

1. **Hypothesis and Specific Aims**

Overarching Hypothesis: Insulin signaling is a critical neurobiological substrate of anhedonia in mood disorders

Specific Hypotheses: In individuals with mood disorders, objective and self-reported measures of anhedonia and reward behaviors are associated with alterations in insulin signaling-related molecular, cellular and neural substrates. Considering the role of insulin in the regulation of energy expenditure, we primarily hypothesize that individuals with mood disorders will display decreased willingness to make effort for rewards, relative to healthy controls; and that this abnormality will be associated with:

- a) Molecular: Differential gene and protein expression of the metabolic (i.e. PI3K/Akt) and mitogenic (i.e. Ras/MAP kinase) components of the insulin signaling pathway, as well as with components of reward-related processes (i.e. dopamine receptors and transporter)
- b) Physiology: Reduced peripheral sensitivity to insulin and corresponding metabolic correlates, including increased body mass index and dyslipidemia (i.e. peripheral insulin resistance)
- c) Neural Circuits: Diminished response to insulin in cortico-striatal networks (i.e. brain insulin resistance)

Overarching Aim: To determine the role of insulin signaling on the neurobiological substrates subserving anhedonia within individuals with mood disorders.

Specific Aims:

- a) Molecular: Assessment of components of the insulin cascade, as well as of anhedonia and reward-related processes, using a proteomics and gene expression approach;
- b) Physiology: Measurement of peripheral sensitivity to insulin and metabolic correlates, including body mass index and dyslipidemia;
- c) Neural Circuits: Evaluation of the insulin sensitivity of prefrontal (e.g. prefrontal cortex) and striatal (e.g. nucleus accumbens, ventral tegmental area) networks in the resting-state and during an effort-based decision making test, using acutely administered intranasal insulin and functional magnetic resonance imaging (fMRI);
- d) Behavioral: Measurement of willingness to make effort for rewards, as well as of other components of reward response and anhedonia, using validated behavioral tasks and clinical scales (e.g. Snaith-Hamilton Pleasure Scale - SHPS).

2. **Background and Rationale**

Mood disorders (i.e. major depressive disorder [MDD] and bipolar disorder [BD]) and metabolic disorders (i.e. type 2 diabetes mellitus [T2DM], obesity) are highly prevalent and impactful conditions. Epidemiological studies have identified depressive disorders and diabetes as leading causes of disability worldwide¹. Mood and metabolic disorders are also major causes of excessive and premature mortality. Mortality studies indicate that with individuals with MDD or BD have markedly reduced life expectation, on average of 10-15 years²; the main driver of excess and premature mortality in this population is cardiovascular disease and/or diabetes complications^{2,3}. Conversely, depressive symptoms, particularly anhedonia, are consistently reported in T2DM populations and have been associated with an increased incidence of complications and all-cause mortality^{4,5}.

Comorbid metabolic disorders are extremely common in individuals with mood disorders. Meta-analytic studies have documented that individuals with MDD or BD have an approximately 2-fold increased risk of T2DM, relative to the general population⁶. It is estimated that the prevalence of

T2DM in individuals with mood disorders is between 8 and 10%⁶, whereas the prevalence of metabolic syndrome is approximately 30%⁷. Convergent lines of inquiry indicate that mood and metabolic disorders have a bidirectional association⁸⁻¹⁰. For example, it is reported that individuals with T2DM have an approximate 2-3 fold greater risk for subsequently declaring MDD or BD¹¹. Nonetheless, the association between metabolic and mood disorders is still poorly understood. Overlapping risk factors (e.g. diet, physical activity, psychotropic medications, sleep, childhood trauma) have an important role⁶; however there is insufficient data on the extent to which these environmental factors are responsible for the mood-metabolic disorders comorbidity. Mechanistic studies have documented a role for the biological substrates of metabolic and mental disorders, such as neurostructural alterations^{12,13}, and differences in peripheral biomarkers¹⁴. A recent genetic study identified multiple genes that are likely shared between mood and metabolic disorders; pathway analysis revealed an over-representation of genes that encode for molecules involved in dopamine signaling¹⁵. There is considerable evidence that dopamine has a core role in anhedonia and reward-related behaviors, indicating that this is a particularly promising area for further advancing the knowledge on the association of mood and metabolic disorders.

1.1. Mood Disorders and Anhedonia

Anhedonia is defined as markedly diminished interests or pleasure in all, or almost all, activities most of the day, nearly every day for an extended period of time. Anhedonia is a core criterion item for the diagnosis of a major depressive episode. Anhedonia is also a robust predictor of poorer longitudinal course of symptoms of MDD and BD¹⁶⁻¹⁹. Existing psychological and pharmacological treatments are relatively ineffective for treating anhedonia, as standard medication treatments have little effect and in some cases may even worsen anhedonic symptoms^{17,20}. As a result, anhedonia has been described as one of the most prevalent residual disturbances following treatment with selective serotonin re-uptake inhibitors^{17,21,22}.

Anhedonia includes elements of multiple reward processes²³⁻²⁵. Reward systems are primarily responsible for responses to positive motivational situations or contexts, such as reward seeking, and reward/habit learning²³⁻²⁵. Reward is a broad and heterogeneous construct. To effectively study it, it is important to consider its three main subconstructs: (1) reward responsiveness, including anticipatory and initial hedonic responses; (2) learning, or the acquisition of information about stimuli, actions and context; and (3) valuation, including the measurement of value and/or incentive salience of a prospective outcome and the willingness to work for it (i.e. effort)^{26,27}.

Evidence indicates that individuals with mood disorders exhibit abnormalities in multiple of these reward subcomponents²⁸⁻³². Anhedonia is closely related to reward anticipation, motivation and decision making^{29,33}. A consistent finding has been a decrease in the willingness to expend effort and/or in the modulation of effort expenditure for rewards^{28-31,33}. It has been demonstrated that individuals with depression display reduced motivation on effort-based decision making²⁹⁻³¹, a dissociation between liking a reward and the willingness to exert effort for it²⁸, an increased subjective feeling of having exerted more effort³¹ and a lower ability to effectively use information about rewards to modify their behavioral choices²⁹.

1.2. Neural Substrates of Anhedonia and Reward

The most well-characterized reward circuit is the mesolimbic pathway, which consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc)^{34,35}. These areas are heavily interconnected with the prefrontal cortex (PFC), amygdala and hippocampus, among other areas³⁴. Specifically, connections between the dorsolateral PFC (dlPFC), NAc and VTA

have a key role in motivated behavior³⁶. Indeed, connectivity between the PFC and striatal areas during reward-related tasks negatively correlates with anhedonia in individuals with MDD^{37,38}.

Dopaminergic neurotransmission has been highlighted as a key component of multiple reward processes³⁹. Dopamine signaling has traditionally been implicated in arousal, motivation and psychomotor activation (i.e. incentive salience models), and reward learning/reinforcement (i.e. prediction error models)⁴⁰⁻⁴². Recent work proposes that dopamine signals value, or the available reward for a certain effort, which is then employed for both learning and motivational functions⁴². Multiple studies from different lines of research, such as genetics, neuroimaging and pharmacology, have documented abnormalities in dopaminergic neurotransmission in individuals with mood disorders (8, 9). For example, neuroimaging studies have demonstrated alterations in dopamine receptor and transporter binding in individuals with MDD⁴³⁻⁴⁵. Conversely, the Val(158)Met polymorphism of the catechol-O-methyltransferase (COMT) gene, which affects dopamine catabolism and modulates prefrontal dopamine levels, has been associated with measures of anhedonia^{46,47}. Overall, abnormalities in reward-related behaviors and dopaminergic transmission are well-established features of mood disorders phenomenology and neurobiology.

1.3. Brain Insulin Signaling Pathway, Dopamine Signaling and Reward

Dopamine signaling within and across brain circuits is in interplay with many systems relevant to reward, including the insulin signaling pathway^{34,48,49}. Insulin acts via the insulin receptor (INSR). Activation of INSR initiates the recruitment of the insulin receptor substrate (IRS) family of proteins, which organize and mediate signaling cascades^{50,51}. IRS1 and IRS2 are the most extensively studied substrates; they are expressed in the brain and have well-documented effects on insulin signaling and glucose homeostasis⁵²⁻⁵⁴. Insulin binding to INSRs activates several divergent signaling pathways, including the metabolic pathway (i.e. PI3K/Akt), which affects glucose and lipids homeostasis; and the mitogenic pathway (i.e. Ras/MAP kinase), which is implicated in the regulation of apoptosis and cell proliferation/differentiation.

Multiple brain regions that are relevant to reward systems have relatively increased expression of INSRs (e.g., PFC, hippocampus, amygdala, substantia nigra)^{22,23}. Specifically, midbrain dopamine neurons widely express INSRs⁴⁹. Imaging studies have reported that, in healthy individuals, insulin modulates brain activity in the hypothalamus, hippocampus and prefrontal cortex³⁴⁻³⁶. Brain insulin signaling has traditionally been implicated in glucose and lipid metabolism, and eating behavior. However, replicated evidence, from multiple studies, has documented that insulin also has a role on emotional processing⁵⁵⁻⁵⁷ and reward processes, including, but not limited to, those pertaining feeding behaviors and energy expenditure^{58,59}.

An important way insulin affect reward processes is via interaction with dopaminergic signaling. Evidence indicated that insulin modulates multiple components of dopamine transmission, including synthesis and transport, receptors function and reuptake and degradation. For example, lower expression and function of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, have been reported in animal models of diabetes^{60,61}. In humans, insulin resistance was associated with less endogenous dopamine at D2/3 receptors in the ventral striatum and NAc, in both obese and non-obese participants^{62,63}. A separate study documented that weight loss following bariatric surgery was associated with an increase in D2 receptor availability⁶⁴.

Termination of dopamine action, which is via reuptake from the synaptic cleft by the dopamine transporter (DAT), or by degradation by monoamine oxidases (MAOs) A/B and COMT, has also been shown to be affected by insulin. Studies with animal models of diabetes have reported lower availability and activity of DAT^{65,66} and lower activity of COMT⁶⁷; whereas a murine model with a

brain specific knockout of the insulin receptor reported that decreased insulin function in the brain resulted in increased expression of MAO A/B in the striatum and NAc⁶⁸. As a result, evidence indicates that insulin sensitivity modulates reward behavior in obese adults, wherein insulin resistance was associated with a stronger preference for immediately receiving a smaller, but certain, monetary reward over delaying the receipt of a larger, but less certain one (i.e. greater delay discounting)⁶⁹.

1.4. Insulin Signaling, Anhedonia and Major Depressive Disorder

The epidemiological, phenomenological and biological associations between mood disorders and metabolic disturbances have been amply documented. The foregoing observations provide the rationale for hypothesizing that there is a neurobiological association between metabolic disorders (e.g. diabetes) and mood disorders. The devastating impact of mood and metabolic disorders on affected individuals underscores the importance of urgently understanding better the basis of this association. Anhedonia is a well-described symptom that similarly occurs in both disorders. The extant evidence of the association between insulin and dopamine signaling and the potential repercussions of disturbances in this system indicates that this is a promising target for clinical and neurobiological investigations. The interface between anhedonia and insulin represents a unique opportunity to integrate innovative and transdisciplinary lines of research in an investigative platform aiming at providing a more accurate and refined assessment of relevant mechanisms in the mood-metabolic disorders comorbidity.

2. Preliminary Data

Using the “Gene Expression in Postmortem DLPFC and Hippocampus from Schizophrenia and Mood Disorders” (dbGaP accession number phs000979.v1.p1) database, which contains microarray data from the post-mortem dLPFC (healthy controls [HC]: n = 209; patients: n = 321) and hippocampus (HC: n = 180; patients: n = 196), we conducted a hypothesis-driven analysis through the a priori selection of 12 dopamine- and 3 insulin-related genes (manuscript under review). Results showed that, in the dLPFC, after adjustment for relevant covariates, expressions of insulin receptors- (i.e. INSR, IRS1/2) and dopamine regulation-related genes (e.g. TH, DRD1-5, DAT) were influenced by body mass index (BMI), with significantly lower expression in high BMI patients with mood or psychotic disorders (Figure 1). In the hippocampus, we observed significantly lower expressions of these genes, which were not affected by BMI.

Genes that were differentially expressed between groups were selected for mediation analyses. Diagnosis by BMI effects on expression of dopamine genes were fully mediated by expression of INSR. Analysis of conditional indirect effects showed interactions between INSR and BMI, indicating significantly stronger indirect effects at higher BMI values. In the hippocampus, we observed that expression of insulin receptor substrate 1 and 2 fully mediated the effects of diagnosis on expression of dopamine genes (Figure 2). All models were significant (e.g. DRD1: $R^2 = 0.31$, $F_{6,385} = 29.519$, $p < 0.001$). These results suggest that the differential expression of dopamine-related molecules in the post-mortem brain of individuals with mental disorders is related to altered expression of insulin signaling genes. We also observed that obesity has region-specific moderating effects on gene expression and co-expression, supporting the hypothesis that metabolic systems are critical mediators of dopaminergic function and providing the rationale for further investigation.

Figure 1 – Mean standardized expression values of insulin and dopamine signaling genes in the dorsolateral prefrontal cortex, according to group (HC vs. MI) and obesity (BMI ≥ 30 kg/m²). HC: healthy controls; MI: mental illness; BMI: body mass index.

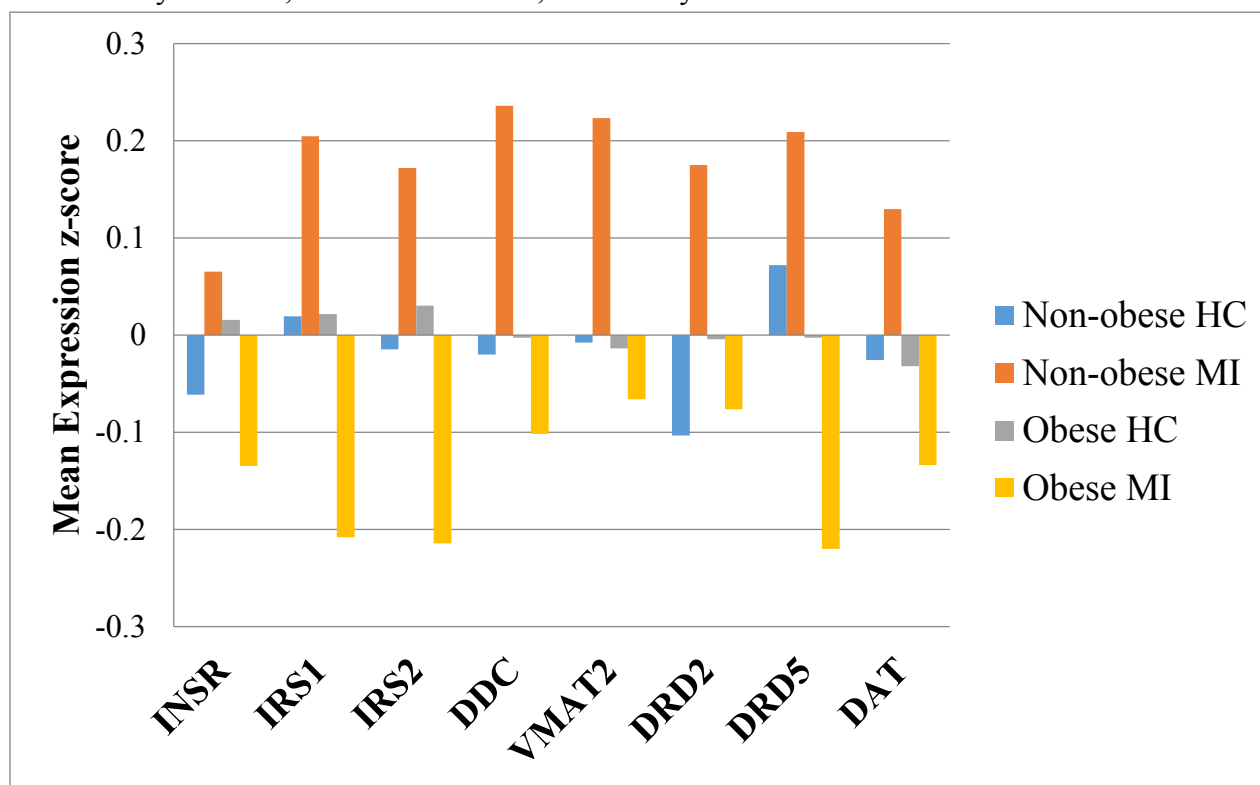
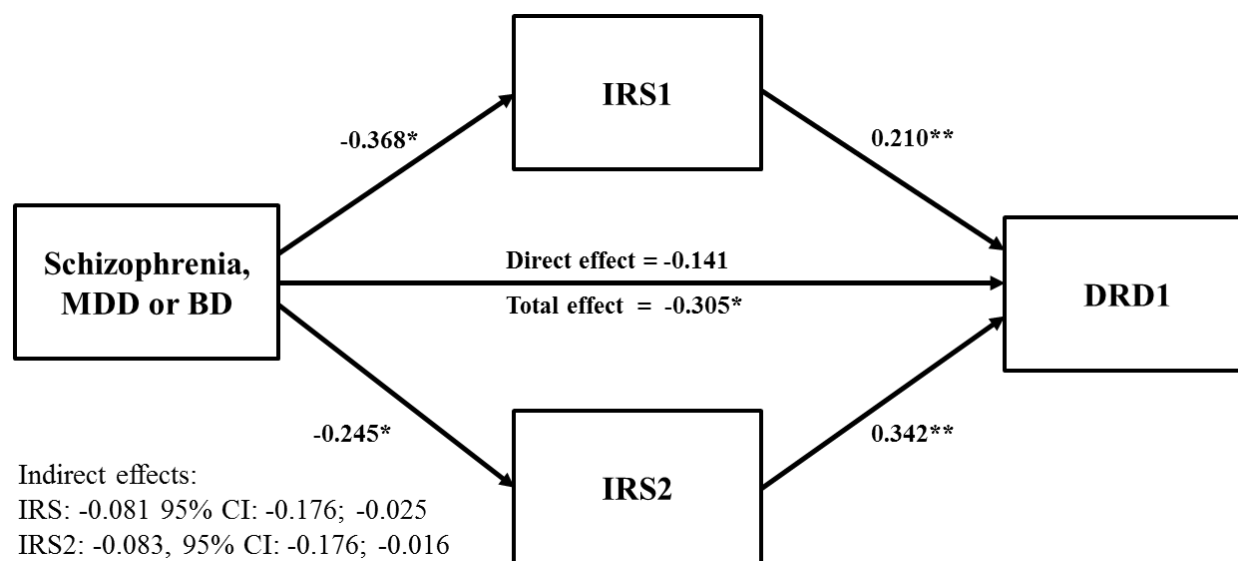


Figure 2 – Unstandardized path coefficients for a mediation model in the hippocampus, adjusted for age, gender, race, ethanol and nicotine/cotinine. * $p < 0.05$; ** $p < 0.001$.



3. Design and Methodology

Study population: We propose to recruit 50 adults between the ages of 18 to 50 years: 25 patients with DSM-5 defined MDD or BDI/II, in a depressive episode, recruited from the Mood Disorder Psychopharmacology Unit, University Health Network, and 25 age- and sex-matched healthy controls recruited through local advertisements in the community.

Inclusion criteria (patients):

1. Age 18-50
2. DSM-5 defined MDD/BD and a total score ≥ 20 on the Montgomery-Åsberg Depression Rating Scale (MADRS) and no history of dementia or intellectual disability
3. A written, voluntary informed consent prior to study enrollment

Exclusion criteria (patients):

1. Use of insulin and/or oral hypoglycemics, due to its confounding effects
2. Diagnosis of possible or probable AD, MCI, or any other dementia
3. History of neurological disorder, or evidence of neurologic or other physical illness that could produce cognitive deterioration
4. Substance use disorder within 3 months before screening or a positive baseline toxicology screen
5. Presence of clinically unstable general medical illness
6. Pregnancy or breastfeeding
7. MRI contraindications

Inclusion criteria (healthy controls):

1. Age 18-50
2. A written, voluntary informed consent prior to study enrollment

Exclusion criteria (healthy controls):

1. Use of insulin and/or oral hypoglycemics, due to its confounding effects
2. Presence of any current or lifetime psychiatric or neurological conditions
3. Substance use disorder within 3 months before screening or a positive baseline toxicology screen
4. Presence of clinically unstable general medical illness
5. Pregnancy or breastfeeding
6. MRI contraindications

Study Procedures: Single-dose, randomized, controlled, double-blinded, cross-over study (50 participants)

Screening and Baseline Assessment: Patients and healthy controls will first meet, in-person or virtually, with a staff psychiatrist for a clinical consultation or a research team member, respectively. After obtaining consent and if inclusion criteria are met, a medical comorbidity questionnaire will be administered to screen for concurrent and lifetime medical conditions. Participant's demographic characteristics and current medications, as well as lifetime psychotropic medications received will be recorded. We will also assess dietary intake with the Diet History Questionnaire III (DHQ III); smoking, by questioning number of cigarettes smoked per day, age when smoking started, and quitting date, if applicable, to calculate a smoking pack-year history; physical activity using the International Physical Activity Questionnaire (IPAQ); and alcohol and/or substance abuse. In addition, we will assess demographics, socioeconomic status, medical history, and family psychiatric history; childhood trauma history with the Childhood Trauma Questionnaire (CTQ) will also be assessed. Participants will also be asked to complete an MRI screening questionnaire to ensure that they have no contraindications. The following self-reported measures will be carried out: Perceived Deficits Questionnaire-Depression (PDQ-D), Sheehan Disability Scale (SDS), UCLA life Stress (Episodic), DeJong Gierveld Loneliness Scale, and Pittsburg Sleep Quality Index (PSQI) (Table 1).

When in-person visits are possible, participants will complete a urine drug screen. Female participants will be asked to do blood work to confirm they are not pregnant.

Physiological Assessment: Anthropometric (i.e. BMI, waist-hip ratio) and metabolic measurements (i.e. fasting glucose, fasting insulin, glycated hemoglobin, lipids and HOMA-IR) will be completed at each study visit (1-3). In addition, physical activity and energy expenditure will be measured using an accelerometer. Participants must wear the accelerometer at all times for one week and return it upon Visit 3 for data collection and analysis. Additionally, a heart rate monitor device will be connected to the accelerometer that will monitor heart rate variability over one day of the week that the subjects wear the accelerometer.

Laboratory Assessment:

Routine: the following laboratory tests will be completed:

Triglycerides
Total Cholesterol
Insulin
Glucose
HbA1c
C-Peptide
IGF-1
TSH
Free T4
Free T3
C-Reactive Protein
hCG

Biomarkers: High sensitivity CRP and TNF α will be measured at both visits (hs-CRP only). Ultrasensitive ELISA will be used to measure high-sensitivity CRP and TNF- α , TNF-RI and TNF-R2 (measured at both visits). For exploratory mechanistic analysis, a 10-plex immunoassay of

inflammatory cytokines will be measured at both visits (e.g. IFN- γ , IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13). Blood will be collected after a 12 hour fast and centrifuged at 1000g for 15 min at 4°C; plasma will be stored at -80 °C.

Behavioral assessment: These tasks are simple, user-friendly tools that provide an opportunity, when used in conjunction with the most widely used questionnaires, to gain novel insights on reward behaviors in mood disorders.

- a) The primary behavioral measurement will be effort-based decision-making, assessed using the Effort-Expenditure for Rewards Task (EEfRT)³³, which will be done in a fMRI paradigm⁷³, as described below during Visits 2 and 3.
- b) Assessments will include the validated anhedonia scale Snaith-Hamilton Pleasure Scale (SHPS), the mood questionnaires Montgomery-Åsberg Depression Rating Scale (MADRS) and Young Mania Rating Scale (YMRS) during each study visit, and the Bandit Task during the first visit.

Neural Circuits Assessment: A promising tool to study the effect of insulin on CNS is intranasal insulin, which is delivered directly into the brain, without relevant effects on peripheral glucose^{76,77}. A single dose of intranasal insulin has been shown to influence brain activation, increasing regional perfusion and functional connectivity between the hippocampus and the PFC⁷⁸⁻⁸⁰. Acute administration of intranasal in randomized, placebo-controlled, cross-over designs has been previously used to determine regional responses to insulin in the resting-state or during tasks^{78,80}.

After screening and baseline assessment, participants will receive a crossover treatment assignment of either insulin or diluent followed by an MRI scan, with a washout period of 1 week. Therefore, participants will receive a total of 2 MRI scans throughout the course of the study- one following the first treatment, and one following the second treatment. The subsample will be enriched for the presence of self-reported anhedonia, using a score of greater than 2 on the SHPS as a cut-off, which provides the best discrimination between “normal” and “abnormal” level of hedonic tone⁸¹. This subsample will also have equal representation of normal weight and obese participants.

To proxy insulin activity within pre-selected brain regions, we will use a provocation paradigm, involving the administration of exogenous intranasal insulin, based on the published work of other research groups⁷⁸⁻⁸⁰. Activation of brain regions will be assessed during the resting state and task-based reward paradigm (i.e. EEfRT), following an intranasal administration of either 160 units of intranasal insulin or diluent (insulin and placebo will be prepared as nasal sprays), in a randomized, double-blinded, cross-over design. Please refer to the pharmacy manual for randomization details. A hypothesis-driven region of interest approach will be used to investigate initially the striatum and the medial PFC (mPFC). Willingness to make effort for rewards will be measured with the EEfRT³³. Briefly, participants have to select between an easy and a hard task. Both tasks involve pressing a key for a predetermined amount of time. The hard task offers more money (variable amounts), but is harder and takes twice as long. Participants make their selection knowingly under different win probabilities (i.e. they might not win money even if they are successful if the win probabilities are low). This task will measure proportions of hard-task choices with varying probabilities of reward and reward magnitudes. To provide stable estimations, the fMRI task will be conducted in three runs⁷³.

Table 1

Assessment	Baseline (V1)	First Spray (V2)	Second Spray (V3)
General Information			
Demographics Questionnaire	✓		
Medical Comorbidity Questionnaire	✓		
Family Psychiatric History Questionnaire	✓		
SES Questionnaire	✓		
MRI Questionnaire	✓	✓	✓
Anthropometric Measures			
Height (ft)	✓		
Weight (kg)	✓	✓	✓
Blood Pressure (mmHg)	✓	✓	✓
Heart Rate (bpm)	✓	✓	✓
Waist/hip Circumference (cm)	✓		
Physical Activity/ Energy Expenditure/ 7-Day Accelerometer Wear Time Log	✓	✓	✓
Laboratory Assessment			
Triglycerides	✓	✓	✓
Total Cholesterol	✓	✓	✓
Insulin	✓	✓	✓
Glucose	✓	✓	✓
HbA1c	✓	✓	✓
C-Peptide	✓	✓	✓
IGF-1	✓	✓	✓
TSH	✓	✓	✓
Free T4	✓	✓	✓
Free T3	✓	✓	✓
C-Reactive Protein	✓	✓	✓
hCG	✓		
Imaging			
fMRI-EEfRT		✓	✓
Dimensional Measures			
Clinician Administered			
YMRS	✓	✓	✓
MADRS	✓	✓	✓
Bandit Task	✓		
Self-Report			

SHPS	✓	✓	✓
PDQ-D	✓		
SDS	✓		
UCLA Life Stress Interview (Episodic)	✓		
CTQ	✓		
DeJong Gierveld Loneliness Scale	✓		
Diet History Questionnaire (III)	✓		
IPAQ	✓		
PSQI	✓		
Neurocognitive Tasks			
DSST	✓	✓	✓
RAVLT	✓	✓	✓
TMT A/B	✓	✓	✓

Imaging procedures will be started with a safety monitoring for glucose and cardiovascular vital signs. An insulin/placebo spray will be administered intranasally and, within 30 min, fMRI measurement will be performed. Venous blood samples, for determination of plasma glucose and insulin concentrations, will be obtained at the time of the insulin/placebo spray administration and immediately after the scan. The 2 scans for each individuals will be acquired on the same scanner. Subjects will be scanned using a 3.0-Tesla Signa HDx scanner with an 8-channel phased-array receiver coil (GE Healthcare, Milwaukee, Wisconsin) consisting of a structural and functional neuroimaging, comprising:

- i. Whole-brain 3-D T1-weighted Inversion-Recovery prepared Fast Spoiled Gradient-Echo (IR-FSPGR) anatomical scan with the following parameters: (TI/TR/TE = 450/8/3 ms, matrix size 256 × 256, field of view 22 × 22 cm, slice thickness = 1 mm, and flip angle = 15°)
- ii. Whole-brain, T2*-weighted BOLD echo planar imaging (EPI) during awake resting state with the following parameters: (TR/TE = 2000/30 ms, 3.5 x 3.5 x 3.5 mm voxel size, field of view 24x24 cm, 39 slices, slice thickness 3.5 mm, matrix size 64x64, number of frames = 405, flip angle = 70°),
- iii. Three runs of whole-brain, T2*-weighted BOLD EPI series during task-based reward paradigm with the same parameters as in ii.

4. Study Treatment

4.1 Treatments Administered

Description of Study Medications

The Investigational Pharmacy Services (IPS, also known as the clinical trials pharmacy) at the University Health Network's (UHN's) Toronto Western Hospital (TWH) will be responsible for all study related preparation, dispensing, and documentation. IPS will be responsible for the following study preparation procedures. All active treatment and placebo will be prepared by an IV certified pharmacy technician, under aseptic conditions, in a sterile room, i.e., centralized intravenous additive services (*CIVA*) area, and verified by a pharmacist.

Dose preparation for the active and placebo nasal sprays follow the same procedure. This will be conducted upon the receipt of an order from the blinded investigator with regard to the number of nasal spray bottles of Humulin® R insulin or diluent to be prepared the pharmacy.

Each product, whether intranasal Humulin® R insulin or diluent, will be prepared under the laminar flow hood one at a time. Using aseptic technique, the protective cap is removed from the vial of Humulin® R insulin or diluent (10 mL/vial, manufactured by Eli Lilly) and the rubber stopper is swabbed with an alcohol swab and allowed to dry. Using an appropriate sterile Luer Lock syringe with an appropriately sized needle attached with the bevel of the needle facing up at a 45 degree angle, the Humulin® R insulin or diluent vial is punctured and 5 mL of insulin or diluent is removed and transferred to a metered dose nasal spray bottle (PharmaSystems, Inc.) provided by the study coordinator overseeing the project herein. The nasal spray bottle is then capped accordingly. In summary, the intranasal solutions are prepared as follows:

- Intranasal Insulin Arm: IPS will obtain commercial supplies of Humulin® R insulin (10 mL/vial, manufactured by Eli Lilly) and aseptically transfer a 5 mL sample into a metered dose nasal spray bottle (PharmaSystems Inc.).

- Intranasal Placebo Arm: IPS will obtain commercial supplies of Humulin® R insulin's diluent (10 mL/vial, manufactured by Eli Lilly) and aseptically transfer a 5 mL sample into a metered dose nasal spray bottle (PharmaSystems Inc.).

This procedure is repeated until the required number of nasal spray bottles has been prepared. Once completed, the nasal spray bottles are wiped with 70% isopropyl alcohol soaked gauze and removed from the laminar flow hood and appropriately labeled.

Both active and placebo nasal spray bottles are kept separate from each other and a worksheet will be completed to document information, including date of preparation, batch #, expiry date, drug name and strength. The IV certified pharmacy technician who has prepared the dose is responsible for completing the documentation and will be affixing the labels identifying what was prepared. The labels, at a minimum, will contain information regarding the lot #, strength, date of manufacture, expiry date, and storage conditions. All of this will be verified by a pharmacist.

Labeling

All labels will contain product name, volume, protocol number, investigational drug warning, dosage instructions, storage conditions, lot number, expiration date, and the pharmacy name.

Storage

Please refer to the pharmacy manual for information on storage.

Study Medication Administration

This will be a single-dose, randomized, double-blinded, cross-over study involving intranasal insulin/placebo solution.

Designated pharmacy staff will dispense study medication, in a double-blind manner, according to the randomization code specific to each subject and dosing period.

Details regarding intranasal insulin and diluent can be referred to in the pharmacy manual.

The investigator and/or designee will instruct the study participant on how the intranasal insulin/placebo spray should be administered. Each study participant is to receive a single dose of Humulin R 160 U / placebo.

- Given:
 - The nasal spray bottles provide a deliverable volume of 0.1 mL/spray.
 - Humulin R insulin will be sourced as 100 units/mL in a vials of 10 mL from Eli Lilly.
 - Diluent will be sourced as 10 mL/vial from Eli Lilly.
- Each spray will offer:
 - 0.1 mL (i.e., 10 units) of Humulin R insulin
 - 0.1 mL of diluent (to serve as placebo)
- To obtain a dose Humulin R 160 U / placebo
 - 16 sprays (0.1 mL/spray) are to be given per dose

- This works out to 16 sprays split between each nostril such that each nostril receives 8 sprays.
- The sprays will be administered between alternating nostrils.

Given this is a crossover study, the study participants will receive a total of two doses in this study. The time between 2 doses is one week.

4.2 Accountability of Study Supplies

Please refer to the pharmacy manual for information on accountability of study supplies.

4.3 Disposition of Used/Unused Product

Please refer to the pharmacy manual for information on the disposition of used/unused product.

5. Methods of Assigning Subjects to Treatment Groups

Please refer to the pharmacy manual for information on the methods of assigning subjects to treatment groups (i.e. randomization, and blinding/masking).

6. Analytic Plan, Power Analysis and Approach to Potential Challenges

Initial data analyses will inspect socio-demographic and clinical group differences as well as correlations between clinical and biological variables and specified behavioral measures. Clinical or demographic variables demonstrating a correlation with both biological and behavioral measures, will be assessed as potential confounders⁸². The EEfRT behavioral data will be analyzed using generalized estimating equation (GEE) models, according to previous studies^{30,33}. For the assessment of associations between the remaining behavioral measurements and biometric data, we will use generalized linear models, due to its greater flexibility and ability to analyze data with non-normal distributions. Group (mood disorders vs. non-psychiatric) effects will be analyzed, with age, gender, and other relevant covariates included in the models. Special considerations will be given to smoking and use psychotropic medications, given its potential effects of both reward processes and insulin signaling.

Mediation and moderation models will be assessed using regression-based, bias-corrected bootstrap tests⁸³. The main objective is to test whether there is a mediated effect of X (i.e. mood disorders) on the outcome Y (i.e. anhedonia and reward behavior) through M (i.e. insulin signaling and dopamine metabolism). If appropriate, moderated mediation models (conditional process analysis) will be used to test whether the mediation effect was contingent on a moderating variable W (e.g. obesity, medications). This method uses bootstrapping to determine bias-corrected (asymmetric) 95% confidence intervals for the indirect effects⁸⁴. Bootstrapping methods have been recently advocated, as they are based on random sampling of the data and do not make assumptions about the shape of the sampling distribution; they are also more powerful and allow for the construction of more accurate confidence intervals, relative to normal theory methods⁸³.

For the imaging analysis, statistical parametric mapping (SPM8, Wellcome Department of Cognitive Neurology, London, U.K., www.fil.ion.ucl.ac.uk/spm) will be used to preprocess the raw fMRI data. A hypothesis-driven region of interest approach will be used to investigate initially the striatum and mPFC. For the resting-state data, BOLD signal in these regions will be compared between conditions (i.e. insulin and placebo) for each subject using GEE. For the task-based data, mean percent BOLD signal change in these regions under varying reward and probability conditions will be extracted, and we will compute a set of contrasts testing the main effects of reward magnitude, reward

probability, and the interaction for each subject⁷³. These first-level individual contrast maps will be fed into a second-level group (i.e. mood disorders vs. controls) and condition analysis, which will also use GEE. Association between behavioral measurements, biometric data and neuroimaging measurements will be assessed with generalized linear models. The false discovery rate (FDR) method will be used to correct for multiple comparisons and significance will be taken at a FDR-corrected threshold of $p < 0.05$ ⁸⁶.

Power Analysis: The primary hypothesis of this study is that anhedonic symptoms in individuals with mood disorders will be mediated by abnormalities in insulin signaling; therefore, the power analysis is based on a bias-corrected bootstrap test of mediation. Results from our preliminary data indicated that the path coefficients were mostly of medium effect sizes (e.g. $\beta \approx 0.25-0.35$); empirical analysis indicated that in this condition a sample sizes of 116 is necessary to achieve 80% power⁸⁵. To account for the possibility of subject attrition and/or inadequate measurements, we will set an initial enrollment goal of 150 subjects.

Potential Challenges and Ways to Overcome Them: The primary challenge in this study is that of recruiting and retaining 150 participants over a 3-year period. Given the experience and volume of the mood disorders clinics involved in this study, we believe that this goal is attainable. There are two potential limitations in our sample composition proposal. First, it is expected that the majority of participants recruited in tertiary mood disorders clinics will report clinically significant anhedonic symptoms. Nonetheless, it is possible that some of the subconstructs analyzed will not display satisfactory psychometric properties for contrast/comparisons. Second, we propose to pre-stratify our sample based on overweight/obesity. There is a well-documented association between adiposity and insulin sensitivity; though these are not perfectly collinear constructs. There are no, however, validated, publicly available, normative data of anhedonic symptoms, whereas gold-standard evaluations of insulin sensitivity are invasive and costly (e.g. hyperinsulinemic euglycemic clamp); therefore approximations are initially necessary. We propose to conduct a preliminary analyses after one third of the sample ($n=50$) is recruited and reevaluate the sample selection procedures.

A key challenge for the interpretation of results will be how to account for the role of potential confounding factors. These factors include, but are not limited to, psychotropic medications, smoking, socio-demographic characteristics, dietary and lifestyle factors. We opted for a “real-life” naturalistic treatment-seeking sample, as we seek to recruit a sample that is representative of other clinical samples. Moreover, ethical considerations preclude us from withdrawing or deferring initiation of medications. Therefore, we propose to systematically measure these confounding factors and use it as control and/or sub-stratification variables, as appropriate.

ADVERSE EVENTS

Definitions

Adverse event

An adverse event is the development of an undesirable medical condition or the deterioration of a pre-existing medical condition following or during exposure to a pharmaceutical product, whether or not considered causally related to the product. An undesirable medical condition can be symptoms (e.g., nausea, chest pain), signs (e.g., tachycardia, enlarged liver) or the abnormal results of an investigation (e.g., laboratory findings, electrocardiogram). In clinical studies, an AE can include an undesirable medical condition occurring at any time, including run-in or washout periods, even if no study treatment has been administered.

Serious adverse event

A serious adverse event is an AE occurring during any study phase (i.e., run-in, treatment, washout, follow-up), and at any dose of the investigational product, comparator or placebo, that fulfils one or more of the following criteria:

- results in death
- is immediately life-threatening
- requires in-patient hospitalization or prolongation of existing hospitalization
- results in persistent or significant disability or incapacity
- is a congenital abnormality or birth defect
- is an important medical event that may jeopardize the patient or may require medical intervention to prevent one of the outcomes listed above.

The causality of SAEs (i.e., their relationship to study treatment) will be assessed by the investigator(s). Note that SAEs that could be associated with any study procedure should also be reported. For such events the casual relationship is implied as "yes".

Recording of adverse events

AEs will be collected during each visit. At each visit, subjects will be asked if they have had any health problems since the previous visit. All AEs will be recorded appropriately, whether or not considered related to the investigational product. This will include AEs spontaneously reported by the patient and/or observed by the staff as well as AEs reported in response to a direct question e.g. "Have you had any health problems since your last visit?"

For each AE, the following parameters be described:

- start and stop date
- action taken with regards to investigational product
- outcome
- if the AE caused the patient to discontinue
- a statement if the AE fulfils the criteria for a SAE or not
- the investigator's assessment of the causal relationship between the event and the investigational product
- intensity of the AE
 - o mild (awareness of sign or symptom, but easily tolerated)
 - o moderate (discomfort sufficient to cause interference with normal activities)
 - o severe (incapacitating, with inability to perform normal activities)

It is important to distinguish between serious and severe AEs. Severity is a measure of intensity whereas seriousness is defined by the criteria in Section. An AE of severe intensity need not necessarily be considered serious. For example, nausea that persists for several hours may be considered severe nausea, but not an SAE. On the other hand, a stroke that results in only a limited degree of disability may be considered a mild stroke but would be an SAE.

Symptoms associated with overdose should be reported as AEs. For further information regarding overdose, see section.

Pregnancy in itself is not regarded as an AE unless there is a suspicion that an investigational product may have interfered with the effectiveness of a contraceptive medication.

Follow-up of adverse events should be based upon the clinical judgement of the investigator.

Reporting of serious adverse events

Reporting of SAEs to regulatory authorities will be done by the investigator in accordance with local regulations. A copy of the report will also be sent to the manufacturer of the investigational product.

7. Anticipated Timeline

Study start-up is planned immediately after REB approval. The last six months will focus on data management, image processing and analyses. This will allow 27 months of subject recruitment and study execution, with a target of an average of 2-3 participants per site per month.

8. Personnel and Facilities

Dr. Rodrigo B. Mansur (co-principal investigator [PI]) is an Assistant Professor of Psychiatry at the University of Toronto and a Clinician-Investigator at the Mood Disorders Psychopharmacology Unit (MDPU) at the University Health Network. His role on the project is to oversee all of the procedures and he will be involved in the design, implementation, and interpretation of all aspects of the study. Dr. Roger S. McIntyre is a Professor of Psychiatry and Pharmacology at the University of Toronto and is Head of MDPU at the University Health Network; he will facilitate the design and implementation of the study and provide clinical and statistical expertise. As clinicians, they will also be involved in subject recruitment and screening. Dr. Jian Chen is the Director of Proteomic Sciences, Indoc Research, and an Assistant Professor at the Department of Pathology and Molecular Medicine, Queen's University. He will be responsible for the proteomic/genomics analysis.

Other Personnel: We will employ part-time research coordinators who will oversee all endeavors related to startup and completion of the study.

Infrastructure and Facilities:

The study will be conducted at the University Health Network (UHN), which is among the largest Health Science Centers in Canada. The investigators will work with a research coordinator, clinicians, and scientists on this project. Investigators all have access to full-service clinical laboratories at UHN. Neuroimaging will be acquired at the Toronto Neuroimaging Facility at the University of Toronto, which has a 3T GE Signa HDx MRI scanners equipped with an 8-channel phased-array head coil.

9. Innovation, Impact and Future Research Plans

The proposed study is the first to integrate an innovative theoretical framework with unique methodological components, which are, nonetheless, extensively supported by the current literature. The study of obesity and insulin sensitivity in mood disorders has often been a secondary concern; this study is among the first to propose approaching metabolic factors as integral to mood disorders pathophysiology. Our proposed methodology includes multiple levels of assessment, using validated tools and techniques, and has the potential to provide a more comprehensive view of insulin's role. We are also proposing a systematic and detailed evaluation of environmental factors, which have a key role on the mood-metabolic disorders comorbidity.

We expect that results from this study will advance the knowledge on the mechanistic basis of anhedonia, which is a critical step for the development of targeted and more effective therapeutic options for individuals with MDD and BD. In addition, by highlighting the role of insulin signaling in the pathophysiology of mood disorders, we hope to refine the conceptual framework on the association between mood and metabolic disorders. Understanding better the impact of insulin signaling disturbances has tremendous potential to reframe how researchers and clinicians conceptualize mood disorders. Ultimately, the aim is to support the development of empirically supported, integrated therapies, such as medications and/or behavioral modifications (e.g. exercise programs) that target both mood and metabolic manifestations. These are urgently necessary to address adequately the multifaceted needs of affected individuals and families.

Future research plans consists of eventually establishing a longitudinal cohort, focused on the interface of metabolic and mood disorders. There is a clear need for longitudinal studies in this area; it is the only design that allows for the exploration of developmental trajectories and for definitive conclusions on causality. Moreover, our research team has ongoing collaborations with international groups, including Brazil, China, Denmark and South Korea, which have generated published articles and collaborative projects; we also intend to further develop these collaborations and explore opportunities for replication studies and cross-cultural comparisons. Finally, our research group has extensive experience with clinical trials, especially with metabolic/inflammatory treatments, such as the immunomodulatory infliximab and the antidiabetic medication liraglutide. We expect that this study will provide opportunities for translational studies, specifically by identifying novel pharmacological targets and/or subgroups of individuals (e.g. obese patients with disturbances in reward valuation) more likely to respond to novel therapies.

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