCATGATAACGGTG
	c	Reverse	gcaCACCGTTATCATGAAGTCTAATTACATCTGTGGCTTCACTAATTAGACTTCATGATAACGGTT
	d	Forward	gcgAACGCACATCCTGAATATACACTAGTGAAGCCACAGATGTAGTGTATATTCAGGATGTGCGTG
	d	Reverse	gcaCACGCACATCCTGAATATACACTACATCTGTGGCTTCACTAGTGTATATTCAGGATGTGCGTT
	e	Forward	gcgAGCCATGCCTATGTTGTATAACTAGTGAAGCCACAGATGTAGTTATACAACATAGGCATGGCC
	e	Reverse	gcaGGCCATGCCTATGTTGTATAACTACATCTGTGGCTTCACTAGTTATACAACATAGGCATGGCT
	f	Forward	gcgCGCTCAGGAGCTGGAAATGGAGTAGTGAAGCCACAGATGTACTCCATTTCCAGCTCCTGAGCT
	f	Reverse	gcaAGCTCAGGAGCTGGAAATGGAGTACATCTGTGGCTTCACTACTCCATTTCCAGCTCCTGAGCG
	g	Forward	gcgCGGAGATGCTGTCAAGCACCAGTAGTGAAGCCACAGATGTACTGGTGCTTGACAGCATCTCCA
	g	Reverse	gcaTGGAGATGCTGTCAAGCACCAGTACATCTGTGGCTTCACTACTGGTGCTTGACAGCATCTCCG
	h	Forward	gcgCCCACTGAACCTGTCCTGGTACTAGTGAAGCCACAGATGTAGTACCAGGACAGGTTCAGTGGA
	h	Reverse	gcaTCCACTGAACCTGTCCTGGTACTACATCTGTGGCTTCACTAGTACCAGGACAGGTTCAGTGGG
D2L	a	Forward	gcgAACCGTTATCATGAAGTCTAATTAGTGAAGCCACAGATGTAATTAGACTTCATGATAACGGTG
	a	Reverse	gcaCACCGTTATCATGAAGTCTAATTACATCTGTGGCTTCACTAATTAGACTTCATGATAACGGTT
	b	Forward	gcgCACCCACCCTGAGGACATGAAATAGTGAAGCCACAGATGTATTTCATGTCCTCAGGGTGGGTA
	b	Reverse	gcaTACCCACCCTGAGGACATGAAATACATCTGTGGCTTCACTATTTCATGTCCTCAGGGTGGGTG
	c	Forward	gcgCCATGAAACTCTGCACCGTTATTAGTGAAGCCACAGATGTAATAACGGTGCAGAGTTTCATGT
	c	Reverse	gcaACATGAAACTCTGCACCGTTATTACATCTGTGGCTTCACTAATAACGGTGCAGAGTTTCATGG
	d	Forward	gcgCTGGGAGTTTCCCAGTGAACAGTAGTGAAGCCACAGATGTACTGTTCACTGGGAAACTCCCAT
	d	Reverse	gcaATGGGAGTTTCCCAGTGAACAGTACATCTGTGGCTTCACTACTGTTCACTGGGAAACTCCCAG
	e	Forward	gcgAACCACTCAAGGGCAACTGTACTAGTGAAGCCACAGATGTAGTACAGTTGCCCTTGAGTGGTG
	e	Reverse	gcaCACCACTCAAGGGCAACTGTACTACATCTGTGGCTTCACTAGTACAGTTGCCCTTGAGTGGTT
	f	Forward	gcgCTATCATGAAGTCTAATGGGAGTAGTGAAGCCACAGATGTACTCCCATTAGACTTCATGATAA
	f	Reverse	gcaTTATCATGAAGTCTAATGGGAGTACATCTGTGGCTTCACTACTCCCATTAGACTTCATGATAG
	g	Forward	gcgCCTCTGCACCGTTATCATGAAGTAGTGAAGCCACAGATGTACTTCATGATAACGGTGCAGAGT
	g	Reverse	gcaACTCTGCACCGTTATCATGAAGTACATCTGTGGCTTCACTACTTCATGATAACGGTGCAGAGG
D2S	a	Forward	gcgATCAAGGATGCTGCCCGCCGAGTAGTGAAGCCACAGATGTACTCGGCGGGCAGCATCCTTGAG
	a	Reverse	gcaCTCAAGGATGCTGCCCGCCGAGTACATCTGTGGCTTCACTACTCGGCGGGCAGCATCCTTGAT
	b	Forward	gcgCAGACACCACTCAAGGATGCTGTAGTGAAGCCACAGATGTACAGCATCCTTGAGTGGTGTCTT
	b	Reverse	gcaAAGACACCACTCAAGGATGCTGTACATCTGTGGCTTCACTACAGCATCCTTGAGTGGTGTCTG
	c	Forward	gcgCGAAGACACCACTCAAGGATGCTAGTGAAGCCACAGATGTAGCATCCTTGAGTGGTGTCTTCA
	c	Reverse	gcaTGAAGACACCACTCAAGGATGCTACATCTGTGGCTTCACTAGCATCCTTGAGTGGTGTCTTCG
	d	Forward	gcgCCCACTCAAGGATGCTGCCCGCTAGTGAAGCCACAGATGTAGCGGGCAGCATCCTTGAGTGGT
	d	Reverse	gcaACCACTCAAGGATGCTGCCCGCTACATCTGTGGCTTCACTAGCGGGCAGCATCCTTGAGTGGG
	e	Forward	gcgCCACCACTCAAGGATGCTGCCCTAGTGAAGCCACAGATGTAGGGCAGCATCCTTGAGTGGTGT
	e	Reverse	gcaACACCACTCAAGGATGCTGCCCTACATCTGTGGCTTCACTAGGGCAGCATCCTTGAGTGGTGG
	f	Forward	gcgAACACCACTCAAGGATGCTGCCTAGTGAAGCCACAGATGTAGGCAGCATCCTTGAGTGGTGTC
	f	Reverse	gcaGACACCACTCAAGGATGCTGCCTACATCTGTGGCTTCACTAGGCAGCATCCTTGAGTGGTGTT
	g	Forward	gcgAAAGACACCACTCAAGGATGCTTAGTGAAGCCACAGATGTAAGCATCCTTGAGTGGTGTCTTC
	g	Reverse	gcaGAAGACACCACTCAAGGATGCTTACATCTGTGGCTTCACTAAGCATCCTTGAGTGGTGTCTTT

Appendix B – Table of primers/probes used in duplex qPCR of striatal tissue

mRNA Target	Primer/Probe	Sequence	Efficiency	R^2
β actin	Forward	CTGGCACCACACCTTCTACA	1.00	1.00
	Reverse	AGGTCTCAAACATGATCTGGGT		
	Probe-Hex or Fam	CTGTGCTGCTCACCGAGGCC		
D2L	Forward	ACTCAAGGGCAACTGTACCC	0.97	0.99
	Reverse	GTGCTTGACAGCATCTCCAT		
	Probe-Hex	CCCTGAGGACATGAAACTCTGCACC		
D2S	Forward	AGCTTTCAGAGCCAACCTGA	0.82	0.99
	Reverse	GTGCTTGACAGCATCTCCAT		
	Probe-Hex	ACACCACTCAAGGATGCTGCCC		
D1R	Forward	TTACCTGATCCCTCATGCTG	0.93	0.99
	Reverse	AGCTTCTCCAGTGGCTTAGG		
	Probe-Fam	CCACCGGCCTCCTCCCTCTT		
TrkB	Forward	GTCTGGCTGCTCCTAACCTC	0.89	0.99
	Reverse	CAACGTCCCAGTACAAGGTG		
	Probe-Fam	CCCACACTGCAGGAAAGGGTCA		
RGS4	Forward	AAGTCCCAAAGCCAAGAAGA	0.98	1.00
	Reverse	CTCTCTGGTGCAAGAGTCCA		
	Probe-Fam	CGAGTTCATCTCAGTGCAAGCAACAA		
RGS9	Forward	GGATCTCTAACCTGGAGGCA	0.84	0.98
	Reverse	TATGGCGTCTGAAATCGGTA		
	Probe-Fam	TCATCCTCAAGCCGGACAGCA		

Appendix C: Primers for insertion of rtTa and Cre into DAT-Bac

Primer Name	Purpose	Sequence
DAT1-GalKf	To amplify Galk with homology to DAT	TATGTTGGTTGAAGTCGAAGAAGAAGGAAACAGACTTCCTCGGGCTCCCGTCTACCC**ATG***CCTGTTGACAATTAATCATCGGCA*
DAT1-GalKr	" "	GGGCTCTTTAGCCGGGGCCACCACAGAAGACATTGGTCCCACGGAGCATTTGCTTTTACT*TCAGCACTGTCCTGCTCCTT*
rtTA-R	To amplify rtTa m2.2	GGCGGTGGAATCGAAATCTCCCGGTCAATCAATCAGAATTGA
SV40-F	" "	TCAATTCTGATTGATTGACCGGGAGATTTCGATTCCACCGCC
DAT1-5′f	To amplify area 5' of DAT	AAAGACACCTGCCAAACTCCTTG
DAT1-5′r	" "	GGAGCCCGAGGAAGTCTGTTT
DAT1-3′f	To amplify area 3' of DAT	AGTAAAAGCAAATGCTCCGTGGGACCAATGTC
DAT1-3′r	" "	ATGTCTCAGCCCAGTCCCTAGC
DAT2-GalKf	To amplify Galk with homology to Lpcat1	AGTTCAGTCCTGCGACCCAAAATTTAAGGTCTCCCTACACATGCAAATTCGCAGGATTCCCCTGTTGACAATTAATCATCGGCA
DAT2-GalKr	" "	GGACCGGGCGGCGCCCAGCCCCTCCCCACCGCATCCCGAGCGCATCCTGGATAGAGTACATCAGCACTGTCCTGCTCCTT
Cre-f	To amplify Cre	AAGTGCCACCTGACGTCGACGG
Cre-r	" "	GCGCTTAATGCGCCGCTAC
DAT-Cre-f1	To amplify area 5' of Lpcat1	TGCTCAAATAAGCTTTGCCTCCA
DAT-Cre-r1	" "	CCGTCGACGTCAGGTGGCACTTaACCTCCCCAAAGTCCAAACTTCC
DAT-Cre-f2	To amplify area 3' of Lpcat1	CGCTCACTGCCCGCTTTCCAACTGTGTGTCTCCGTGGCCTAGT
DAT-Cre-r2	" "	GCGGGCTATATGAGACTCCCAGT

Appendix D: Primers for insertion of rtTa and Cre into GAD67-Bac

Primer Name	Purpose	Sequence
GAD-GalKf	To amplify Galk with homology to GAD67	CCTGCGCCCAGTCTGCGGGGGACCCTTGAACCGTAGAGACCCCAAGACCACCGAGCTG**ATG**CCTGTTGACAATTAATCATCGGCA
GAD-GalKr	" "	TGGTAGTATTAGGATCCGCTCCCGCGTTCGAGGAGGTTGCAGGCGAAGGAGTGGAAGATGCTCAGCACTGTCCTGCTCCTT
GAD-rtTAf	To amplify rtTa m2.2	CCAAGACCACCGAGCTGATGTCTAGACTGGACAAGAGCAAAGTC
GAD-sv40pAr	" "	GTTGCAGGCGAAGGAGTGGAAGATGCCAGGCTTTACACTTTATGCTT
GAD-5′f	To amplify area 5' of GAD67	GCGGGGTCTTAGATTTACCCAGA
GAD-5′r	" "	CATCAGCTCGGTGGTCTTGG
GAD-3′f	To amplify area 3' of GAD67	CTTCCACTCCTTCGCCTGCAAC
GAD-3′r	" "	AGAATTTTGGGAGAGGGGTGGTG
hpg-GalKf	To amplify Galk with homology to hpg	TCTCATTTCTACTTCACTTAAGCAAAGCCCCCTTTGGACGCAGGCCGAGATTATCAAACACCTGTTGACAATTAATCATCGGCA
hpg-GalKr	" "	CTTACTTTGCTGAGAATGCATTAGCTCCACCCGGGATCTGCGCTGGAGAAGTATAAAGCTTCAGCACTGTCCTGCTCCTT
Cre-f	To amplify Cre	AAGTGCCACCTGACGTCGACGG
Cre-r	" "	GCGCTTAATGCGCCGCTAC
hpg-Cre-f1	To amplify area 5' of hpg	CTGGACAGGGGAGCAGAACACT
hpg-Cre-r1	" "	CCGTCGACGTCAGGTGGCACTTATAATCTCGGCCTGCGTCCAA
hpg-Cre-f2	To amplify area 3' of hpg	CGCTCACTGCCCGCTTTCCAAGCTTTATACTTCTCCAGCGCAGAT
hpg-Cre-r2	" "	CTCGTGCCTTTAATCCCAGCAC

Appendix E: Predicted changes in GAD67-Bac throughout recombineering

Length of Bac fragment (base pairs)				
PstI Digestion				
OLD	Galk1	rtTa	Galk2	Cre
8696	8696	8696	8696	8696
7769	7769	7769	7769	7769
7535	7535	7535	7535	7535
5947	5947	5947	5947	5947
5645	5645	5645	5645	5645
	5509			
5446	5446	5446	5446	5446
				5414
5119	5119	5119	5119	5119
			4885	
		4882	**4882**	**4882**
4763	4763	4763	4763	4763
4735	4735	4735	4735	4735
4323	4323	4323	4323	4323
4279				
4224	4224	4224	4224	4224
4049	4049	4049	4049	4049
3663	3663	3663	3663	3663
3655	**3655**	**3655**		
3653	3653	3653	3653	3653
3548	3548	3548	3548	3548
3337	3337	3337	3337	3337
3110	3110	3110	3110	3110
3086	3086	3086	3086	3086
3018	3018	3018	3018	3018
3009	3009	3009	3009	3009
2892	2892	2892	2892	2892
2858	2858	2858	2858	2858
2770	2770	2770	2770	2770
2724	2724	2724	2724	2724
2668	2668	2668	2668	2668
2664	2664	2664	2664	2664
2628	2628	2628	2628	2628
2491	2491	2491	2491	2491
2437	2437	2437	2437	2437
2353	2353	2353	2353	2353
2345	2345	2345	2345	2345
2339	2339	2339	2339	2339
2197	2197	2197	2197	2197

2021	2021	2021	2021	2021
1952	1952	1952	1952	1952
1916	1916	1916	1916	1916
1714	1714	1714	1714	1714
1701	1701	1701	1701	1701
1576	1576	1576	1576	1576
1576	1576	1576	1576	1576
1541	1541	1541	1541	1541
1497	1497	1497	1497	1497
1463	1463	1463	1463	1463
1295	1295	1295	1295	1295
		1261	**1261**	**1261**
1252	1252	1252	1252	1252
1235	1235	1235	1235	1235
1202	1202	1202	1202	1202
1161	1161	1161	1161	1161
1008	1008	1008	1008	1008
983	983	983	983	983
880	880	880	880	880
794	794	794	794	794
675	675	675	675	675
570	570	570	570	570
513	513	513	513	513
510	510	510	510	510
473	473	473	473	473
458	458	458	458	458
403	403	403	403	403
399	399	399	399	399
230	230	230	230	230
133	133	133	133	133

SmaI Digestion				
OLD	Galk1	rtTa	Galk2	Cre
48422	48422	48422	48422	48422
16359	16359	16359	16359	16359
15395	15395	15395	15395	15849
				15395
14431	14431	14431	14431	14431
9752	9752	9752	9752	9752
			8951	
8067	8067	8067	8067	8067
7833	7833	7833	7833	7833
7721	**7721**	**7721**		
6681	6681	6681	6681	6681
6370	6370	6370	6370	6370
6369	6369	6369	6369	5499
5499	5499	5499	5499	
4993	4993	4993	4993	4993
		4581	**4581**	**4581**

2717	**3947**			
2616	2616	2616	2616	2616
1392	1392	1392	1392	1392
1306	1306	1306	1306	1306
880	880	880	880	880
665	665	665	665	665
376	376	376	376	376
132	132	132	132	132
70	70	70	70	70
43	43	43	43	43

KpnI Digestion				
OLD	Galk1	rtTa	Galk2	Cre
18418	18418	18418	18418	18418
16793	16793	16793	16793	16793
14064	14064	14064	14064	14064
13409	13409	13409	13409	13409
12294	12294	12294	12294	12294
		10827	**10827**	**10827**
	10193			
9836	9836	9836	9836	9836
9543	9543	9543	9543	9543
9497	9497	9497	9497	9497
				9185
8963				
			8656	
7426	**7426**	**7426**		
7034	7034	7034	7034	7034
6687	6687	6687	6687	6687
6635	6635	6635	6635	6635
5645	5645	5645	5645	5645
5136	5136	5136	5136	5136
5039	5039	5039	5039	5039
4441	4441	4441	4441	4441
3084	3084	3084	3084	3084
2795	2795	2795	2795	2795
1350	1350	1350	1350	1350

Appendix F: Predicted changes in DAT-Bac and SV40pA recombination

HindIII Digestion Fragment sizes (in bp)		
rtta-Cre	**polyA recombination**	**rtta-galk2**
10281		10281
9996		9996
8881	8881	8881
8755	8755	8755
8140	8140	8140
7956		7956
7949		7949
7447		7447
7213	7213	7213
6924		6924
6582	6565	6565
6565	6511	6511
6511	6131	6131
6131		
	6047	
5783	5783	5783
		5747
5579	5579	5579
5153		5153
4879	4879	4879
4647		4647
4329	4329	4329
4255	4255	4255
4148	4148	4148
4052	4052	4052
4018	4018	4018
3178	3178	3178
3172	3172	3172
3159	3159	3159
2974		2974
2890	2890	2890
2413	2413	2413
2366	2366	2366
2331	2331	2331
2328	2328	2328
2183	2183	2183
2149	2149	2149
2109	2109	2109
2092	2092	2092
2010	2010	2010
1943		1943
1847		1847
1798	1798	1798
1733	1733	1733
1705		1705
1594	1594	1594

1386	1386	1386
1227		1227
1196	1196	1196
1195	1195	1195
1112	1112	1112
1088	1088	1088
907	907	907
667	662	667
662	658	662
658		658
614	614	614
575	575	575
567	567	
510	510	510
490	490	490
449	449	449
440	440	440
375	375	375
327		327
302	302	302
277	277	277
247	247	247
163	163	163
163		163
154	154	154
147	147	147
113	113	113
99	99	99
72	72	72

Appendix G: Primers for genotyping Bac-inserted mice

Primer Name	Purpose	Sequence
rtTA-GT-F5	To screen for presence of rtTa	AATGTGAATGAGGGCTTTGG
rtTA-GT-R5	" "	AGCGGAATGACTTGGCGTTG
Cre-GT-F4	To screen for presence of Cre	CTCCACAGCCTTCTTAGCCC
Cre-GT-R4	" "	TGCGATCTGACGGTTCACTA
CMR-F	To screen for presence of cmr	GACATGGAAGCCATCACAAA
CMR-R	" "	CCTATAACCAGACCGTTCAGC

Appendix H: qPCR primers for genotyping Bac-inserted mice

Primer	Purpose	Sequence
rtTAgt-F	Genotype for presence of rtTa	CTGGGAGTTGAGCAGCCTAC
rtTAgt-R	" "	CGATGTGAGAGGAGAGCACA
CREgt-F	Genotype for presence of Cre	TGCAACGAGTGATGAGGTTC
CREgt-R	" "	ATGTTTAGCTGGCCCAAATG
cmr-f	Genotype for presence of cmr	CCGAATTGACTAGTGGGTAGG
cmr-r	" "	GGGTTCTTTGTTCTCTTTGAATG
DAT-F	Detect an increase in DAT copy #	GGCAGGAAAGAGGATGTGTC
DAT-R	" "	GGATAAAAGATAGCTAAGGGATGTG
BacRegA-F	Detect an increase in copy #	CAAAGGCACAGCACAGAAAA
BacRegA-R	" "	GGAAAAGTTTGGGCTCACAG
BacRegB-F	Detect an increase in copy #	TGCAAAAGAGACGCACATTC
BacRegB-R	" "	CTGGCAAAGCTTCATGTTCA
BacRegC-F	Detect an increase in copy #	CAGTGGCAGCATGTCCTCTA
BacRegC-R	" "	GACGTGTTCACCATGCAAAC
GenomActin-F	Act as a control for approximate copy #	CTGTATTCCCCTCCATCGTG
GenomActin-R	" "	ACCATCACACCCTGTGGAAG