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TITLE: Central and Peripheral Mechanisms of Antipsychotic Medication-Induced Metabolic Dysregulation

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<b>14. ABSTRACT</b> Antipsychotic drugs (APDs) are widely used psychotropic medications, though they have significant metabolic side effects. While the mechanisms for these metabolic disturbances are poorly understood, the single known unifying property of all APDs is their blockade of the dopamine D <sub>2</sub> (D2R) and D <sub>3</sub> (D3R) receptors. We therefore hypothesize that D2R and/or D3R mediate the metabolic side effects of APDs both centrally in the hypothalamus and peripherally in pancreas, areas critical for metabolic regulation. In Year 1 of this award, we have completed the design of a D3R-flox mouse in order to selectively knock out expression of D3R in the hypothalamus and pancreatic beta cells. The resulting transgenic mice are being tested to confirm the successful production of the strain. In parallel, we have completed construction of novel inducible transgenic hypothalamic- and pancreatic beta cell-specific D2R knockout (KO) mice. Additionally, using pancreatic islets isolated from beta cell-selective D2R KO mice and complete D3R KO mice, we found diminished inhibition of stimulated insulin secretion in both strains relative to littermate controls, suggesting a role for both receptors in mediating insulin secretion.					
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## 1. INTRODUCTION

Antipsychotic drugs (APDs) are widely used psychotropic medications for numerous psychiatric illnesses including schizophrenia, posttraumatic stress disorder and depression. However, these medications also have significant metabolic side effects characterized by substantial weight gain, glucose intolerance, insulin resistance, hypertension and dyslipidemia as well as increased risks for type 2 diabetes and cardiovascular disease. Indeed, the prevalence of these APD-induced metabolic side effects in Veterans is more than twice that of the general population. However, the mechanisms for these metabolic disturbances are not well understood. Significantly, all APDs cause these side effects to differing degrees and ultimately result in life-shortening morbidity. A potentially important clue is that the single known unifying property of all APDs is their blockade of the dopamine D<sub>2</sub> (D2R) and D<sub>3</sub> (D3R) receptors, suggesting a role for these receptors in APD metabolic side effects. Consistent with this, D2R and D3R are expressed both centrally in the hypothalamus in regions mediating appetite and feeding behavior as well as peripherally in insulin-releasing pancreatic beta cells, key regulators of metabolism. We previously showed that activation of pancreatic beta cell D2R and D3R inhibited glucose-stimulated insulin secretion (GSIS) and that APD-induced receptor inhibition disrupted this regulatory mechanism. Thus, our central hypothesis is that D2R and/or D3R are critical regulators of metabolism and mediate the metabolic side effects of APDs both centrally in the hypothalamus and peripherally in pancreas. However, the relative contributions of peripheral and central D2R and D3R to APD-induced metabolic dysregulation are unknown. To disentangle these mechanisms, in partnership with PI Dr. Zachary Freyberg, we will aim to do the following: (1) to identify contributions of hypothalamic D2R and D3R action in APD-induced weight gain and metabolic dysregulation *in vivo*; (2) to identify the relationship of peripheral D2R and D3R to APD-induced weight gain and metabolic dysfunction *in vivo*; and (3) to identify APD-mediated effects on insulin and DA release in pancreatic beta cells using real-time imaging. Key to these aims is the generation of tissue-specific D2R and D3R knockout (KO) mice targeting either hypothalamus or pancreatic beta cells. In the short term, our work will elucidate the anatomical and functional mechanisms of APD-induced metabolic side effects. In the longer term, we will use our findings to develop better-targeted APDs that can selectively reverse these drugs' metabolic side effects while preserving their clinical efficacy.

## 2. KEYWORDS

Keywords relevant to the work proposed here include:

1. Antipsychotic drug (APD)
2. Dopamine (DA)
3. Dopamine D<sub>2</sub> Receptor (D2R)
4. Dopamine D<sub>3</sub> Receptor (D3R)
5. Insulin
6. Glucose-stimulated insulin secretion (GSIS)
7. Diabetes
8. Metabolism

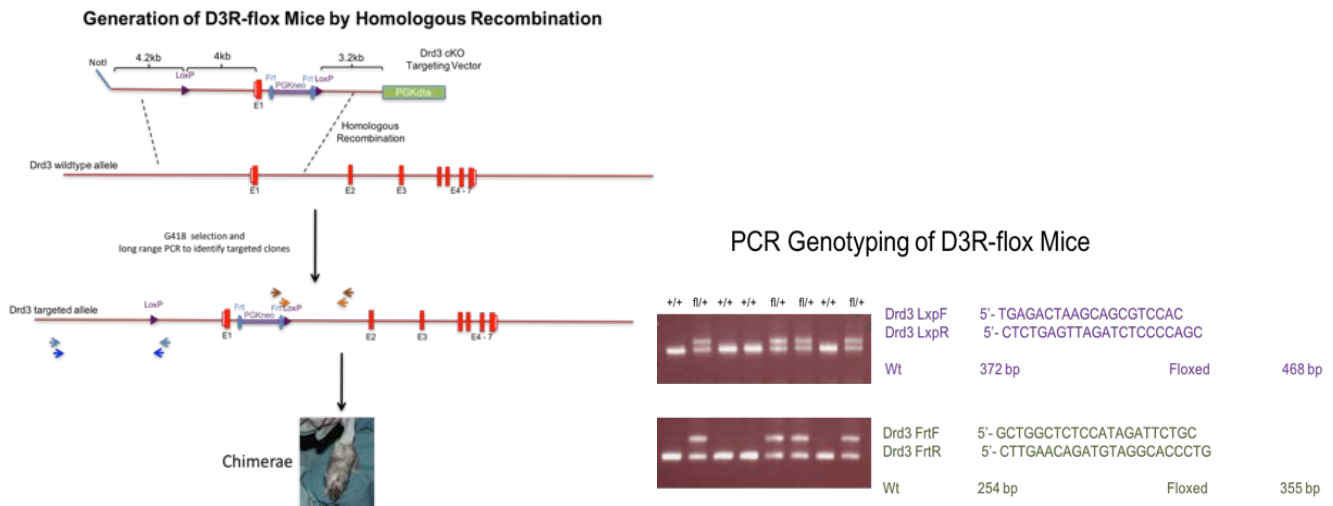
## 3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

The major goals of the project as stated in the approved SOW are as follows:

- A. Metabolic characterization of hypothalamus-specific D2R and D3R knockout mice in the presence or absence of APD treatment
- B. Metabolic characterization of pancreatic beta cell-specific D2R and D3R knockout mice in the presence or absence of APD treatment
- C. Treatment with domperidone to determine whether peripheral D2R/D3R blockade alone can produce relevant metabolic disease
- D. Determine the precise contributions of D2R and D3R to glucose-stimulated insulin and dopamine release using pancreatic islets from pancreatic beta cell-selective D2R and D3R knockout mice as well as wildtype controls
- E. Determine effects of APDs on kinetics of real-time glucose-stimulated insulin and dopamine release in wildtype and beta cell-specific D2R or D3R knockout mouse pancreatic islets.

- **What was accomplished under these goals?**



**Figure 1. Construction of targeted D3R-flox transgenic mouse strain.** We employed a recombinering approach to insert LoxP cassettes that flank the exon 1 coding region of the Drd3 gene that encodes the D3R protein. Insertion of these cassettes has resulted in the generation of chimeric mice.

**Figure 2. PCR genotyping to confirm insertion of D3R LoxP sites.** We collected tail clippings from our newly generated D3R-flox chimeric mice and confirmed the presence of the LoxP sites flanking exon 1 of the Drd3 gene using two independent PCR strategies (see primers in purple and green, respectively). The successful insertion of the LoxP elements was confirmed by the presence of two PCR bands versus a single band in the wildtype littermates.

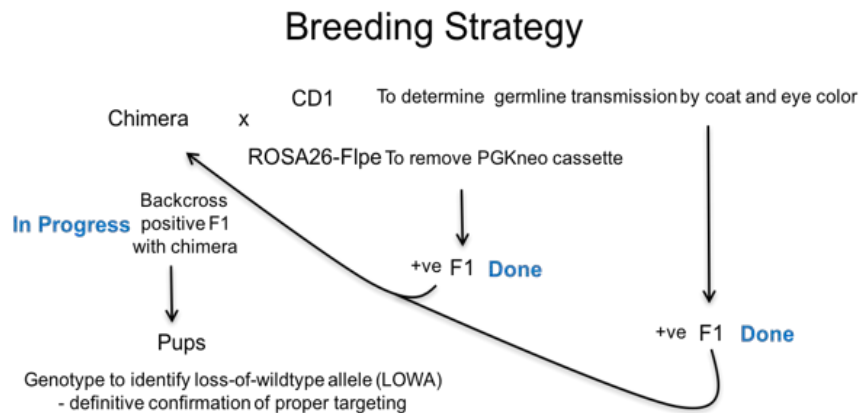
In the course of the reporting period for Year 1 of this award, we conducted studies to address each of the major goals of the project as follows:

**I. Metabolic characterization of hypothalamus-specific D2R and D3R knockout mice in the presence or absence of APD treatment**

- In order to characterize the metabolic consequences of hypothalamus-specific knockout of D2R and D3R, we commenced construction of the transgenic D3R-flox mouse strain required for tissue-selective knockout of D3R. Consequently, in consultation with Dr. Claudia Schmauss (a consultant on this award) and Dr. Siu-Pok Yee of the University of Connecticut at Storrs, we examined the genomic sequences associated and flanking the Drd3 gene corresponding to D3R. We then established a strategy to insert LoxP cassettes flanking the first exon of Drd3. In practice, the presence of these flanking cassettes permits Cre-recombinase mediated excision of this exon and therefore prevents transcription and translation of the entire D3R protein. The first step in constructing this D3R-flox mouse strain was the design of a genomic targeting vector. The targeting vector was prepared by recombinering according to Lee et al. [Lee *et al.*, (2001) Genome Research 13:476–484]. Briefly, we first retrieved approximately 12.2 kb of Drd3 genomic sequence containing 8.3 kb of 5'-upstream, exon 1 and of 3.5 kb intron 1 sequence from the BAC, RP24-135K7, into the pDTA vector containing the PGK-DTA negative selectable marker by gap repair. We then inserted the 5' LoxP site approximately 4 kb upstream of the Drd3 transcriptional start site in exon 1, followed by insertion of Frt-PGFneo-Frt-LoxP approximately 500 bp 3' downstream of exon 1. The final vector contains 5' and 3' arms of 4.2 and 3.2 kb, respectively. The vector was then linearized by NotI digestion, purified and electroporated into mouse ES cells derived from F1(129Sv/C57BL6j) blastocysts. Electroporated cells were cultured in the presence of the G418 antibiotic 48 hours post-electroporation. Drug-resistant colonies were picked and screened by long range PCR using primers corresponding to sequences outside the arms and specific to the 5' and 3' LoxP sites to identify targeted ES clones. Targeted ES clones were then expanded and further analyzed by long-range PCR for confirmation prior to

using them for ES<->morula aggregation to generate chimeric animals (Figure 1). We further confirmed the presence of the inserted LoxP sites within the chimeric animals by additional PCR assays (Figure 2).

At present, the chimeric animals that we have generated are being bred with ROSA26-Flpe mice to remove the PGK neo cassette to generate the final conditional D3R-flox mouse strain (Figure 3).



**Figure 3. Breeding strategy to complete construction of the D3R-flox transgenic mouse strain.** Using our newly generated D3R-flox chimeric mice, we have conducted two separate crosses in parallel: (1) to CD1 mice to determine whether we have established successful germline transmission of the LoxP sites; and (2) to ROSA26Flpe mice to remove the PGKneo cassette. Presently, we are conducting the final backcrosses to complete the final targeted D3R-flox mice.

Upon generation of the strain, we will begin breeding them to Nkx2.1-cre mice which selectively express the Cre recombinase throughout the hypothalamus. The resulting Nkx2.1-cre hemizygous/D3R-flox homozygous mice will have selective knockout of D3R across the hypothalamus.

- In order to generate hypothalamus-specific D2R knockout mice, we bred our existing D2R-flox and Nkx2.1-cre hypothalamic expression driver mice to one another over the course of the year. This series of breeding steps required 12 months of breeding (concluding in September 2016) and permitted us to generate the final Nkx2.1-cre hemizygous/D2R-flox homozygous mice.
- Significantly, we have successfully secured IRB approval for all of our animal work during this reporting period.

## II. Metabolic characterization of pancreatic beta cell-specific D2R and D3R knockout mice in the presence or absence of APD treatment

- As of September 2016, we have completed construction of a transgenic mouse strain with transgenes for inducible D2R knockout specifically in pancreatic beta cells. In the course of designing the breeding strategy, we were able to use a new pancreatic beta cell-specific expression driver, Mip1-cre/ERT which has two important advantages over previous beta cell cre driver strains: (1) it is more specific to beta cells with virtually no off-target expression in other organ systems including brain; and (2) it is inducible, allowing us to avoid possible developmental effects that could confound our interpretation of the results. Following approximately 12 months of crosses, we have now successfully generated the final desired genotype of hemizygous cre; homozygous D2R-flox mice required to completely knockout expression of D2R selectively in pancreatic beta cells. With this transgenic mouse strain established, they can now be amplified in numbers sufficiently powered to resolve potential effects of D2R absence on APDs' effects on glucose tolerance, insulin resistance, glucose stimulated insulin secretion, adiposity and weight gain.
- The Mip1-cre/ERT mice are also being prepared for crosses to the new D3R-flox mouse strain in order to begin construction of an inducible beta cell-specific D3R knockout mouse line. We expect the crosses to begin in December 2016.

- Both male and female cohorts of D3R floxed mouse lines required for the above mentioned crosses have been maintained on the high fructose, medium fat diet for 16 weeks. As these are a critical control strain, it was essential to perform metabolic characterization and surgical preparations in of this line of mouse prior to crosses. During the 16 week high fructose medium fat diet maintenance regimen, the D3 floxed animals become obese, glucose and insulin intolerant, show reduced energy expenditure after an initial hyperphagia and weight gain phase, and reduced locomotor activity. In this regard they do not differ significantly from wild type C57B6J mice maintained on this diet, which we have phenotyped in parallel. Furthermore, there are no apparent differences between males and females in either the quality or magnitude of the metabolic deficiencies that result from the high fructose diet maintenance. Importantly, we have performed chronic intravenous and intra-arterial catheterization in these animals in preparation for insulin clamp studies of hepatic insulin action; these obese, glucose and insulin intolerant animals survive the surgery well, and have stable elevated basal glucose levels, and are thus amenable to the planned clamp procedures.

### **III. Treatment with domperidone to determine whether peripheral D2R/D3R blockade alone can produce relevant metabolic disease**

- We have been working closely with the Initiating PI of this project, Dr. Zachary Freyberg, to optimize the dietary conditions responsible for inducing the development of insulin resistance. Initial studies with standard 60% high fat diet, while promoting significant adiposity over the course of 16 weeks, failed to reliably drive glucose intolerance as a critical baseline condition for observation of the effects of D2R/ D3R blockade on diabetic features of obesity, including reduced glucose intolerance, insulin insensitivity, and reduced hepatic insulin action. Consequently we have identified and employed a 12-16 week trial of high fructose, medium fat diet supplied by Research Diets, that promotes fatty liver and glucose intolerance in C57B/6J mice. We find that it not only promotes significant adiposity but also significant glucose intolerance and insulin resistance comparable to those seen in clinical populations, including in those treated with APDs. Currently these animals require an oral glucose gavage of 1.5- 2 mg/kg for oral glucose tolerance testing, due to their chronic basal elevated levels that range from 180-225 mg/dl. Consequently, we will henceforth use this diet and strain as a baseline with which to compare effects of domperidone on development of metabolic disease both in wildtype as well as in beta cell-specific and hypothalamic D2R or D3R knockout mice. Importantly, we have performed chronic intravenous and intra-arterial catheterization in these animals in preparation for insulin clamp studies of hepatic insulin action; these obese, glucose and insulin intolerant animals survive the surgery well, and have stable elevated basal glucose levels, and are thus amenable to the planned clamp procedures well as the clamp level for both hyperinsulinemic- euglycemic and euinsulinemic- hyperglycemic clamps.

- **What opportunities for training and professional development has the project provided?**

Nothing to Report.

- **How were the results disseminated to communities of interest?**

Work resulting from this award were presented at an international meeting, Dopamine 2016, (held in September 2016, Vienna, Austria). At the Dopamine 2016 meeting, one abstract based on these studies were published. Presently, we are preparing three manuscripts based on our characterization of the mechanisms by which dopamine and dopamine D<sub>2</sub> and D<sub>3</sub> receptors mediate glucose-stimulated insulin secretion. We hope that the publication of these manuscripts will facilitate the dissemination of the experimental data derived from the aims of this project.

- I. **What do you plan to do during the next reporting period to accomplish the goals?**

#### **I. Metabolic characterization of hypothalamus-specific D2R and D3R knockout mice in the**

### **presence or absence of APD treatment**

- In the next reporting period, we will conduct weekly measurement of weights and food consumption in hypothalamus-specific D2R (and wildtype littermate controls) treated with either with first-generation APD haloperidol or second-generation APD olanzapine (via i.p. administration). We will also measure serum fasting glucose and insulin levels in hypothalamus-specific D2R knockout mice and wildtype littermate control mice in the presence or absence of APD treatment; serum will be collected at weeks 13 and 26 of APD treatment.
- With the completion of construction of the D3R-flox mice, we will also begin crosses to establish hypothalamus-specific D3R knockout mice (Nkx2.1-cre hemizygous, D3R-flox homozygous mice). This process is expected to take approximately 10-12 months.

## **II. Metabolic characterization of pancreatic beta cell-specific D2R and D3R knockout mice in the presence or absence of APD treatment**

- We will first characterize the quantity and duration of tamoxifen necessary to induce successful deletion of D2R in our inducible pancreatic beta cell-specific D2R knockout mice. Once we have confirmed deletion of D2R in pancreatic islets isolated from beta cells, we will begin characterizing the metabolic status of these animals from week 3 of life onwards following completion of weaning. Specifically, we will conduct weekly measurement of weights and food consumption in beta cell-specific D2R (and wildtype littermate controls) treated with either with first-generation APD haloperidol or second-generation APD olanzapine (via i.p. administration). We will also measure serum fasting glucose and insulin levels in hypothalamus-specific D2R knockout mice and wildtype littermate control mice in the presence or absence of APD treatment; serum will be collected at weeks 13 and 26 of APD treatment.
- In parallel with generation of beta cell-specific D2R knockout mice, we will also begin crosses to establish inducible pancreatic beta cell-specific D3R knockout mice (Mip1-cre/ERT hemizygous, D3R-flox homozygous mice). This process is expected to take approximately 10-12 months.

## **III. Treatment with domperidone to determine whether peripheral D2R/D3R blockade alone can produce relevant metabolic disease**

- As reported above, once we and our Initiating PI have finalized the dietary conditions necessary to induce insulin resistance, we will use this diet to compare effects of domperidone on the rate of development of insulin resistance both in wildtype as well as in beta cell-specific D2R or D3R knockout mice. Besides insulin resistance, we will look at other markers of metabolic disease including adiposity, fatty liver and pancreatic beta cell mass.
- As each of these three above aims proceeds with the new strains available, we will perform insulin clamp studies to assess the efficacy of hepatic insulin action as well. We will then also be able to examine cognitive, affective, emotional and autonomic regulation of feeding and weight in these beta-cell and hypothalamic D2R/ D3R knockout models.



#### 4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**  
The presentation of our preliminary results at scientific meetings including at an international conference, Dopamine 2016, were instrumental in advancing the concept that APDs may act on peripheral dopaminergic targets. In presenting this work, our findings were broadly disseminated to a broad scientific audience whose expertise spans multiple disciplines including neuroscience, endocrinology, cell biology and clinical medicine.
- **What was the impact on other disciplines?**  
In the longer term, the knowledge resulting from our work may directly lead to development of better APDs free of metabolic side effects. This could significantly reduce serious morbidity and mortality from medication-associated type II diabetes and cardiovascular disease. Moreover, better understanding the mechanisms by which dopamine and dopamine receptors mediate insulin release may also significantly contribute to our fundamental understanding of obesity and lead to novel treatments. Since APD-induced metabolic disturbances also increase risks of developing type II diabetes and Alzheimer's disease, further elucidating the mechanisms of APD-induced weight gain may also lead to fundamental insights into the mechanisms for development of these disorders.
- **What was the impact on technology transfer?**  
Nothing to Report.
- **What was the impact on society beyond science and technology?**  
Nothing to Report.

#### 5. CHANGES/PROBLEMS

Nothing to Report.

#### 6. PRODUCTS

- **Publications, conference papers, and presentations**

##### **Journal publications**

No peer-reviewed articles or papers appeared in scientific, technical or professional journals. However, presently three manuscripts are under preparation based on work resulting from this award. We expect to submit these manuscripts in the next 3-6 months.

##### **Books or other non-periodical, one-time publications**

Nothing to report.

##### **Other publications, conference papers, and presentations**

Data based on the studies originally proposed for this award were presented at an international meeting (Dopamine 2016 meeting, September 2016, Vienna, Austria).

Abstracts based on the above poster presentations were published as follows:

1. Bonifazi A, Ellenberger M, **Freyberg Z**, Deschamps J, Janowsky A, Schwartz GJ, Pommier E, Rais R, Slusher B, Newman AH. (2016) Novel tools to investigate the role of dopamine D2/D3 receptors in antipsychotic drug-induced metabolic disease. Abstract and talk presented at the Dopamine 2016 Conference, September 5th – 8th 2016 in Vienna, Austria.
- **Website(s) or other Internet site(s)** Nothing to Report.
  - **Technologies or techniques** Nothing to Report.
  - **Inventions, patent applications, and/or licenses** Nothing to Report.
  - **Other Products** Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

• Name:	Gary J. Schwartz, Ph.D.
• Project Role:	Principal Investigator
• Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0003-0446-5553
• Nearest person month worked:	4
• Contribution to Project:	Dr. Schwartz has designed and analyzed all experimental data in the areas of metabolic phenotyping, including body weight, adiposity, glucose tolerance, insulin tolerance, and preliminary food intake and calorimetric assessments of energy expenditure.
• Funding Support:	National Institutes of Health/R01 NIDDK

• Name:	Licheng Wu
• Project Role:	Technician D
• Researcher Identifier (e.g. ORCID ID):	N/A
• Nearest person month worked:	12
• Contribution to Project:	Mr. Wu has performed dietary testing, glucose and insulin injections and gavage, animal maintenance and genotyping, blood sampling for glucose and insulin tolerance tests, vascular surgical procedures for clamps and initial metabolic phenotyping.
• Funding Support:	N/A

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to Report.
- **What other organizations were involved as partners?**

Nothing to Report

## 8. SPECIAL REPORTING REQUIREMENTS

### • Collaborative Awards

We have worked closely with the Initiating PI of this award, Dr. Zachary Freyberg. Dr. Freyberg has submitted a separate report independently of the Partnering PI that summarizes his progress over the course of the last reporting period (10/1/2015-09/30/2016).

## 9. APPENDICES

Below is the abstracts presented from a talk at an international meeting (Dopamine 2016 meeting, September 2016, Vienna, Austria) (see the **Products** section).

### A. Abstract, talk and poster presented at the Dopamine 2016 meeting, September 2016, Vienna, Austria:

Novel tools to investigate the role of dopamine D2/D3 receptors in antipsychotic drug-induced metabolic disease

Alessandro Bonifazi<sup>1</sup>, Michael Ellenberger<sup>1</sup>, Zachary Freyberg<sup>2</sup>, Jeffrey Deschamps<sup>3</sup>, Aaron Janowsky<sup>4</sup>, Gary J. Schwartz<sup>5</sup>, Elie Pommier<sup>1</sup>, Rana Rais<sup>6</sup>, Barbara S. Slusher<sup>6</sup>, Amy Hauck Newman<sup>1</sup>

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Antipsychotic drugs (APDs) are the most widely prescribed medications for treatment of major psychiatric illnesses. Despite their clinical utility, APDs cause significant metabolic side effects and increased risks for type II diabetes with consequent high rates of treatment discontinuation. The ubiquitous trait amongst effective clinical APDs is their antagonism at dopamine D2R and D3R playing a role in the mediation of APD-induced metabolic side effects. Evidence suggests that dopamine (DA) signaling through D2R and D3R in the central nervous system (CNS) mediates appetite and feeding behavior. Because both D2R and D3R are also expressed peripherally in both human and rodent insulin secreting pancreatic beta cells, DA signaling outside the CNS may provide an additional mechanism for systemic metabolic regulation and development of APD-induced metabolic disease. Activation of these receptors in beta cells mediates a negative feedback where DA co-released with insulin inhibits further insulin secretion. Chronic blockade of pancreatic D2R or D3R by APDs may result in hyperinsulinemia and diminished insulin sensitivity. Stimulation of beta cell D2R and/or D3R may mitigate or reverse some of these APD-induced effects. To avoid exacerbating psychosis in clinical populations by countering APDs' actions in the CNS, we have sought to design peripherally-limited D2R or D3R agonists that do not penetrate the blood-brain barrier via quaternization at the basic nitrogen. In binding studies using [<sup>3</sup>H]7-OHDPAT in HEK293 cells stably transfected with hD2R and hD3R most of the quaternary ammonium salts showed only a 4 to 10-fold loss of affinity at the D2R retaining agonist profiles in mitogenesis studies when compared with their parent molecules. The quaternary salt of bromocriptine (D2R/D3R agonist recently approved by the FDA to improve glycemic control in type II diabetes) retained very high affinity ( $K_i=2.9$  nM) for D2R when compared with bromocriptine itself ( $K_i=0.7$  nM). We examined these quaternary salts and their parent drugs in beta cell islet assays to determine their effects on glucose-stimulated insulin release. Bromocriptine and its methiodide analogue were also evaluated for mouse microsomal metabolism as well as for i.v. pharmacokinetic and blood/brain plasma ratios. Bromocriptine methiodide is currently being evaluated in *in vivo* metabolic analyses in mice including indirect calorimetry, food intake and body weight in the presence or absence of APD treatment.