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14. ABSTRACT

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_2 (D2R) and D_3 (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. We have completed construction of novel inducible transgenic hypothalamic- and pancreatic beta cell-specific D2R knockout (KO) mice and are finalizing construction of hypothalamic and pancreatic beta cell-selective D3R KO mice. Additionally, using pancreatic islets isolated from beta cell-specific 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. In parallel, we have developed novel assays for measurement of pancreatic alpha cell glucagon and shown that APDs act directly on alpha cells to significantly disturb glucagon release.

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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 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_2 (D2R) and D_3 (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 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 D_2 Receptor (D2R)
4. Dopamine D_3 Receptor (D3R)
5. Insulin
6. Glucagon
7. Glucose-stimulated insulin secretion (GSIS)
8. Diabetes
9. 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 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 3 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 knockout (KO) of D2R and/or D3R, as a first step, we have successfully constructed a D3R-flox mouse strain to knock out D3R expression selectively in the hypothalamus. Because the D3R-flox strain was generated on a mixed genetic background, we have been systematically back-crossing the D3R-flox mice to C57BL/6J mice in order to make the D3R-flox strain congenic to the C57BL/6J genetic background, a widely accepted background widely used in metabolic studies. The goal is to backcross the D3R-flox mice for 10 generations; presently we are in the N8 generation of the backcrosses. Once the mice are at the intended N10 backcrossed generation, we will commence metabolic testing of the uncrossed D3R-flox line. Specifically, we will breed our D3R-flox mouse to the Nkx2.1-Cre mouse strain to create hypothalamus-selective D3R KO mice. Once sufficient numbers of hypothalamus-selective D3R KO mice and littermate controls are generated, we will commence metabolic testing of the KO mice and littermate controls. We will also confirm 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.
- We successfully generated a pan-hypothalamic KO of D2R by crossing D2R-flox mice with the established Nkx2.1-Cre pan-hypothalamic driver mouse strain. We are currently working closely with the Partnering PI, Dr. Gary Schwartz, to characterize the potential metabolic consequences of the resulting loss of hypothalamic D2R expression within this mouse strain. Furthermore, together with Dr. Schwartz, we are determining effects of this hypothalamus-specific D2R KO on APD-induced metabolic disturbances by treating the hypothalamic KO mice and their wildtype littermate controls with either olanzapine, haloperidol or vehicle control.
- We have successfully renewed IACUC 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 an inducible pancreatic beta cell-specific D2R KO mouse strain using the pancreatic beta cell-specific expression driver, Mip1-Cre/ERT, crossed to D2R-flox mice. Following 12 months of crosses, we generated a sufficient number of mice with the intended genotype: Mip1-Cre/ERT hemizygous and D2R-flox homozygous. Consequently, with the mice in hand, we have commenced both *in vivo* and *ex vivo* experiments.
- We have initiated studies to characterize our pancreatic beta cell-specific D2R KO mice by examining effects of beta cell-specific D2R KO on basal and glucose-stimulated insulin secretion

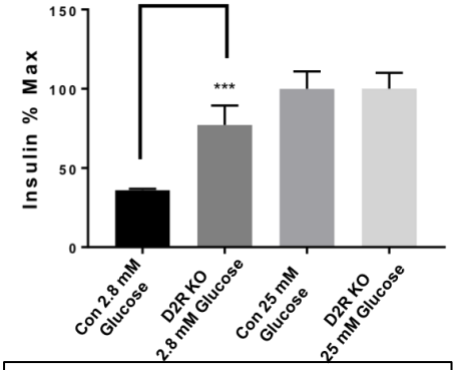

Figure 1. Beta cell-specific D2R knockout increases basal insulin secretion. We examined effects of beta cell-specific D2R knockout (KO) on basal and glucose-stimulated insulin secretion (GSIS) in pancreatic islets from D2R KO (n=8) vs wildtype littermate control mice (n=8). Basal insulin release under low glucose (2.8 mM) conditions were doubled in KO islets compared to islets from littermate controls ($p<0.001$). Under high glucose (25 mM) conditions, there was no significant difference between D2R KO and control islets on GSIS ($p>0.05$). All data conducted in triplicate and normalized to % maximal insulin secretion. Results are represented as mean ± SEM.

(GSIS). To avoid potential developmental confounds, we induced D2R KO in 8-10-week-old adult mice by administering tamoxifen and then waiting 3-4 weeks prior to provide sufficient time for turnover of existing D2R expressed in beta cells (D2R protein half-life = ~7 days). Using islets from the D2R KO mice and matched wildtype (WT) littermate controls, we found that the D2R knockdown caused an approximate doubling of basal insulin secretion compared to the WT controls under low glucose conditions (**Figure 1**). This finding is consistent with our earlier work showing that D2R signaling in beta cells is responsible for inhibition of insulin secretion and that beta cell D2R blockade results in increased insulin secretion. Furthermore, upon high glucose stimulation (25 mM) to induce GSIS, we found no significant differences in insulin secretion between WT and D2R KO islets (**Figure 1**). These data suggest that D2R may play a new, important role in controlling insulin release under resting conditions.

- We next tested effects of APDs on GSIS in pancreatic islets isolated from the beta cell-specific D2R KO mice and WT littermate controls (**Figure 2**). In WT islets, we observed that DA treatment significantly diminished GSIS by ~50%. However, co-treatment with typical APD haloperidol alongside DA largely reversed DA's GSIS inhibition (**Figure 2A**). Importantly, we found that haloperidol's effects were attenuated in the D2R KO islets (**Figure 2A**). Similarly, while the atypical APD olanzapine prevented DA-induced GSIS inhibition in WT islets, olanzapine's effects were no longer evident in beta cell-specific D2R KO islets (**Figure 2B**). Overall, our results show that APDs can act directly on peripheral targets in islets to disturb insulin secretion. Furthermore, these findings suggest that both typical and atypical APDs require beta cell D2R expression for their effects on GSIS.

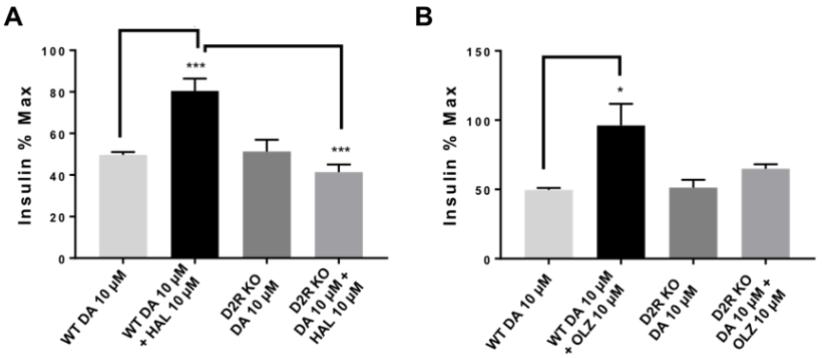

Figure 2. Beta cell-specific D2R KO attenuates antipsychotic drug (APD)-induced effects on insulin secretion. (A) Treatment of pancreatic islets from wildtype (WT) littermate controls (n=8) with dopamine (DA, 10 µM) significantly decreased glucose-stimulated insulin secretion (GSIS) by 50% relative to vehicle. Co-treatment with typical APD haloperidol (HAL, 10 µM) eliminated DA-induced GSIS inhibition in the WT islets compared to WT ($p<0.001$). Though islets from beta cell-specific D2R KO mice (n=8) also responded to DA inhibition of GSIS, there was no significant difference between DA-treated vs DA+HAL treatment ($p>0.05$). **(B)** Co-treatment of WT islets with 10 µM + atypical APD olanzapine (OLZ, 10 µM) reversed DA's GSIS inhibition and OLZ's effects were attenuated in the D2R KO islets. All data conducted in triplicate and normalized to % maximal insulin secretion. Results are represented as mean ± SEM.

- We examined the metabolic consequences of beta cell-specific D2R KO *in vivo*. Using our beta cell-specific D2R KO mice, we found that beta cell-selective D2R KO animals show significantly higher postprandial serum insulin compared to matched littermate WT controls,

particularly following meal challenge (**Figure 3**). These data suggest that beta cell D2R modulation of insulin release is especially sensitive to acute food intake (which is a key source of pancreatic L-DOPA and DA). Over time, these insulin elevations may contribute to development of insulin resistance, suggesting the importance of DA signaling in pancreatic islet physiology.

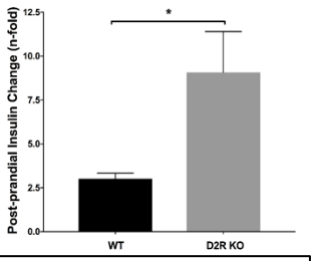

Figure 3. D2R KO elevates postprandial insulin secretion. Postprandial elevation in serum insulin levels was 3-fold higher in beta cell-specific D2R KO mice (grey, n=12) compared to WT littermate controls (black, n=9; p=0.038). Postprandial serum insulin values were normalized to subjects' pre-meal fasting serum insulin levels. Results represented as mean± SEM.

- The Mip1-Cre/ERT mice used to drive beta cell-specific expression of the Cre recombinase are also being prepared for crosses to the new D3R-flox mouse strain once the D3R-flox strain is fully isogenic with C57BL/6J (see above). The aim will be to begin construction of an inducible beta cell-specific D3R knockout mouse line. Once ready, the D3R-flox strain we will initiate the crosses to generate hemizygous Mip1-Cre/ERT; homozygous D3R-flox mice.

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

- With the development of both hypothalamus-specific and beta cell-specific D2R KO mice, we have continued working closely with the Partnering PI of this project, Dr. Gary Schwartz, to commence metabolic studies examining effects of peripherally-limited D2R blocker domperidone on metabolism. Metabolic studies in conjunction with Dr. Schwartz, including metabolic cage experiments, will be expected to commence in the next 6 months.

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

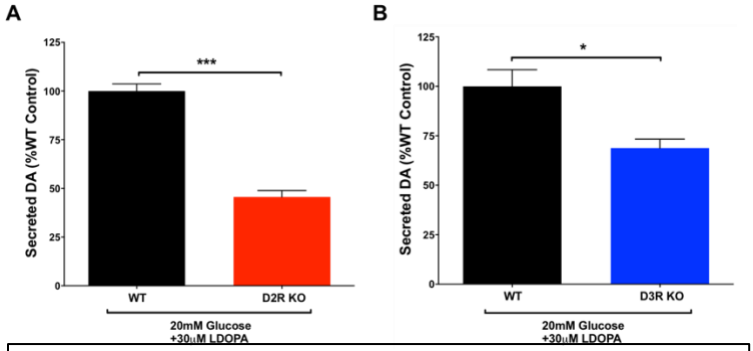

Figure 4. Beta cell D2R KO and global D3R KO diminishes pancreatic islet DA secretion. (A) Pancreatic islets from homozygous beta cell-specific D2R KO mice secreted 55% less DA compared to wildtype (WT) littermate controls ($p<0.0001$; n=5 for D2R KO and WT) in response to stimulation with 20 mM glucose and 30 μM L-DOPA. **(B)** Pancreatic islets isolated from homozygous global D3R KO mice secreted significantly less DA (32% reduction) compared to wildtype (WT) littermate controls (P=0.012; n=6 D3R KO, n=8 WT). For Panels **A** and **B**, all mean DA values were normalized to % secreted DA in the WT control. All data conducted in triplicate. Results are represented as mean ± SEM.

- We have continued elucidating the precise roles of pancreatic beta cell D2R and D3R in modulation of DA secretion by conducting DA secretion assays using pancreatic islets from beta cell-specific D2R knockout mice and their wildtype littermate controls. Previously, we found that treatment of wildtype islets with the dopamine precursor, L-DOPA, led to enhanced DA secretion which in turn produced a concentration-dependent decrease in GSIS. 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 observed that the absence of pancreatic D3R expression using a global D3R KO also significantly diminished L-DOPA-induced inhibition of GSIS in a concentration-dependent manner compared to the control ($p>0.05$). Taken together, our data suggested that both D2R and D3R work in concert to mediate dopaminergic inhibition of GSIS and led us to ask whether these D2R- and D3R-driven effects were based on changes in local DA production and release. Consequently, we have now directly

examined the DA-mediated mechanisms for these effects. We compared the capacity of WT and respective D2R and D3R KO islets to secrete *de novo*-synthesized DA during glucose stimulation using pancreatic islets pre-treated with L-DOPA from global D3R KO and β-cell-specific D2R KO mice versus WT littermate controls. We found that D3R KO islets secreted significantly less DA during glucose stimulation (32% reduction; p=0.012) relative to the WT littermate controls (**Figure 4A**). Similarly, D2R KO islets secreted 55% less DA compared to WT (p<0.0001; **Figure 4B**). These findings explain the greater impairment of L-DOPA-mediated GSIS relative to the direct actions of DA in the KO islets, and provide an important new mechanistic target for future investigation of DA-driven regulation of GSIS.

- Since pancreatic beta cells take up L-DOPA and produce DA, we have now examined whether glucagon-secreting pancreatic alpha cells also produce and secrete DA, particularly given recent evidence that alpha cells express machinery for DA production and signaling. In our studies, we have used alphaTC1 clone 6 (alphaTC1-6) cells, a glucagon-secreting mouse alpha cell line. Whereas rodent beta cells primarily rely on uptake of pre-existing L-DOPA to make DA, we found

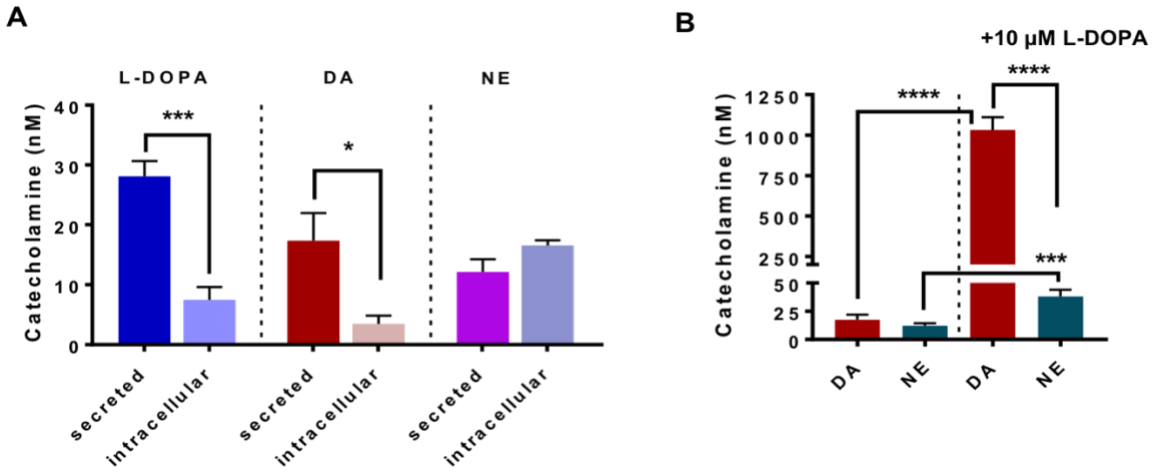

Figure 5. Pancreatic alpha cells preferentially produce and secrete DA. (A) HPLC analyses of supernatants and lysates from mouse alpha cell-derived alphaTC1-6 cells demonstrating synthesis of L-DOPA, DA and NE *de novo* in the absence of catecholamine precursor supplementation. Cells secrete most intracellular L-DOPA and DA with significantly lower L-DOPA (P=0.0002) or DA (P<0.05) in lysates compared supernatants. **(B)** HPLC analyses show that pre-incubation with 10 µM L-DOPA significantly enhanced DA and NE production and secretion. L-DOPA supplementation preferentially boosted DA production over NE with DA levels 27-fold more compared to NE (P<0.0001). Assay points were carried out in triplicates from n≥2 independent experiments. All bars represent the mean ± SEM.

alpha cells produced and secreted L-DOPA *de novo* as well as converted this endogenous L-DOPA to DA and NE (**Figure 5A**). Furthermore, exogenous L-DOPA supplementation (10 µM) promoted a substantial 60-fold increase in alpha cell DA secretion (p<0.0001). In contrast, NE was increased only 3-fold in response to L-DOPA addition compared to untreated cells (p=0.0004; **Figure 5B**). These results suggest alpha cells can adjust catecholamine production based on precursor availability, and that this mechanism is preferentially geared towards DA synthesis and release.

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 examined the kinetics of DA synthesis by beta cells by monitoring uptake of DA precursor L-DOPA into beta cell-derived INS-1E cells during glucose stimulation. To do so, we used [3H]L-DOPA to monitor beta cell intracellular accumulation of L-DOPA (**Figure 6**). [3H]L-DOPA uptake almost doubled within 30 min of glucose stimulation relative to the unstimulated control (1.7-fold increase, p=0.0005; **Figure 6**). Total DA uptake over the entire 120 min experiment was increased

2.5-fold compared to the control (p=0.002). We also observed a progressive decrease in accumulated intracellular [3H]L-DOPA over time (**Figure 6**). We investigated whether this decrease in tracer signal was due to conversion of [3H]L-DOPA to [3H]DA, which could then be

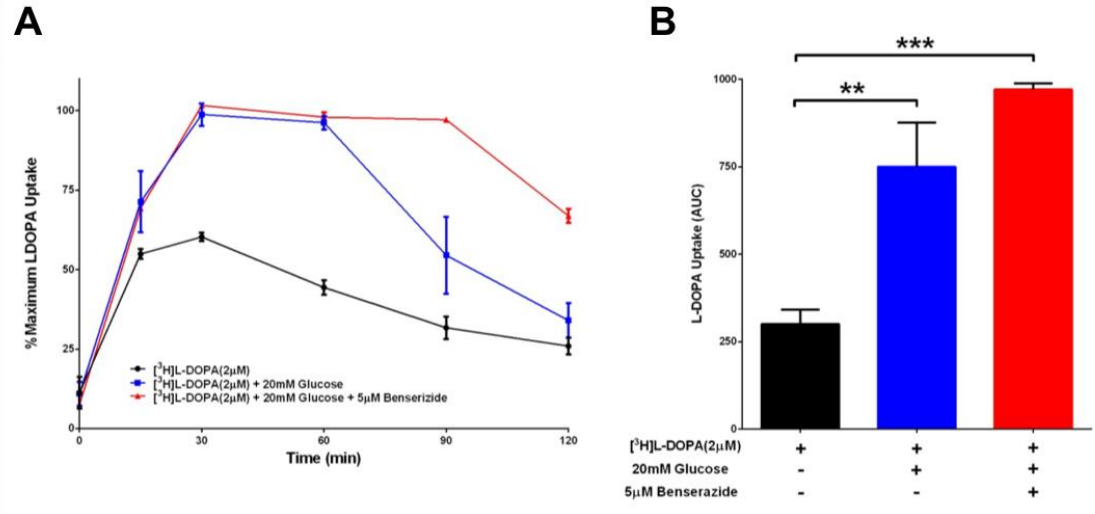

Figure 6. Glucose stimulation enhances L-DOPA uptake in INS-1E cells. (A) Time course of [3H]L-DOPA (2 μM) uptake in the presence (blue square) or absence (black circle) of 20 mM glucose stimulation. Glucose stimulation caused a 40% increase in [3H]L-DOPA uptake within 30 min relative to the unstimulated condition (p=0.0005). The AADC inhibitor benserazide (5 μM, red triangle) inhibited decreases in [3H]L-DOPA accumulation at later time points (60-120 min). Data was normalized to the point of maximal [3H]L-DOPA uptake. **(B)** Area under the curve (AUC) analysis of [3H]L-DOPA uptake for the 3 conditions shown in **Panel A**. Glucose stimulation caused a significant overall AUC change [$F(2,5)=42.72$, $p=0.001$]. There was a 2.5-fold increase in the glucose-stimulated condition compared to the unstimulated condition (p=0.002). Benserazide (5 μM) further enhanced this AUC increase (p=0.001). All measurements were performed in triplicate and represent the mean of n≥3 independent experimental days. All points represent the mean ± SEM.

released out of the cell. Therefore, we treated cells with 5 μM benserazide, a potent inhibitor of aromatic acid decarboxylase (AADC), to block conversion of L-DOPA to DA. Benserazide treatment significantly attenuated the decrease in accumulated intracellular [3H]L-DOPA we observed during glucose stimulation, with 2-fold more intracellular [3H]L-DOPA remaining at the study conclusion relative to the benserazide-untreated control (p=0.001; **Figure 6**). Overall, our data demonstrate that glucose stimulation primes *de novo* DA biosynthesis in beta cells by enhancing DA precursor uptake followed by AADC-dependent conversion to DA, which can be released to initiate signaling at DA receptors in the plasma membrane. receptor level in order to resolve effects of APD blockade of D2R and D3R.

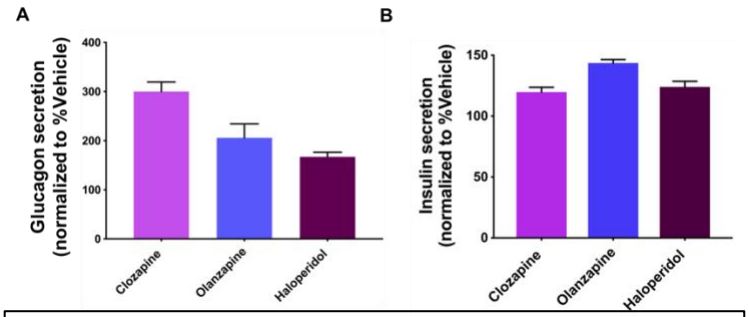

Figure 7. APDs increase secretion of glucagon and insulin in human islets. (A) Treatment with either atypical APDs clozapine and olanzapine, or typical APD haloperidol (1 μM) significantly raised glucagon secretion; and **(B)** elevated GSIS relative to vehicle controls. Results normalized relative to vehicle. Results= mean ± SEM.

- Lastly, we have examined whether APDs disrupt coordinated secretion of glucagon and insulin during glucose stimulation of human islets. We tested olanzapine and clozapine, atypical APDs that produce significant metabolic disturbances, and found that both drugs substantially increase alpha cell glucagon secretion relative to vehicle controls. In contrast, typical APD haloperidol also raised glucagon secretion, albeit less compared to olanzapine and clozapine (**Figure 7A**). All three APDs also significantly increased GSIS from the same islets

(**Figure 7B**). Our results suggest that APDs enhance not only insulin secretion but also produce substantial increases in glucagon release. Given the growing evidence that glucagon plays a key role in systemic glycemic control, our findings suggest that these APD-induced increases in glucagon and insulin may contribute significantly to the clincally-observed systemic metabolic dysfunction including hyperglycemia and insulin resistance produced by these medications.

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

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

  Our results were disseminated to communities of interest at national and international scientific meetings. These meetings include the Science 2018 meeting (2018), Pancreatic Beta Cell Consortium Annual Meeting (2018), National Center for Brain Mapping Neural Probes meeting (2019), 74th Annual Meeting of the Society of Biological Psychiatry (2019), 7th Annual Molecular Psychiatry Meeting (2019), 5th Annual Sleep & Circadian Science Research Meeting (2019), and 58th annual meeting of the American College of Neuropsychopharmacology meeting (2019). Collectively, presenting recent findings stemming from this project 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. Furthermore, we have also published our results extensively. 8 publications detailing our results from this project were published in high-impact journals including *Molecular Psychiatry*. In addition to the work already published, we presently have five 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

- Once the D3R-flox mice are fully isogenic to the C57BL/6J background, we will begin crosses to establish hypothalamus-specific D3R knockout mice (Nkx2.1-cre hemizygous, D3R-flox homozygous mice). This process is expected to take approximately 6-8 months. Once complete, we will conduct weekly measurement of weights and food consumption in hypothalamus-specific D2R (and WT 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 6, 13 and 26 of APD treatment.

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

- Once the D3R-flox mouse strain is fully isogenic with the C57BL/6J strain in the next 4-6 months, we will begin crosses to establish inducible pancreatic beta cell-specific D3R knockout mice (Mip1-cre/ERT hemizygous, D3R-flox homozygous mice).

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

- We and our Partnering PI will use a diet-induced obesity model to induce insulin resistance and obesity and then 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.

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 KO 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 KO mice. Moreover, we will generate beta cell-selective D2R and D3R double KO mice to examine the combined effects of both dopamine receptors on GSIS. In parallel, we will measure DA secretion from these double D2R/D3R KO islets and the WT 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

- Our studies examining APD treatments' effects on GSIS in pancreatic islets from KO and WT mouse islets are currently underway. In future work, we will continue to treat pancreatic islets from beta cell-specific D2R KO mice with additional APDs including aripiprazole. We will also sample insulin and DA levels every 10 min which will permit determination of a kinetic curve for the respective release events. Once the beta cell-selective D3R KO mouse strain is generated, we will repeat the above experiments in islets from these mice. Furthermore, we will validate our findings in human islets including comparisons with islets from individuals with insulin resistance and/or type 2 diabetes to better model APD effects in a background of preexisting metabolic dysfunction.

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

We presented our preliminary results at scientific meetings including at national and international conferences: Science 2018 meeting (2018), Pancreatic Beta Cell Consortium Annual Meeting (2018), National Center for Brain Mapping Neural Probes meeting (2019), 74th Annual Meeting of the Society of Biological Psychiatry (2019), 7th Annual Molecular Psychiatry Meeting (2019), 5th Annual Sleep & Circadian Science Research Meeting (2019), and 58th annual meeting of the American College of Neuropsychopharmacology meeting (2019). These presentations were instrumental in advancing the concept that APDs may act on peripheral dopaminergic targets which is a topic directly relevant to endocrinology and psychiatry – the two principal disciplines associated with this project. This has begun generating considerable interest within these two fields.

- **What was the impact on other disciplines?**
  In presenting results from this project, our findings were also broadly disseminated to a broad scientific audience beyond endocrinology and psychiatry. The results were communicated to diverse audiences whose expertise spanned multiple disciplines including neuroscience, cell biology and clinical medicine. By appealing to a broader audience, this may foster in the longer-term new knowledge that leads to development of better APDs free of metabolic side effects. Ultimately, such a development could significantly reduce serious morbidity and mortality from medication-associated type II diabetes and cardiovascular disease. Moreover, better understanding the mechanisms by which DA and DA 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:

1. Chipchura D, **Freyberg Z**, Edwards C, Leckband SG, McCarthy MJ. Does timing of drug administration alter the metabolic risk of aripiprazole? Frontiers in Psychiatry 2018. 9:494; doi: 10.3389/fpsyt.2018.00494. PubMed PMID: 30364286.

2. Guo J, Zhou B, Zeng X, **Freyberg Z**, Xu M. Model compression for faster structural separation of macromolecules captured by cellular electron cryo-tomography. in: Campilho A., Karray F., Romeny B. (eds.) Image Analysis and Recognition. 2018. Springer International Publishing. DOI: 10.1007/978-3-319-93000-8.

3. **Freyberg Z**, Logan RW. The intertwined roles of circadian rhythms and neuronal metabolism fueling drug reward and addiction. Curr Opin in Physiology 2018; Oct 5: 80-89. doi.org/10.1016/j.cophys.2018.08.004. PubMed PMID: 30631826.

4. Sorzano COS, Jimenez A, Mota A, Vilas JL, Maluenda D, Martinez M, Ramirez-Aportela E, Majtner T, Segura J, Sanchez-Garcia R, Rancel Y, del Cano L, Conesa P, Melero R, Jonic S, Vargas J, Cazals F, **Freyberg Z**, Krieger J, Bahar I, Marabini R, Carazo JM. Survey of the analysis of continuous conformational variability of biological macromolecules by electron microscopy. Acta Crystallographica. Section F Struct Biol Commun. 2019. F75 (Pt 1): 19-32. doi: 10.1107/S2053230X18015108. PubMed PMID: 30605122.

5. Farino ZJ, Morgenstern TJ, Maffei A, Quick M, De Solis A, Wiriyasermkul P, Freyberg RJ, Aslanoglou D, Sorisio D, Inbar B, Free R, Donthamsetti P, Mosharov E, Kellendonk C, Schwartz GJ, Sibley D, Schmauss C, Zeltser L, Moore H, Harris PE, Javitch JA, **Freyberg Z**. New roles for dopamine D_2 and D_3 receptors in pancreatic beta cell insulin secretion. Mol Psychiatry 2019. doi:10.1038/s41380-018-0344-6. PubMed PMID: 30626912.

6. Li R, Zeng X, Siegmund SE, Lin R, Zhou B, Liu C, Wang K, Jiang R, **Freyberg Z (co-corresponding author)**, Lv H, Xu M. Automatic localization and identification of mitochondria in cellular electron cryo-tomography using faster-RCNN. BMC Bioinformatics 2019. 20 (Suppl 3): 75-85. doi: 10.1186/s12859-019-2650-7. PubMed PMID: 30925860.

7. Han F, Konkalmatt P, Mokashi C, Zhang Y, Ko A, Farino ZJ, Asico L, Xu G, Gildea G, Zheng X, Felder R, Lee REC, Jose P, **Freyberg Z (co-corresponding author)**, Armando I. Dopamine D_2 receptor modulates Wnt expression and control of cell proliferation. Scientific Reports 2019. 9, 16861. doi:10.1038/s41598-019-52528-4. PubMed PMID: 31727925.

8. Wei H, Zapata R, Lopez-Valencia M, Farino ZJ, Aslanoglou D, Benner V, Osborn O, **Freyberg Z (co-corresponding author)**, McCarthy MJ. Dopamine D_2 receptor signaling modulates pancreatic beta cell circadian rhythms. Psychoneuroendocrinology *In Press*

   In addition to the work already published, we presently have five 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:

1. Kumar M, Asico L, Konkalmatt P, **Freyberg Z**, Jose PA, Armando I. (2018) Role of dopamine D_2 receptors in the regulation of cell proliferation in renal injury. Abstract and poster to be presented at the 2018 Hypertension meeting, Chicago, IL.

2. Johnson MA, Koppes E, Warshafsky MG, Moresco JJ, Diedrich JK, Aslanoglou D, Park HJ, Walsh DR, Luppi P, Gollin SM, Yates JR, **Freyberg Z**, Peter Drain, Nicholls RD. (2018) Cellular and molecular basis of insulin-secretion deficiency in Prader-Willi syndrome. Abstract and presentation presented at the 2018 Foundation for Prader-Willi Syndrome Scientific Day, Las Vegas, NE.

3. Kumar M, Asico L, Konkalmatt P, **Freyberg Z**, Jose PA, Armando I. (2018) Regulation of cell proliferation in renal injury by dopamine D_2 receptors. Abstract and poster presented at the American Heart Association Scientific Sessions 2018, Chicago, IL.

4. Li R, Zeng X, Jiang R, **Freyberg Z** (**co-corresponding**), Lu H, Xu M. (2019) Automatic identification and localization of mitochondria in Cellular Electron Cryo-Tomography using Faster RCNN. Paper presented at the 2019 17th Asia Pacific Bioinformatics Conference, Singapore.

5. McCarthy M, Reagan L, Hahn M, **Freyberg Z**. (2019) Central and peripheral actions of insulin: Implications for psychiatric disorders. Abstract and presentation presented at the 2019 Society of Biological Psychiatry annual meeting, Chicago, IL.

6. Teresi CE, Bocarsly ME, Dobbs LK, **Freyberg Z**, Alvarez VA. (2019) Behavioral characterization of mice with selective dopamine D_3 receptor knockout on striatal D1 projection neurons. Abstract and poster presented at the Society for Neuroscience 2019 Annual Neuroscience meeting, Chicago, IL.

7. Aslanoglou D, Bertera S, Sanchez-Soto M, Lee J, Yechoor V, Brissova M, Free RB, Sibley D, **Freyberg Z**. (2019) New peripheral dopaminergic mechanisms of antipsychotic drug-induced metabolic disturbances. Abstract and poster presented at the 58th Annual Meeting of the American College of Neuropsychopharmacology, Orlando, FL.

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

1. Invited speaker, Department of Molecular Biochemistry and Biophysics, Columbia University, New York, NY; 2018
2. Invited speaker, Departments of Neurobiology and Computational Neuroscience seminar series, University of Chicago, Chicago, IL; 2019
3. Invited speaker, Department of Neurobiology and Behavior, University of Pennsylvania, Philadelphia, PA; 2019

- **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:	Zachary Freyberg M.D., Ph.D.
• Project Role:	Principal Investigator
• Researcher Identifier (*e.g.* ORCID ID):	ORCID ID: 0000-0001-6460-0118
• Nearest person month worked:	5
• Contribution to Project:	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.

• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292); The Pittsburgh Foundation Rising Star Research Award

• Name:	Corey Foreman
• Project Role:	Research Technician
• Researcher Identifier (*e.g.* ORCID ID):	N/A
• Nearest person month worked:	7
• Contribution to Project:	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 *in vitro* functional assays measuring insulin and dopamine secretion from pancreatic islets and insulin-secreting pancreatic beta cell-derived cell lines.
• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292); The Pittsburgh Foundation Rising Star Research Award

• Name:	Despoina Aslanoglou, Ph.D.
• Project Role:	Postdoctoral Researcher
• Researcher Identifier (*e.g.* ORCID ID):	N/A
• Nearest person month worked:	12
• Contribution to Project:	Dr. Aslanoglou has performed cloning and molecular biological studies, tissue culture as well as *in vitro* and *ex vivo* functional assays measuring insulin, glucagon and DA secretion from mouse and human pancreatic islets and hormone-secreting pancreatic cell-derived cell lines.
• Funding Support:	Department of Defense Peer Reviewed Medical Research Program Investigator-Initiated Research (PR141292)

- **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

Below are abstracts presented at two international meetings including the 2019 58th Annual Meeting of the American College of Neuropsychopharmacology, and the 2019 Society of Biological Psychiatry annual meeting.

1. Abstract and poster presented at the 2019 58th Annual Meeting of the American College of Neuropsychopharmacology, Orlando, FL:

   Title: New Peripheral Dopaminergic Mechanisms of Antipsychotic Drug-Induced Metabolic Disturbances

   Authors: Despoina Aslanoglou, Suzanne Bertera, Marta Sanchez Soto, Jeong Lee, Vijay Yechoor, Marcela Brissova, R. Benjamin Free, David Sibley, Rita Bottino, **Zachary Freyberg[1]**

   Background:
   Antipsychotic drugs (APDs) are used to treat highly prevalent psychiatric illnesses including schizophrenia, bipolar disorder and major depressive disorder, making them among the most widely prescribed psychiatric medications today. However, these drugs also cause profound metabolic disturbances including weight gain, glucose intolerance, and insulin resistance and increase the risks of developing type 2 diabetes (T2D) and cardiovascular disease. Significantly, all APDs cause metabolic side effects to differing degrees and current treatments to reduce these metabolic symptoms have only limited efficacy. To date, the mechanisms for APD-induced metabolic disturbances are poorly understood. Nonetheless, the single unifying property of all APDs is their blockade of dopamine (DA) D2-like receptors including D2 (D2R) and D3 (D3R) receptors, suggesting a potential role for these receptors in APD-induced metabolic dysfunction. Importantly, APD-induced changes in glucose homeostasis occur even in the absence of increased food intake, or psychiatric disease. Indeed, as little as a single administration of olanzapine is sufficient to alter glucose homeostasis independent of weight changes in healthy human subjects. This raises the possibility that APDs may act directly on metabolically-relevant peripheral targets to cause metabolic disturbances. Consistent with this, we and others discovered that D2R and D3R are expressed peripherally in both human and rodent insulin-secreting pancreatic beta cells, key regulators of glucose metabolism. While considerably less studied then beta cells, there is also evidence that D2R and D3R are also expressed in glucagon-secreting pancreatic alpha cells. We therefore hypothesize that APD-induced metabolic disturbances are driven by the direct actions of APDs on pancreatic alpha cell and beta cell D2-like receptors.

   Methods:
   Human pancreatic islet transcriptome analysis: De-identified human islet alpha cells and beta cells (n=5: 3 females, 2 males) were purified by FACS sorting; alpha cells and beta cells were distinguished via indirect antibody labeling with alpha- and beta cell markers (Brissova et al., 2018).
   For DA measurements, mouse alpha cell-derived alpha TC1-6 cell supernatants and cell lysates were collected and run on HPLC (Farino et al., 2019).
   Insulin and glucagon homogenous time-resolved resonance energy transfer (HTRF) assays: De-identified cadaveric human islets and BALB/c mouse islets were collected and cultured overnight prior to use. Islets were glucose-stimulated and supernatants collected for insulin and glucagon measurement via HTRF (Farino et al. 2019; Aslanoglou et al., 2019).

All human and mouse islet studies were IRB- and IACUC-approved. All studies were conducted in triplicate on n>3 experimental days.

Results:
We conducted a comprehensive transcriptome analysis to characterize the DA signaling and biosynthetic machinery in human pancreatic alpha and beta cells followed by RNA-sequencing analysis (RNAseq). We found that human alpha and beta cells express the complete DA biosynthetic, catabolic and signaling machinery. Consistent with this, HPLC analyses demonstrated that alpha cells both synthesize and secrete DA. Unlike beta cells, however, alpha cells also secrete DA precursor L-DOPA. Our transcriptome data also showed that both human alpha and beta cells express all five DA receptors (D1-D5), with D2R and D3R being the predominantly expressed DA receptors. These data suggest that APDs may target these receptors in beta cells and alpha cells.

To study APD actions on alpha and beta cell D2R and D3R, we developed a novel rapid optical glucagon detection assay based on HTRF technology, similar to our existing HTRF insulin assay (Farino et al., 2016). This approach eliminates all washing steps, making our assays rapid and high-throughput. We first examined the roles of DA signaling on glucagon release from human pancreatic islets, discovering that low DA concentrations potently decreased glucagon release, in addition to DA's inhibition of beta cell glucose stimulated insulin secretion (GSIS). These data suggest that DA modulates both glucagon and insulin secretion in islets. We next examined whether APDs disrupt coordinated secretion of glucagon and insulin during glucose stimulation of human islets. We showed that both clozapine and olanzapine substantially increased alpha cell glucagon secretion relative to vehicle controls; haloperidol also raised glucagon secretion, albeit to a lesser degree compared to olanzapine and clozapine. All three APDs also significantly increased GSIS from the same islets. Our results suggest that APDs enhance insulin and glucagon release, contributing to systemic metabolic dysfunction.

Conclusions:
Overall, we show that pancreatic alpha cells may provide a key source of pancreatic DA which signals locally at alpha and beta cell receptors to modulate insulin and glucagon release. APDs disrupt this peripheral DA signaling at alpha and beta cells to significantly disturb secretion of key hormonal regulators of metabolism. Specifically, APDs disrupt dopaminergic inhibition of GSIS in beta cells, leading to excessive insulin secretion in islets – a driver of insulin resistance in type 2 diabetes. Similarly, APD blockade of alpha cell D2R/D3R also profoundly elevates glucagon secretion – a key driver of hyperglycemia. Ultimately, our work suggests that APDs act directly on both alpha cell and beta cell DA signaling to significantly disturb metabolism.

2. Abstract and talk presented at the 2019 Society of Biological Psychiatry annual meeting, Chicago, IL:

Title: Central and peripheral actions of insulin: Implications for psychiatric disorders

Authors: Michael McCarthy, Lawrence Reagan, Margaret Hahn, **Zachary Freyberg**

Overall synopsis: There have been numerous observations demonstrating significant metabolic dysfunction in psychiatric illnesses such as schizophrenia, major depression and bipolar disorder. Individuals with these disorders are at markedly increased risk of developing disturbed glucose homeostasis, insulin resistance and type 2 diabetes which ultimately lead to significant cardiometabolic co-morbidities and shortened lifespan. Though antipsychotic drugs (APDs) are increasingly recognized as important contributors to these metabolic disturbances, many of the metabolic findings pre-date the APD era and, along with recent data from drug-naïve patients, suggest that psychiatric illnesses also confer significant intrinsic risk for metabolic dysfunction. To date, the biological mechanisms

underlying both these intrinsic and APD-induced metabolic disturbances are poorly understood and somewhat under-investigated. An important clue may lie in dysfunctional insulin signaling both in the central nervous system and in the periphery associated with schizophrenia and other psychiatric disorders. Here, we will present cutting-edge findings that reveal new connections between the actions of insulin both centrally and in the periphery. We will cover the molecular actions of insulin in the brain, dopamine in islet cells, and how the circadian clock may integrate metabolic and central functions. In addition, we will focus on how these mechanisms may underlie metabolic disorders, cognition, mood and their implications on treatment outcomes/side effects with APDs.