AWARD NUMBER: W81XWH-15-1-0556

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

PRINCIPAL INVESTIGATOR: Zachary Freyberg, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213-3320

REPORT DATE: July 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Central and Peripheral Mechanisms of Antipsychotic Medication-Induced Metabolic Dysregulation

Zachary Freyberg M.D., Ph.D.

E-Mail: freyberg@pitt.edu

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 D2 (D2R) and D3 (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.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Keywords</td>
<td>1</td>
</tr>
<tr>
<td>3. Accomplishments</td>
<td>1</td>
</tr>
<tr>
<td>4. Impact</td>
<td>9</td>
</tr>
<tr>
<td>5. Changes/Problems</td>
<td>10</td>
</tr>
<tr>
<td>6. Products</td>
<td>10</td>
</tr>
<tr>
<td>7. Participants &amp; Other Collaborating Organizations</td>
<td>11</td>
</tr>
<tr>
<td>8. Special Reporting Requirements</td>
<td>13</td>
</tr>
<tr>
<td>9. Appendices</td>
<td>13</td>
</tr>
</tbody>
</table>
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 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 D2 (D2R) and D3 (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 Partnering PI Dr. Gary Schwartz, 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. Moreover, in focusing on the peripheral contributions of pancreatic D2R and D3R, we have also developed new and highly sensitive optical and biochemical assays to study D2R- and D3R-mediated effects on insulin and DA release in real-time. We have applied these new assays to an experimentally tractable model using the well-characterized rat beta cell-derived INS-1E cell line for our in vitro studies, in addition to our work in the D2R and D3R KO pancreatic islets. 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 D2 Receptor (D2R)
4. Dopamine D3 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?**

In the course of the reporting period for Year 2 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**

- To characterize the metabolic consequences of hypothalamus-specific knockouts of D2R and/or D3R, as a first step, we have now successfully constructed a D3R-flox mouse strain to knock out D3R expression selectively in the hypothalamus. Consequently, we have begun the next stage of preparing our mouse strains for metabolic characterization which is to make these D3R-flox mice congenic to the C57BL/6J genetic background. To date, the initially-generated D3R-flox mice were produced in a mixed 129Sv/C57BL/6J background, which may introduce potential physiological and behavioral confounds that are specific to this mixed background. Because all other mouse strains used in our studies are already fully isogenic with the C57BL/6J background, the introduction of a mixed genetic background via D3R-flox crosses may thus lead to potentially confounded conclusions, especially in terms of metabolic phenotypes. Therefore, to address this, we have begun systematic back-crossing of the D3R-flox strain to C57BL/6J wildtype mice for 10 generations which is expected to make the genetic background of this essential strain fully uniform. Presently, we are in the N2 generation of the backcrosses. Once the animals are backcrossed for 5 generations (resulting in >98% isogenicity with C57BL/6J), we expect to begin pilot metabolic testing of the partially back-crossed D3R-flox mice by crossing them to the Nkx2.1-cre pan-hypothalamic driver strain. We are also in the process of confirming by quantitative RT-PCR (qPCR) that the undriven D3R-flox strain does not have any non-specific changes in D3R expression by testing for D3R expression in relevant tissues including in brain (hypothalamus, and striatum) as well as in pancreatic beta cells.

- Since the D2R-flox strain is already isogenic with the C57BL/6J genetic background, we crossed the D2R-floxed mice to the Nkx2.1-cre pan-hypothalamic driver mouse strain. Consequently, we have successfully obtained Nkx2.1-cre hemizygous, D2R-flox homozygous mice which represent a complete hypothalamus-selective D2R knockout mouse. We are in the process of working with the Partnering PI, Dr. Gary Schwartz, to commence metabolic characterization of this mouse strain.

- We have successfully renewed 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**

- We have now 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 Mip1-cre/ERT; homozygous D2R-flox mice required to completely knockout expression of D2R selectively in pancreatic beta cells in an inducible manner using tamoxifen injection to initiate the gene deletion. This strain is currently being amplified in numbers sufficient to power the requisite number of experiments to resolve potential effects of D2R absence on APDs’ effects on pancreatic islet insulin and dopamine release. Moreover, we are optimizing the tamoxifen induction protocol to ensure successful and efficient gene deletion of D2R, confirming deletion by qPCR analyses.

- 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. Once the D3R-flox strain is sufficiently isogenic with the C57BL/6J control strain (N5 or greater), we will initiate the crosses to generate Mip1-cre/ERT hemizygous; D3R-flox homozygous mice.

While breeding to generate the respective beta cell-selective D2R and D3R knockout mouse strains has been ongoing, Partnering PI Dr. Gary Schwartz and our lab have been optimizing our experimental system to effectively examine metabolic consequences of modulating D2R/D3R signaling in vivo in preparation for the above experiments using beta cell-specific D2R and D3R knockout mice. Consequently, we have treated insulin-resistant, obese C57BL/6J mice with D2R/D3R agonist bromocriptine (10 mg/kg) or vehicle for 10 weeks and at the conclusion conducted oral glucose tolerance testing (OGTT). We found that the bromocriptine-treated group exhibited a 21.5% improvement in insulin sensitivity relative to the vehicle-treated group (Figure 1). Significantly, these data are consistent with bromocriptine’s recent FDA approval for treatment of type 2 diabetes. Interestingly, while ordinarily improvements in insulin sensitivity are concomitant with decreases in body weight or adiposity, we found no such effect in the bromocriptine treatment group; there was no significant difference in either total body weight or in adiposity in the bromocriptine treated group compared to the vehicle control (Figure 2). We will use recently developed peripherally-limited D2R/D3R agonists next to discern whether these bromocriptine-induced improvements in insulin sensitivity are due to the drug’s direct effects on peripheral beta cell dopamine receptors.

III. Treatment with domperidone to determine whether peripheral D2R/D3R blockade alone can produce

![Figure 1. Bromocriptine improves insulin sensitivity in insulin-resistant, obese mice.](image1)

![Figure 2. Bromocriptine treatment has no effect on body weight or adiposity in insulin-resistant, obese mice.](image2)
relevant metabolic disease
● We have continued working closely with the Partnering PI of this project, Dr. Gary Schwartz, to optimize the dietary conditions responsible for inducing the development of insulin resistance (see Partnering PI report for further details). At present, we find that the 5TJN diet combining high fat and high fructose, together with our C57BL/6J genetic background, reliably induces insulin resistance comparable to that seen in clinical populations, including in those treated with APDs. With these dietary conditions in place, we will compare effects of domperidone on development of metabolic disease both in wildtype as well as in beta cell-specific D2R or D3R knockout mice.
● In parallel to our optimization of the diet-induced insulin resistance conditions, we have begun examining whether domperidone has direct effects on pancreatic beta cell insulin secretion. We found that increasing concentrations of domperidone, a peripherally-limited D2R/D3R blocker, had direct effects on glucose-stimulated insulin secretion (GSIS). Indeed, domperidone raised levels of insulin secretion in wildtype C57BL/6J pancreatic islets by 25% at its higher concentrations (Figure 3). These data suggest that, even in the absence of actions on the central nervous system, acute blockade of DA D2-like receptors (including D2R and D3R) by domperidone is sufficient to disrupt dopaminergic regulation of GSIS and lead to metabolic
disturbances. We intend on repeating these experiments in both beta cell-selective D2R and D3R knockout mice in the coming year.

IV. 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

- We have continued elucidating the precise roles of pancreatic beta cell D2R and D3R in modulation of GSIS by conducting insulin secretion assays using pancreatic islets from beta cell-selective D2R knockout mice and their wildtype littermate controls. Previously, we found that treatment of wildtype islets with the dopamine precursor, L-DOPA, had a concentration-dependent increase in GSIS inhibition. By comparison, there was an attenuation of GSIS inhibition caused by L-DOPA treatment in the pancreatic islets from beta cell-selective D2R knockout mice compared to the wildtype control islets (p>0.05). In parallel, we seek to assay the contributions of D3R on pancreatic islet GSIS. However, while we await completion of the tamoxifen-inducible beta cell-selective D3R knockout mouse strain (see above), we used global D3R knockout mice which also lack D3R expression in the pancreas. Consequently, we found that absence of pancreatic D3R expression also significantly diminished L-DOPA-induced inhibition of GSIS in a concentration-dependent manner compared to the control (p>0.05). Our data therefore suggest that both D2R and D3R work in concert to mediate dopaminergic inhibition of GSIS. Dopamine secretion assays using pancreatic islets from D2R and D3R knockout mice are also currently underway and we hope to have complementary data in the coming months. In our follow up studies, we have now directly treated wildtype (WT), D2R knockout and D3R knockout mouse pancreatic islets with increasing concentrations of exogenous dopamine in order to determine the contributions of the remaining dopamine receptors on GSIS in the respective knockout backgrounds (Figure 4). WT islets exhibited significant dose-dependent GSIS inhibition across the range of dopamine (DA) concentrations tested (100 nM-10 µM, P<0.001; Figure 4a). Islets from beta-cell-specific D2R knockout or global D3R knockout mice were less sensitive to DA’s inhibitory effects on GSIS. While there was no significant GSIS inhibition following treatment with the lowest DA concentration in D2R or D3R knockout mouse islets with increasing concentrations of exogenous dopamine in order to determine the contributions of the remaining dopamine receptors on GSIS in the respective knockout backgrounds (Figure 4). WT islets exhibited significant dose-dependent GSIS inhibition across the range of dopamine (DA) concentrations tested (100 nM-10 µM, P<0.001; Figure 4a). Islets from beta-cell-specific D2R knockout or global D3R knockout mice were less sensitive to DA’s inhibitory effects on GSIS. While there was no significant GSIS inhibition following treatment with the lowest DA concentration in D2R or D3R KO islets (100 nM, P>0.05; Figures 4b, c), both D2R knockout and D3R knockout islets exhibited significant GSIS inhibition at higher DA concentrations (Figure 4b, c). We therefore infer that this GSIS inhibition is likely due to dopaminergic signaling at the remaining D2-like receptors.

V. 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
In preparation for examining the kinetics of glucose-stimulated insulin secretion in pancreatic islets, we have continued our studies examining effects of APDs including sulpiride on GSIS in the pancreatic beta cell-derived INS-1E cell experimental system and extended them to pancreatic islets as well. We found that D2R/D3R blockade by sulpiride or a similar blocker, raclopride, were most effective in blocking dopamine’s inhibitory effects on GSIS following pre-incubation of the cells with DA precursor L-DOPA (Figure 5). We extended these observations further, by taking advantage of recently developed pharmacological tools that can distinguish between D2R and D3R. This is especially significant since most pharmacological tools have limited receptor selectivity amongst the different DA D2-like receptor subtypes, it has remained unclear which of these receptors is responsible for mediating DA’s inhibition of GSIS. To determine the relative contributions of D2R and D3R in mediating GSIS, we used recently developed D2R-selective and D3R-selective compounds to block the respective activities of these two receptors. We observed that as little as 300 nM of the D3R-selective blocker R22 successfully reduced L-DOPA’s inhibition of GSIS (p<0.0001), albeit to a lesser degree than raclopride or sulpiride which block both D2R and D3R. Likewise, the D2R-selective inhibitor ML321 (3 µM) also partially reduced L-DOPA-induced GSIS inhibition (p<0.0001; Figure 5). Taken together, these data suggest that both D2R and D3R mediate GSIS inhibition, and that greater inhibition is achieved through joint receptor action. These data also suggest that beta cells require a priming step of the DA signaling machinery including at the receptor level in order to resolve effects of APD blockade of D2R and D3R.

We have also begun to assay effects of commonly used APDs including olanzapine and haloperidol on GSIS in wildtype mouse islets. We found that acute treatment of islets with olanzapine elevated GSIS by ~50% while haloperidol increased GSIS by 25% (Figure 6). These data are consistent with the prediction that APD blockade of these receptors would attenuate D2 and/or D3 receptor-mediated GSIS inhibition and ultimately lead to elevated levels of insulin release. Such APD-induced increases in GSIS thus may provide a putative mechanism for APD-induced insulin resistance since chronic elevations of insulin, as observed during APD treatment, may lead to desensitization of insulin-sensitive organs including skeletal muscle, liver and adipose tissue to circulating insulin. Furthermore, given that APD-induced metabolic disturbances are
clinically more prevalent in response to olanzapine treatment compared to haloperidol, our results showing olanzapine’s greater effects on islet GSIS fit within this clinical framework.

Lastly, we have also started to examine the role of DA signaling in modulating GSIS in cadaveric human pancreatic islets. In parallel, we also measured glucagon secretion from the same islets (Figure 7). Using de-identified islets from a non-diabetic 51 year old female, we found that, as in mouse islets and rat INS-1E cells, DA inhibited GSIS in a concentration-dependent manner. Moreover, the IC50 of DA-induced GSIS inhibition in the human islets (12.5 µM) is within the range of that observed for rodent islets and cells (~3-10 µM; Figure 7a). Furthermore, we show for the first time that DA treatment has a highly potent inhibitory effect on pancreatic alpha cell secretion of glucagon (IC50=0.07 nM), concurrent with insulin secretion during glucose stimulation (Figure 7b).

- **What opportunities for training and professional development has the project provided?**
  Nothing to Report.

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

The presentation of our preliminary results at scientific meetings including at national conferences, the 56th Annual Meeting of the American College of Neuropsychopharmacology (2017), and the 2018 Behavior, Biology, and Chemistry: Translational Research in Addiction Conference (2018) were instrumental in advancing the concept that APDs may act on peripheral dopaminergic targets. In presenting this work during talks, abstracts and poster presentations, our findings were broadly disseminated to a broad scientific audience whose expertise spans multiple disciplines including neuroscience, endocrinology, cell biology and clinical medicine. In addition to the work already published, we presently have three manuscripts under preparation directly based on work resulting from this award. We expect to submit these manuscripts in the next 6-12 months.

**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**

- Since the breeding of the hypothalamus-specific D2R mouse strain (Nkx2.1-cre hemizygous, D2R-flox homozygous mice) has just been successfully completed, 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.

- Once the D3R-flox mice are successfully backcrossed to the C57BL/6J background (now in the N2 generation), 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

- With the completion of the tamoxifen-inducible beta cell-selective D2R knockout mouse strain, we have begun characterization of the quantity and duration of tamoxifen necessary to induce successful deletion of D2R in our inducible pancreatic beta cell-specific D2R knockout mice. We are in the process of confirming successful tamoxifen-induced knockout of D2R using i.p. tamoxifen administration using a consecutive 5-day tamoxifen dosing regimen and will confirm by qPCR of D2R mRNA expression in isolated islets from these animals. We will then confirm efficiency of gene deletion using an alternate route of tamoxifen injection by oral gavage to determine whether this can help avoid potential metabolic confounds due to systemic i.p. administration. 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.

- We will compare our findings from the current tamoxifen-inducible beta cell-selective D2R knockout mouse strain which was constructed using the Ins1-Cre/ERT beta cell-specific Cre driver strain originating from the laboratory of Dr. Louis Philipson with a newer version of this driver line obtained from the laboratory of Dr. Bernard Thorens. The rationale for this comparison is that the Philipson version of the strain contains a human growth hormone (hGH) minigene embedded within the Cre driver cassette. The presence of this hGH minigene may introduce potential confounds in terms of beta cell proliferation which may ultimately affect the interpretability of some of the metabolic data. We will therefore control for this by using the newer Thorens version of this expression driver which does not have the hGH minigene – something that was only brought to light relatively recently in the literature.

- 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, with the finalization of the dietary conditions necessary to induce insulin resistance, we have now begun metabolic characterization of D2R and D3R signaling in the diet-manipulated mice. As a first step, we are using a potent agonist, bromocriptine, to ensure that we have sufficient signal to see potential changes in metabolism (including changes in insulin
IV. 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

- Using pancreatic islets from tamoxifen-inducible pancreatic beta cell-specific D2R and D3R knockout mice, we will validate our earlier findings demonstrating attenuation of L-DOPA inhibition of glucose-stimulated insulin secretion from global D3R knockout mice and constitutive beta cell-specific D2R knockout mice. Moreover, we will generate beta cell-selective D2R and D3R double knockout mice in order to examine the combined effects of both dopamine receptors on GSIS. In parallel, we will measure DA secretion from these knockout islets and the wildtype controls.

V. 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

- We will complete the validation of our insulin secretion findings obtained from mouse pancreatic islets in human cadaveric pancreatic islets including assessment of effects of D2R agonists (dopamine, quinpirole, bromocriptine) versus APD antagonists (clozapine and aripiprazole).
- As we are in the process of competing our studies examining APD treatments’ effects on GSIS in wildtype pancreatic islets, we will next treat pancreatic islets from beta cell-specific D2R and D3R knockout mice with APDs (haloperidol, sulpiride, aripiprazole, olanzapine and clozapine). Specifically, we will sample insulin and dopamine levels every 10 minutes which will permit determination of a kinetic curve for the respective release events.

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 national conferences: the 56th Annual Meeting of the American College of Neuropsychopharmacology (2017), and the 2018 Behavior, Biology, and Chemistry: Translational Research in Addiction Conference (2018) were instrumental in advancing the concept that APDs may act on peripheral dopaminergic targets. In presenting this work during talks and poster presentations, 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**

There have been no changes in the scope of work since the last reporting periods and therefore the SOW remains the same as originally defined.

6. **PRODUCTS**

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

  **Journal publications**
  Data based on the work resulting from this award has appeared in the following publications:
  

  In addition to the work already published, we presently have three manuscripts in preparation and expect to submit these manuscripts in the next 6-12 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 the following meetings and presentations:


5. Harvill E, **Freyberg Z**. Pathogen manipulation of host metabolism: a common strategy for immune evasion. Abstract to be presented at the 2018 The Metabolism of Infectious Diseases meeting, Fort Collins, CO.

Additionally, Dr. Freyberg was an invited speaker at the following seminars where he presented the work produced from this funded work:

1. Invited speaker, Institute of Cellular Therapeutics, Allegheny Health Network, Pittsburgh, PA; 2017
2. Invited speaker, Department of Infectious Diseases Seminar series, University of Georgia, Athens, GA; 2017

• **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?**
<table>
<thead>
<tr>
<th>Name:</th>
<th>Zachary Freyberg M.D., Ph.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role:</td>
<td>Principal Investigator</td>
</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
<td>ORCID ID: 0000-0001-6460-0118</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>5</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Freyberg has designed and analyzed all experimental data in the areas of construction of the D3R-flox transgenic mouse strain and glucose-stimulated insulin secretion assays in beta cell-selective D2R knockout mice.</td>
</tr>
<tr>
<td>Funding Support:</td>
<td>Department of Defense Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292); The Pittsburgh Foundation Rising Star Research Award</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name:</th>
<th>Emily George</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role:</td>
<td>Research Technician</td>
</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
<td>N/A</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>7</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Ms. George has performed mouse colony maintenance and crosses to generate the required transgenic strains (including back-crossing new transgenic mouse strains), cloning and molecular biological studies, and tissue culture as well as assistance with <em>in vitro</em> functional assays measuring insulin and dopamine secretion from pancreatic islets and insulin-secreting pancreatic beta cell-derived cell lines.</td>
</tr>
<tr>
<td>Funding Support:</td>
<td>Department of Defense Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292); The Pittsburgh Foundation Rising Star Research Award</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name:</th>
<th>Despoina Aslanoglou, Ph.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role:</td>
<td>Postdoctoral Researcher</td>
</tr>
<tr>
<td>Researcher Identifier (e.g. ORCID ID):</td>
<td>N/A</td>
</tr>
<tr>
<td>Nearest person month worked:</td>
<td>12</td>
</tr>
<tr>
<td>Contribution to Project:</td>
<td>Dr. Aslanoglou has performed cloning and molecular biological studies, tissue culture as well as <em>in vitro</em> functional assays measuring insulin secretion from pancreatic islets and insulin-secreting pancreatic beta cell-derived cell lines.</td>
</tr>
<tr>
<td>Funding Support:</td>
<td>Department of Defense Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292)</td>
</tr>
</tbody>
</table>

- **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 Partnering PI of this award, Dr. Gary Schwartz. Dr. Schwartz has submitted a separate report independently of the Initiating PI that summarizes his progress over the course of the last reporting period.

9. APPENDICES


1. Abstract and poster presented at the 2016 56th Annual Meeting of the American Society of Cell Biology, San Francisco, CA:

**Development of Cryo-CLEM Methods to Study Mechanisms of Intracellular Trafficking and their Relationships to the Secretory Pathway.**

Stephen D. Carter¹, Shrawan K. Mageswaran¹, Zachary J. Farino², Joao I. Mamede³, Tom J. Hope³, Joachim Frank⁴,⁵, Zachary Freyberg²,⁶, Grant J. Jensen¹,⁷

¹Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA; ³Department of Cell and Molecular Biology, Northwestern University, Chicago, IL 60611-3008; ⁴HHMI; Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York 10032, USA; ⁵Department of Biological Sciences, Columbia University, New York, New York 10027, USA; ²Departments of Psychiatry and ⁶Cell Biology, University of Pittsburgh, Pittsburgh, PA, 15213, USA; ⁷Howard Hughes Medical Institute (HHMI), California Institute of Technology, Pasadena, CA 91125, USA.

The application of cryogenic electron microscopy (cryo-EM) to the study of cellular ultrastructure provides a resolution several orders of magnitude better than light microscopy. However, though this approach is increasingly applied in situ, it suffers from limitations in our ability to target imaging to specific intracellular targets including the subcellular localization of specific structural events of interest inside the cell. Cryogenic correlated light and electron microscopy (cryo-CLEM) helps to overcome this problem by spatially locating areas of interest inside cells using fluorescence from genetically tagged or stained cellular molecules and allows for the visualization of localized fluorescently-tagged proteins down to the level of individual organelles. Here, we attempted to study the secretory pathway in a specialized mammalian cell line, insulin-secreting INS-1E cells, expressing genetically-encoded fluorophores as a model system to develop a cryo-CLEM methodology. We discovered that there are many bright sources of autofluorescence in frozen cells. Based on our initial observations, we
hypothesized that autofluorescence from endogenous cellular substrates exhibits a broader spectrum of fluorescence than the fluorescence range of our expressed fluorescent proteins. To test this, we developed a quantitative approach to discriminate between autofluorescence and the fluorescent signal from genetically-encoded fluorophores by looking at both fluorescent intensities across different channels. To validate this new methodology, we visualized multiple fluorophore-tagged organelle markers in our experimental cell system. We found that DsRed2-cytochrome c oxidase and chromogranin A-GFP proteins were targeted in INS-1E cells to mitochondria and secretory granules by cryo-CLEM, consistent with their respective well-established intracellular localizations. Moreover, these fluorescent signals were clearly distinguishable from autofluorescence emanating from endogenous structures including insulin crystals and multilamellar bodies. Overall, our novel cryo-CLEM methods open the door to the study of cellular phenomena and structures with a new degree of specificity.

2. Abstract and poster presented at the 2017 56th Annual Meeting of the American College of Neuropsychopharmacology, Palm Springs, CA:

D2 Dopamine Receptors Alter Circadian Rhythms in Pancreatic Islet Cells: Implications for the Metabolic Side Effects of Antipsychotic Drugs.

Danielle Chipchurra1, Heather Wei1, Zachary Freyberg3, Michael J. McCarthy1,2

1 VA San Diego Healthcare System, Research Service
2 University of California, San Diego, Department of Psychiatry
3 University of Pittsburgh, Department of Psychiatry

Background: Antipsychotic drugs (APDs) are used widely in the treatment of psychiatric disorders. However, these drugs also cause unwanted side effects including weight gain and metabolic abnormalities. Accordingly, patients receiving APDs are at elevated risk of cardiac disease, type 2 diabetes and shortened life span. The pharmacological mechanisms for APD-induced metabolic dysfunction is poorly understood but may involve the blockade of dopamine D2 receptors (D2R). In addition to their expression in brain, D2R is located in pancreatic beta cells where they negatively regulate insulin secretion. As a critical regulator of blood glucose, insulin release follows a circadian rhythm to anticipate food intake and prolonged periods of fasting. Under healthy conditions, insulin release peaks during the day and is low at night. However, disruptions of circadian rhythms, and the insulin rhythm in particular may cause hyperglycemia, weight gain and lipid abnormalities. Increasing evidence suggests that dopamine and D2R modulate circadian rhythms. Because APDs block D2R signaling, we hypothesize that APDs, particularly when taken at night to promote sleep, may disrupt 24 h rhythms in beta cells, and interfere with insulin regulation by dopamine through a circadian mechanism.

Methods: We examined laboratory and anthropometric data from patients treated over a 12-month period with a partial D2 agonist (aripiprazole) either in the morning (n=90) or at bedtime (n=53). Differences in metabolic parameters and BMI were examined in the two groups who got the medication at different times of day. We then established an in vitro model of pancreatic beta cell circadian rhythms using beta cell-derived INS-1E cells. To measure circadian gene expression rhythms, INS-1E cells were transduced with a lentivirus containing the Per2-luc circadian reporter and blasticidin resistance element. Per2-luc rhythms were measured for 3-4 days under constant conditions in the resulting Per2-Luc stable cell line. To measure circadian rhythms in insulin release, we used a lentiviral construct to deliver a proinsulin-luc reporter. Insulin release was then measured for 1-2 days under constant conditions.

Results: Patients treated with aripiprazole in the evening had significantly higher levels of serum triglycerides and lower levels of HDL cholesterol compared to patients who took the drug in the morning. There were no changes in hemoglobin A1c, fasting glucose levels, or BMI. In vitro, INS-1E
cells showed circadian rhythms in gene expression. Stimulation of D2R with agonists including bromocriptine or L-DOPA decreased the rhythm amplitude, whereas dopamine D1 receptor stimulation did not have a major effect. In contrast, sulpiride, a D2R-selective antagonist, reversed the effects of D2R stimulation by bromocriptine or L-DOPA on rhythms. Insulin release was rhythmic and in anti-phase with Per2-luc expression, with highest levels of insulin release occurring at times of low levels of Per2-luc (corresponding to daytime). D2R antagonism by sulpiride significantly altered the rhythm of insulin release, causing higher levels of insulin during the nadir.

**Discussion:** Dopamine signaling in pancreatic beta cells alters circadian rhythms in gene expression and insulin release. APDs that block D2R interfere with these rhythms and may lead to increased insulin secretion over 24 hr cycles, especially at night when insulin levels are typically low. This can translate clinically in patients treated with aripiprazole (or similar APDs), where time of dosing affects likelihood of metabolic risk. Patients taking APDs at night may have additional metabolic risk factors compared to those who take the medication during the day. Circadian interventions may therefore reduce the adverse consequences on metabolism of APDs.

3. Abstract and poster presented at the 2018 Keystone Symposium on Cryo-EM from Cells to Molecules: Multi-Scale Visualization of Biological Systems, Tahoe City, CA:

Ribosome-Associated Vesicles: a dynamic vesicular endoplasmic reticulum in secretory cells.

Stephen D. Carter¹, Cheri M. Hampton², Robert Langlois³,³, Roberto Melero⁴, Zachary J. Farino⁵, Wen Li⁶, Ngoc Han Tran⁶, Robert A. Grassucci³,⁷, Stephanie Siegmund⁸, Joshua Pemberton⁹, William J. Rice¹⁰, Christoph Wigge¹⁰, Peter Walter⁶, Tamás Balla⁹, Jose Maria Carazo⁴, Grant J. Jensen¹¹*, Joachim Frank²,³,⁷*, Zachary Freyberg⁵*

¹Division of Biology and Biological Engineering, California Institute of Technology
²Howard Hughes Medical Institute (HHMI); Department of Biochemistry and Molecular Biophysics, Columbia University
³Department of Biochemistry and Molecular Biophysics, Columbia University
⁴Biocomputing Unit, Centro Nacional de Biotecnología – CSIC
⁵Department of Psychiatry, University of Pittsburgh
⁶Department of Biological Sciences, Columbia University
⁷HHMI; Department of Biochemistry and Biophysics, University of California, San Francisco
⁸Department of Neurology, Columbia University
⁹Section on Molecular Signal Transduction, Program for Developmental Neuroscience, National Institute of Child Health and Human Development, National Institutes of Health
¹⁰New York Structural Biology Center
¹¹HHMI; Division of Biology and Biological Engineering, California Institute of Technology

§These authors contributed equally to this work.

*Corresponding authors.

The endoplasmic reticulum (ER) communicates with other organelles, including mitochondria, at discrete contact sites along an extensive network of membranes. However, the structural basis for forming and maintaining these contacts is poorly characterized. Here we combine confocal microscopy in living cells with in situ cryo-electron microscopy, tomography and correlative light and electron microscopy (CLEM) to directly visualize the interactions between ER and mitochondria at high resolution under near-native conditions. Using these imaging approaches, we identify a novel, mobile form of ER, Ribosome-Associated Vesicles (RAVs), found primarily in the cell periphery. RAVs exist as distinct structures separate from the intact ER reticular architecture and form dynamic associations with mitochondria via direct intermembrane contacts. We also show that RAVs are conserved across
multiple cell types and species. Ultimately, our analyses further establish the diversity of ER subtypes within cells and provide the basis for a better understanding of how ER communicates with other organelles.

4. Abstract and poster to be presented at the 2018 Behavior, Biology, and Chemistry: Translational Research in Addiction Conference, San Antonio, TX:

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

Bonifazi, Alessandro,¹ Ellenberger, Michael P.,¹ Schwartz, Gary J.,² Freyberg, Zachary,³ Newman, Amy, H.¹

¹Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, NIDA-IRP, NIH, Baltimore, MD 21224. ²Departments of Medicine and Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461. ³University of Pittsburgh, Department of Psychiatry, Pittsburgh, PA 15213.

Antipsychotic drugs (APDs) cause significant metabolic side effects and increase risk for type II diabetes (T2D) with consequent high rates of treatment discontinuation. The ubiquitous trait amongst all APDs is their antagonism at dopamine D₂ (D2R) and D₃ (D3R) receptors which may mediate APD-induced metabolic side effects. Dopamine (DA) signaling through D2R and D3R in the central nervous system (CNS) mediates appetite and feeding behaviors. Because both D2R and D3R are also expressed peripherally in insulin-secreting pancreatic beta cells, DA signaling outside the CNS may be involved in systemic metabolic regulation. Activation of these receptors in beta cells mediates an autocrine/paracrine negative feedback circuit where DA co-released with insulin inhibits further insulin secretion via D2R/D3R signaling. We hypothesized that agonism of peripheral D2R/D3R, may counter APD actions on beta cells and counter APD-induced metabolic disturbances. To avoid exacerbating psychosis by countering APD actions in the CNS, we have designed peripherally-limited D2R or D3R agonists via quaternization at the basic nitrogen. We examined these quaternary salts and their parent drugs in glucose-stimulated insulin secretion assays using a beta cell-derived cell line and in islets. Bromocriptine methiodide (BroMeI), the most promising quaternary analogue, was compared to its parent, bromocriptine, an FDA-approved drug used in T2D treatment, and evaluated for metabolic stability in mouse microsomes as well as for blood/brain plasma ratios. BroMeI 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. In total, these studies will further illuminate mechanisms underlying APD-induced metabolic syndrome and may lead to improved APD design.

5. Abstract to be presented at the 2018 The Metabolism of Infectious Diseases meeting, Fort Collins, CO:

Pathogen manipulation of host metabolism: a common strategy for immune evasion.

Harvill E¹, Freyberg Z²

¹University of Georgia, Georgia, USA. ²University of Pittsburg, PA, USA

Successful pathogens use a diverse arsenal of factors to modify the immune response, acting on numerous host organ systems. Here we examine how highly diverse pathogens including Plasmodium falciparum, Trypanosoma cruzi, and Bordetella pertussis cause significant clinically relevant metabolic disturbances during infection by disrupting glucose homeostasis in the host. We propose the hypothesis
that pathogen manipulation of metabolism to modulate the host immune response is a common strategy shared by highly divergent pathogens. We use *Bordetella pertussis* (*Bp*) as a model system to examine the relevance of infection-induced hypoglycemia both to bacterial pathogenicity and its relevance to the host immune response. Significantly, *Bp* toxins including pertussis toxin (PTX), and adenylate cyclase toxin (ACT) specifically target and modify intracellular signaling pathways that are critical to insulin secretion and metabolism. Together the data support a view of metabolic manipulation as a mechanism of immune suppression common to these, and likely other, pathogens and support the use of the *Bp* infection model to better understand these pathogen-host interactions. Understanding how pathogens manipulate host metabolism may provide fundamental mechanistic insights into pathogenesis and opens the door to new clinical approaches to significantly reduce the duration and severity of disease.