Neuroimaging of Tryptophan Challenge in People with Schizophrenia and Healthy Controls

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Overview of Research Design:
The study will be a double-blind, placebo-controlled, cross-over, tryptophan (TRYP) challenge study to examine the effects of increased kynurenic acid (KYNA) on neuropsychological test performance; patterns of fMRI activation and connectivity during the performance of a relational memory task; and ^{1}H-MRS measures of medial prefrontal cortex (mPFC) glutamate. The effects of increased KYNA on fMRI default network activity and peripheral markers of the kynurenine pathway, HPA axis, and inflammatory system will also be explored. Participants will include people with DSM-5/DSM-IV-TR schizophrenia or schizoaffective disorder (SRDs) and healthy controls. The healthy control group will be used to estimate the normal response parameters to the TRYP challenge. Participants will be randomly assigned to either TRYP (6 grams) or placebo. They will take the study medication 120 min prior to MRI scanning. TRYP administration will be used to increase peripheral kynurenine levels, which will lead to increased CNS production of KYNA.

Primary Aims:
1) To determine if TRYP-induced elevated KYNA levels impair performance on neuropsychological measures of attention, processing speed verbal and visual memory, and working memory.
2) To determine if TRYP-induced elevated KYNA levels alter neural circuit activation and connectivity between the dorsolateral prefrontal cortex (DLPFC) and hippocampus (HPC) during the performance of the Relational and Item-Specific Encoding (RISE; 90) task.
3) To determine if TRYP-induced elevated KYNA levels alter glutamate levels in the medial (mPFC), a brain region hypothesized to be important in the performance of cognitive tests.

Secondary Aims:
1) To examine if TRYP-induced elevated KYNA levels alter resting patterns of default network activation and connectivity.
2) To examine if baseline and/or post-challenge HPA axis and inflammatory indices moderate or mediate the response of behavioral or functional measures to TRYP-induced elevated KYNA levels.

Inclusion/Exclusion Criteria:
Inclusion Criteria (Participants with Schizophrenia)
1) Males and females between the ages of 18 and 55 years
2) Has met DSM-IV-TR criteria for schizophrenia, schizoaffective disorder or schizophreniform disorder (documented by SCID)
3) Prescription of antipsychotic medication for at least 60 days and constant dose for 30 days prior to study entry (either first or second generation antipsychotics permitted)
4) Female participants must agree to use a medically accepted means of contraception

5) Women must be in the first half of their menstrual cycle at the time of the 2 challenge visits.

Inclusion Criteria (Healthy Controls)

1) Males and females between the ages of 18 and 55 years
2) No DSM-IV-TR Axis I Disorder (documented by SCID)
3) Female participants must agree to use a medically accepted means of contraception
4) Women must be in the first half of their menstrual cycle at the time of the 2 challenge visits.

Exclusion Criteria (all participants)

1) DSM-IV-TR substance abuse in the last month or substance dependence in the last 6 months (documented by SCID)
2) Calgary Depression Scale total score ≥ 10 at baseline
3) Current smoker (expired CO ≥ 10 ppm)
4) Current use of nicotine replacement therapy or other nicotine products
5) Pregnancy or breast feeding
6) Excessive self-reported daily caffeine intake, defined as intake exceeding 1000 mg or the equivalent of 8 cups of coffee
7) Active disorders that have been reported to affect tryptophan metabolism or interfere with absorption will be excluded (Acute Intermittent Porphyria, Celiac Disease, Crohn's Disease, Irritable Bowel Syndrome
8) History of an organic brain disorder; mental retardation; or a medical condition, whose pathology or treatment could alter cognition
9) Claustrophobia
10) Metal in body that will interfere with MR imaging
11) Treatment with monoamine oxidase inhibitors, migraine headache medications (triptans) and dextromethorphan.

Recruitment:

Healthy Controls:

Participants will be recruited from the Maryland Psychiatric Research Center (MPRC) Healthy Control Pool (HP-00042350), MPRC screening protocol (HP-xxxxx) or by advertisements (approved by IRB prior to using). All persons who qualify for this study are referred to the recruiter (PI and/or PI staff) and then contacted and asked if they would like to participate in this study. Interested persons undergo a brief telephone screening followed by an in-person interview. After the initial telephone screen, all potential healthy controls undergo a SCID interview. We will use random digit dialing as our primary source for identifying healthy controls. For random digit dialing, we will utilize contact information lists purchased from Survey Sampling International (SSI), a leading provider of sampling, data collection and data analytic solutions for survey-based research. SSI compiles contact information lists based on census and market-based research data.

Participants with SRDs:

Potential participants with SRDs will be recruited from three sources: the Maryland Psychiatric Research Center (MPRC) clinical programs: the Outpatient Research Program (ORP), the Treatment Research Program (TRP), and the First Episode Clinic (FEC); local outpatient mental health centers; and a standing recruitment database, from which we have enrolled participants in previous MPRC clinical trials.

Potential MPRC and community participants will be identified primarily through chart review or nomination by their primary clinicians who are aware of study entry criteria and demands, and have been asked to identify clinically stable patients who may be interested in research

participation. MPRC and community participants may also be self- or peer-referred for this study.

Healthy controls will be matched to participants with SRDs by age, gender and parental SES level. The planned total enrollment is 50 participants with SRDs and 50 healthy controls, which, under the assumption of 10% attrition, will result in 45 completers in each group. We will enroll 18 participants per year. In each year, we will plan to recruit an equal number of participants with SRDs and healthy controls to avoid potential cohort effects.

Assessments: All of the following assessments will be conducted blind to treatment assignment.

Clinical Assessments: The following rating scales will be used: Brief Psychiatric Rating Scale (BPRS; 98); Scale for the Assessment of Negative Symptoms (SANS; 99); Calgary Depression Scale (CDS; 95); and Clinical Global Impression Scale (CGI; 100).
BPRS: the BPRS total score will be used to measure global psychopathology. The four BPRS positive symptom items - conceptual disorganization, suspiciousness, hallucinatory behavior, and unusual thought content - will be used to measure positive psychotic symptoms.
SANS: the SANS total score, minus the global items, inappropriate affect, poverty of content of speech, and attention items, will be used to measure negative symptoms.
CDS: the CDS total score will be used to measure depressive symptoms. The CDS was specifically designed to assess depressive symptoms in people with schizophrenia.
CGI: the CGI severity of illness item will be used to assess global changes.

Cognitive Assessments: The following assessments, drawn from the MATRICS battery (101), will be used: Brief Assessment of Cognition in Schizophrenia (BACS): Symbol-Coding, Hopkins Verbal Learning Test–Revised (HVLT-R), Brief Visuospatial Memory Test–Revised (BVMT-R), Continuous Performance Test-Identical Pairs (CPT-IP), Maryland Letter-Number Sequencing (LNS), and WMS®-III Spatial Span. These tests were selected, because they assess cognitive processes hypothesized to be under the regulation of α-7 nicotinic or NMDA receptors (34-38).
BACS Symbol-Coding: is a timed paper-and-pencil test in which the participant uses a key to write digits that correspond to nonsense symbols; the total score will be used to asses processing speed.
HVLT-R: is an orally administered test in which a list of 12 words from three taxonomic categories is presented and the participant is asked to recall as many as possible after each of three learning trials; the total score will be used to assess verbal learning.
BVMT: is a test that involves reproducing six geometric figures from memory; the total score will be used to assess visual learning.
CPT-IP: is a computer-administered measure of sustained attention in which the respondent presses a response button to consecutive matching numbers; the d-prime score will be used to assess attention/vigilance.
LNS: is an orally administered test in which the participant mentally reorders strings of number and letters and repeats them to administrator; the total score will be used to assess verbal working memory.
WMS®-III Spatial Span: uses a board on which 10 cubes are irregularly spaced, and the respondent taps cubes in the same (or reverse) sequence as the test administrator; the total score will be used to assess nonverbal working memory.

Laboratory Assessments: The following laboratory assessments will be collected:

Kynurenine, KYNA, and 3-HK: After thawing, serum (100 µl) will be acidified with 50 µl of 6% perchloric acid and centrifuged in a microfuge (10 min). The kynurenine pathway metabolites will be determined in the supernatant (20 µl) using high performance liquid chromatography (HPLC) methods.
Cytokine Profile: The immune panel consists of 12 cytokines: interferon-γ, IL-1β, IL-1RA, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, CXCL8, GMCSF, and TNF-α. These cytokines are part of the Type 1 and Type 2 immune responses, have been previously implicated in schizophrenia (102-105), and have been linked to changes in KYNA levels (106-109). The cytokine samples will be collected in tubes containing EDTA, immediately cooled on and centrifuged under refrigeration and stored at -80$_{o}$C until analyzed. The cytokines will be analyzed by the University of Maryland Cytokine Core Laboratory using either Multiplex Luminex technology or ELISA.
Cortisol and ACTH: The neuroendocrine blood samples will be collected in EDTA-containing tubes, immediately placed on ice, centrifuged under refrigeration and stored at -80$_{o}$C until analyzed by LabCorp®. To reduce the possible effect of stress on cortisol levels, participants will remain in the same room in a calm environment. To control for menstrual effects, challenge studies for females will occur in the first half of the menstrual cycle. If women are oligomenorrheic, they will be enrolled between 1-14 days of their last menstrual period, and amenorrheic women will be enrolled without regards to hormonal status.

Medical and Safety Assessments: In the 2-week Evaluation Phase, participants will receive a complete medical history, including the use of concomitant medications and the use of caffeine, alcohol, and illicit substances; and physical examination, including blood pressure, heart and respiratory rates, height and weight. We will also baseline EKG; CBC; complete metabolic panel, including electrolytes, BUN/Creatinine, LFTs, lipid panel, and TSH); U/A; expired CO, and Toxicology screen assessments. Female participants will have a pregnancy test during the Evaluation Phase and on each challenge test day. Female participants will also be asked to agree to the use of medically accepted means of birth control. Participants will given a breathalyzer test on the morning of each challenge visit. Menstrual cycle will be assessed by patient report of the last day of the previous menstrual cycle. A calendar will be shown for participants.

The Side Effect Checklist (SEC) is designed to assess medication side effects commonly associated with pharmacological treatments. In addition, if not already assessed, the SEC is modified for the purpose of the assessment of specific side effects associated with the particular medication under study; in this case TRYP. The SEC version to be used in the proposed study rates 32 potential side effects, and comprehensively covers the side effects that have been previously reported with TRYP administration, i.e., nausea, vomiting, diarrhea, abdominal pain, and sedation. In addition, there are three “other” spaces for idiosyncratic participant complaints, which are not usually associated with medication treatment. We will use the self-rated Standford Sleepiness Scale (SSS) to supplement our assessment of sedation (110). The SSS is a 7-point Likert-type scale.

Assessment Schedule: The clinical assessments will be administered prior to administration of the study medication, immediately prior to the MRI scan, upon completion of the MRI scan, and at the follow-up safety assessment, which will be conducted 75 min after the completion of the MRI scanning. The cognitive assessments will be conducted prior to the administration of the study medication and after the MRI scanning is completed and the participant has had lunch. The laboratory assessments will be collected at pre-TRYP challenge baseline, and 30 min, 60 min and 90 min post ingestion. The final laboratory assessment will be collected at the time of the second administration of the cognitive battery. The safety assessments will be administered

prior to administration of the study medication, every 30 min after medication administration, upon completion of the MRI scan, and at the follow-up safety assessment.

Table 1. Assessment Schedule.

	Study Lead In (up to 2 weeks)	**Challenge Visit: Estimated Time of Assessment**	**Challenge Visit: Actual Time of Assessment**	**Initials**
Informed Consent, ESC	X			
SCID and diagnostic interview, MRI screening	X			
Medical Screening Medical Hx, physical exam, blood draw, urinalysis, toxicology screen, pregnancy test (females), EKG, medication use screening, caffeine use screening, Birth Control Selection (females)	X			
Clinical Stability Determination (schizophrenia group only seen on two visits, healthy controls one visit) 2 unchanged CGIs	X			
Mock scanning session	X			
Scheduling of 2 challenge visits and NPO order for 12 hours in advance of admission on challenge study days	X			
Participant Admission/BSU Arrival Review of Study Procedures and retrospective recall of food intake (past 12 hours). Collect personal items not safe during MRI testing.		7:30 am		
Urine pregnancy (females), expired CO, urine toxicology and blood alcohol testing, MRI screening		7:45 am		
Insertion of indwelling IV line, fluids, period of relaxation		8:00 am		
Laboratory Assessments (1): cortisol, ACTH, kynurenine, KYNA, 3-HK, cytokines		8:15 am		
Cognitive Measures (1): HVLT-R, BACS Symbol Coding, BVMT, CPT-IP, WMS®-		8:30 am		

III, LNS				
Vital signs, SEC, SSS Clinical Assessments (1): BPRS, SANS, CDS, CGI		9:15 am		
Oral Administration of Medication (Tryptophan 6 gram/ Placebo)		10:00 am		
Protein Free Snack		10:15		
Vital signs, SEC, SSS Laboratory Assessments (2)		10:30		
Vital signs, SEC, SSS Laboratory Assessments (3)		11:00 am		
Clinical Assessments (2; modified version)		11:15 am		
Vital signs, SEC, SSS Laboratory Assessments (4)		11:30 am		
Arrive at Imaging Center		11:45 am		
Neuroimaging Studies		12:00 pm		
Arrive Back at BSU Vitals, SEC, SSS		1:40 pm		
Lunch		1:45 pm		
Cognitive Measures (2) Laboratory Assessments (5)		2:00 pm		
Clinical Assessments (3; modified version) Vital signs, SEC, SSS		2:30 pm		
Participant discharged. If participant has not returned to pre-		2:45 pm		

tryptophan challenge baseline, they will be reassessed every 30 minutes until ready				

Neuroimaging Procedures: All MRI data will be acquired using a 3-T Siemens Trio scanner equipped with a 32 channel head coil at the University of Maryland Center for Brain Imaging Research (CBIR), located at the Maryland Psychiatric Research Center. A T1-weighted structural image (MP-RAGE: 1 mm isotropic voxels, 256 X 256 mm FOV, TR/TE/TI = 1900/3.45/900ms) will be acquired for spectroscopic voxel prescription and anatomical reference.

fMRI Studies: the fMRI activation study will use the Relational and Item Specific Encoding (RISE) task (89,90) to assess brain activation and connectivity patterns underlying item and relational memory. The task, which was developed by the NIMH-funded CNTRACS consortium, is comprised of 3 runs: 1) item and relational encoding; 2) item recognition; and 3) relational recognition. The encode run will comprise presentation of 54 visual object pairs. Pairs will be presented for 4 sec with a variable inter-trial interval of 1-10 sec (4 sec average). Item and relational encoding conditions will be administered in blocks, within a run of 9 trials each. For item encoding, participants will respond via button press yes or no as to whether both objects presented are “living”. During the relational encoding, participants will respond yes or no if one of the objects could fit inside the other. For item recognition participants will view one object from the pairs presented during encoding along with foils. Objects will be presented for 3 seconds with a variable inter-trial interval of 0-10 sec (3 sec average). All objects (108 objects) presented during encoding will be intermixed with 54 foils. Participants will rate their confidence using a 6-point scale as to whether the object is “old” (i.e., previously presented) or “new” (i.e., not previously presented). For relational recognition, participants will view 27 intact pairs intermixed with 27 re-arranged pairs taken from the encoding task. Objects will each be presented for 3 seconds, with a variable inter-trial interval of 0-10 seconds (3 seconds average). Participants will respond “yes” if the pairs were intact from the encoding and “no” if the pair was re-arranged. For resting state fMRI, participants will be given a simple instruction to rest but hold still and keep their eyes open for 15 minutes (allowing for blinks).

MRS Study: Glutamate (Glu) and glutamine (Gln; the major metabolite of synaptic glutamate), will be detected in a 4x3x2 cm mPFC voxel using very short TE phase rotation stimulated echo acquisition mode (STEAM: TR/TM/TE= 2000/10/6.5 ms, NEX=256, 2500-Hz spectral width, 2048 complex points, and $\Delta\varphi1 = 135°$, $\Delta\varphi2 = 22.5°$, $\Delta\varphi3 = 112.5°$, and $\Delta\varphi ADC = 0°$) (111). We chose the mPFC voxel placement, because previous studies have demonstrated: a) the effect of increased KYNA on glutamate in this region (23); and b) the importance of this region in the performance of cognitive tests (112). Water suppression will be automated using a water suppression enhanced through T1 effects sequence (WET). For measurement of T2*-weighted BOLD effects in whole-brain functional images, we will use echo-planar image (EPI) pulse sequence (TR=2000 ms, TE = 27 ms, flip angle = 80, slice thickness = 4.0 mm, interleaved, and FOV = 220 x 220).

Neuroimaging Processing and Analyses: fMRI analyses: These analyses will be carried out in AFNI, MATLAB, and SPM8. Image preprocessing will include motion correction by coregistration to the first image in each series, correction for differences in slice acquisition times, normalization to a standard Montreal Neurological Institute template, and smoothing by convolution with a Gaussian kernel function (FWHM = 6 mm). Resting state will be low-pass filtered (f cutoff = 0.1 Hz). Task: For the task statistical analysis, for each participant, each of the conditions (item and relational encoding; item and relational recognition) will be modeled as a time series of discrete events that is convolved with a function that accounts for the

hemodynamic delay. The condition-specific responses will be assessed by performing regression analyses against these functions. Within condition responses, which reflect a condition minus baseline contrast, will be assessed for each of the conditions. Between-condition differences will be assessed by contrasting the task-specific responses with each other. A fixed effects analysis will be performed by entering whole run sequences for all the participants into a single analysis and regressing each sequence against all conditions. Random effects analysis will be used to assess differences between group fMRI data. For this analysis, the contrasts from the first level analysis for each individual will be used to represent mean response patterns for that individual, and between-condition or between-group analyses will be performed. Due to hypothesis regarding anterior cingulated/medial frontal, dorsolateral frontal and hippocampal involvement, between-group differences for BOLD activation within these regions will be examined with an ROI mask approach. In addition, the BOLD signal change will be extracted from these ROIs for each group to examine the hemodynamic time course. In order to obtain the functional connectivity characteristics, we will first manually draw the ROIs for each individual based on anatomic landmarks. Manually traced ROIs nicely overcome the issue of group structural differences. We will use these ROIs to extract the eigenvariates of time series in each participant. The second step will be to elucidate the functional connectivity of each ROIs with the whole brain by a seed-voxel analysis (extracting the weighted mean time series and then entered as covariates in a whole-brain regression analysis). The individual connectivity images created will be taken to a second-level analysis to obtain the connectivity characteristics of these 3 regions at the group level. The next step will be to determine if our a priori ROIs are correlated to each other by calculating the correlations between eigenvariates of each ROI. DMN: For resting state fMRI analysis we will use white matter time course as a regressor, assuming it will contain similar spurious fluctuations as in the gray matter. We will use SPM segmentation module to segment the whole brain white matter. The time course of white matter will be averaged. We will regress out the six head motion parameters and the average white matter time course before cross correlation analysis. Cross correlation maps are then converted to z score maps using an AFNI built-in function. Correlation analyses will be performed by calculating the correlation coefficient between each voxel's time course and the averaging time courses of all the voxels in the defined regions of interest. We will use ROI seed based analyses for all hypothesis testing. Manually traced ROIs overcome the issue of group structural differences. Independent component analysis (ICA), small-world network, SEM, and Granger causality methods can be used to analyze resting state fMRI data. These methods may extract additional circuit information. However, there are still challenges in these methods (113). We will use ICA primarily for extracting the default mode network component for additional exploratory analyses. The Granger causality and small-world brain network methods require some a priori definition of brain regions and the resultant topologic properties of brain networks are highly dependent on the number of brain regions, which remains to be fully evaluated for multiple group x drug comparisons (114,115). A seed-based method is preferred here for testing a priori hypotheses that address specific brain regions. It also takes advantage of this method's higher sensitivity and ease of interpretation (116). 4.4.3.2 MRS: Spectra will be analyzed using the fully automated, standard curve-fitting software, LCModel. Metabolites will be referenced to water and only metabolites of good fits (CRBs< 20) will be included. Voxels will be segmented into CSF, gray, and white matter tissue using SPM8 and in house Matlab code; metabolites will be corrected according to Gasparovic et al. (117). This method produces excellent reliability for glutamate and glutamine as determined by a test-retest reproducibility study in 10 healthy controls scanned twice separated by about 1 week. All spectra were of excellent quality (the average FWHM was 0.021 ppm and the average SNR was 44). Spectral quantification yielded excellent fits as illustrated with Cramer–Rao lower bounds (CRLB) less than 10 for Glu and Glu+Gln and 23 for Gln and test-retest coefficients of variation (CVs) of 9.3 for Gln and 4.2 for

Glu, absolute percent differences of 13.8 for Gln and 3.8 for Glu, and intra-class correlations (ICCs) of 0.82 for Gln and 0.89 for Glu.

Study Design:
Evaluation Phase: There will be an approximate 2-week Evaluation Phase, during which potential participants will undergo baseline assessments to determine whether they meet inclusion criteria. Participants will be clinically stable, with clinical stability defined as two consecutive CGI ratings with no change in score.

Double-Blind Challenge Phase: The Double-Blind Challenge Phase will utilize a placebo-controlled, cross-over design. Participants who continue to fulfill entry criteria will be entered into the Double-blind Challenge Phase. There will be two Challenge Phase visits. On the day of each visit, the participants will arrive at the Brief Stay Unit (BSU) at 7:30AM; all participants will have fasted since midnight. At the BSU, an indwelling I.V. will be inserted for the repeated laboratory assessments and the initial clinical, cognitive, laboratory, and safety assessments (including urine pregnancy test for female participants) will be completed. Upon completion of the initial assessments, participants will receive the challenge medication. Participants will be randomly assigned to receive TRYP (6 grams) or placebo. They will receive the other study medication on the second visit (i.e., placebo or TRYP). The 6 gram dose was chosen based on previous work showing cognitive effects to be associated with doses above 5 grams (118) and our pilot work, in which we have demonstrated that, in ten healthy controls TRYP (6 grams) produces a robust increase in kynurenine. TRYP and placebo will be administered in oral slurry. A non-blind pharmacist will dispense all study medications. The blind will be broken only if a medical emergency requires this information. If this occurs, the participant will be withdrawn from the study. Two hours after ingestion of TRYP or placebo, participants will be transferred to the CBIR, which is in a building adjacent to the BSU, where they will undergo the following MRI assessments: 1) spectroscopy for the assessment of glutamate, 2) fMRI resting scan; and 3) fMRI during relational and item memory tasks. We chose to begin scanning 120 min after study medication ingestion, because our preliminary data suggests that serum kynurenine initially becomes elevated 30 min after TRYP ingestion, but continues to increase over the next three hours; we wanted to ensure sufficient time for kynurenine to cross the BBB and be converted to KYNA. Kynurenine levels will remain elevated for at least 3.5 hours, which is sufficient time to complete the MRI assessments and post-imaging cognitive assessments. The cognitive assessments will be conducted prior to the administration of the study medication and after the MRI scanning is completed. The post-baseline clinical, laboratory, and safety assessments will be conducted as described above.

Tryptophan Acquisition and Study Dosing: TRYP is a dietary supplement, whose use is regulated under the FDA Dietary Supplement Health and Education Act of 1994 (DSHEA). We will obtain TRYP powder from Ajinomoto, North American Inc and we have approval under an IND for use in this population. On the morning of the challenge studies, TRYP or placebo (lactose monohydrate) powder will be diluted in water (250 mL) into a slurry immediately prior to oral intake (119). The participant will be instructed to drink the full 250 mL over a one minute time period. The unblinded pharmacist, not participating in assessment procedures, will prepare and dispense the study medications.

Randomization and Blinding:
Treatment assignment (placebo and TRYP) will be randomly assigned, with each participant receiving each condition on separate occasions. Participants will be randomly assigned using a permuted block randomization system with separate randomization sequences for healthy

controls and schizophrenia patients. Treatment assignment order is random within each block, and an equal number of subjects are assigned to each treatment within a block. The block sizes will vary in random sequence and randomization will be matched by age and gender. Thus, it will be difficult to ascertain the next treatment assignment, even if a subject becomes unblinded, while the imbalance of numbers between the treatment groups is kept within tight limits. Randomization requests are sent to the study statistician, who sends a coded treatment assignment to an unblinded pharmacist. Separate emergency unblinding envelopes for each subject will be kept in a locked cabinet at each dispensing pharmacy. All raters, investigators and other staff will be blind to treatment assignment except for the pharmacist. The pharmacist does not participate in study assessments and conveys no information about treatment assignment to subjects or staff except in a medical emergency.

Payment:
Participants will be paid $15 per hour for each visit (approximately 3 hours for Evaluation and approximately 7 hours for each Challenge visit) as well as $100 for completion of each Challenge visit. The total payment for the study will be approximately $455.

Statistical Analysis: Initially, we will examine responses to TRYP or placebo challenge separately in healthy controls and participants with schizophrenia-related disorders (SRD), anticipating that different basal states of KYNA metabolism may result in distinct responses to TRYP challenge in the two groups. Secondary analyses will compare responses to TRYP challenge in the two groups.

Primary Aims:

1): To determine if TRYP-induced elevated KYNA levels impair performance on neuropsychological measures of attention, processing speed verbal and visual memory, and working memory.

Analysis plan: To facilitate comparison of the magnitude of effects across the six cognitive tests from the MCCB, we will utilize the MCCB T-scores for each test. For each cognitive measure, we will fit a mixed model for repeated measures ANCOVA: post-study drug score on i-th day = pre-study drug score on i-th day +treatment on i-th day + day + treatment x day, where day (=1,2) is an indicator of whether the scores were collected on the first or second day of testing. In this model, the treatment effect estimates the average TRYP - placebo difference across the two possible orders of testing, and the treatment x day effect estimates how much this difference might depend on whether TRYP or placebo was administered first. Learning effects from repeated cognitive testing could produce diminishing increases across the four repeated administrations of each test, resulting in larger pre-post differences in scores on the first versus second day of testing; these learning effects could be different following dosing with TRYP versus placebo, masking the existence of treatment x day interactions. Since the MCCB tests were chosen to have small learning effects ($d<0.1$; 101), we anticipate these learning effects and possible interactions with treatment will be small. To take account of multiple testing with six cognitive tests, we will use the Benjamini-Hochberg False Discovery Rate (FDR; 120) modification of the Bonferroni method: we will compare the six p-values from largest to smallest to successively stricter critical values $c_j = 0.05 \times (6-j+1)/6$; if the j-th p-value is less than c_j, we will reject that null hypothesis and all null hypotheses corresponding to smaller p-values. To compare the magnitude of T-score differences between TRYP and placebo among cognitive tests, we will add terms for cognitive test and test x treatment interactions to the model described above.

2): To determine if TRYP-induced elevated KYNA levels alter neural circuit activation and connectivity between the dorsolateral prefrontal cortex (DLPFC) and hippocampus (HPC) during the performance of the Relational and Item-Specific Encoding (RISE; 90) task.

Analysis Plan: In healthy controls and individuals with SRD, we will compare intensity of BOLD signal activation in DLPFC and hippocampus, using the mixed model for repeated measures ANOVA: BOLD activation = treatment + ROI + task condition (item encoding, relational encode, item/relational recall) + treatment x task condition + treatment x ROI + treatment x ROI x task condition + order of treatments + interactions with order, with analyses performed separately in each group. A similar model will be used to examine functional connectivity or correlations between fluctuations in BOLD signal activation between DLPFC and HPC in each task condition, using similar methods previously used to test nicotine challenge effect on resting state functional connectivity (121). To assist in meeting normality assumptions, Fisher's z transformation will be applied to correlation coefficients measuring task-related connectivity prior to modeling**.**

3): To determine if TRYP-induced elevated KYNA levels alter glutamate levels in the medial (mPFC), a brain region hypothesized to be important in the performance of cognitive tests.

Analysis plan: We will estimate the effect of TRYP or placebo on glutamate in healthy controls or SRD separately and compare whether it is different in these two groups by fitting the mixed model for repeated measures ANOVA: glutamate in mPFC = group + treatment + order + treatment x group + interactions with order, where group indicates healthy controls or individuals with SRD, treatment indicates receiving TRYP or placebo on a given day, and order indicates whether the active treatment is given on the first or second session. In the event of significant interactions between order and treatment, we will examine treatment effects for each order separately; otherwise, order effects will be dropped from the model.

Secondary Aims:

1): To examine if TRYP-induced elevated KYNA levels alter resting patterns of default network activation and connectivity.

Analysis Plan: For exploratory analysis of changes in neural activation and functional connectivity in the default mode network (i.e., medial temporal lobe, mPFC, posterior cingulate cortex, ventral precuneus and medial, lateral and inferior parietal cortex) in healthy controls and participants with SRDs given TRYP or placebo, we will use models similar to those outlined for Specific Aim 2.

2): To examine if baseline and/or post-challenge HPA axis and inflammatory indices moderate or mediate the response of behavioral or functional measures to TRYP-induced elevated KYNA levels.

Analysis Plan: We will conduct exploratory analyses at unadjusted two-sided alpha = 0.05. People with SRD have been reported to have higher brain KYNA levels than healthy controls, and have major differences in behavioral and functional measures. Accordingly, we will analyze mediating and moderating effects of KYNA separately in the two groups, following the approach of Kraemer et al (118). To test moderation, we will examine the repeated measures models: change (Post-TRYP - Pre-TRYP) in behavioral outcome = treatment + pre-TRYP KYNA on test day + treatment x pre-TRYP KYNA, where the treatment x pre-TRYP KYNA on test day measures the change of slope for pre-treatment KYNA in predicting change in behavioral outcome. If this difference in slopes = 0, there is no mediator effect for pre-TRYP KYNA. Similarly, to assess mediation, we will fit a similar model to assess the difference between treatments (TRYP versus placebo) in slopes predicting change in the behavioral response from change in KYNA to whether change in KYNA mediates the treatment effect, noting that this is not the same as assessing causality (123).

Risks to Human Subjects

a. Human Subject Involvement, Characteristics and Design. The proposed project meets the definition of a "Clinical Trial." This challenge study will enroll both healthy controls and people with schizophrenia. Following a period of baseline evaluation and assessment, participants (healthy controls and schizophrenia patients) will randomly participate in two visits. On one of the two visits, the participant will receive L-tryptophan (TRYP), and on the other visit the participant will receive placebo as an oral slurry. At each of the two visits, all participants will undergo morning baseline assessments followed by medication dosing, assessments and imaging procedures.

Potential Risks to Human Subjects:

This study involves a medication challenge to healthy controls and to people with schizophrenia. We have pilot data on the use of TRYP (6 grams) given to healthy controls (n=10; see accompanying memo) and have had few side effects reported. On beginning, we will carefully monitor for side effects and study risks.

The risks associated with MRI are extremely low. They include the possibility that undetected metal in the participant's body could be displaced by the magnetic field of the MRI scanner. Participants could become claustrophobic while in the scanner. Participants could experience discomfort in attempting to remain still for up to 90 min in the scanner. The tasks could cause boredom or anxiety in some participants. Credit cards, watches or other items could be damaged by the magnetic field if they are inadvertently brought into the scan room. Headphones and earplugs are provided to the participants to protect from scanner gradient noise. There will be no cost to the participant or the participant's insurance company for the MRI scans performed specifically for this study.

There have not been any serious consequences associated with TRYP administration. TRYP is sold over the counter (FDA Dietary Supplement Health and Education Act of 1994 (DSHEA)) and has been available for over 50 years. TRYP is mostly acquired through dietary consumption, but increasing tryptophan is possible with using pure TRYP as in this study which is intended to produce kynurenine. TRYP loading has been widely used in clinical research. A recent review covers 43 studies that assessed TRYP loading. The dosing in these studies varied, with many studies using aggressive loading doses (>10 g/day) (126-130), while others have used dosing of 5-10 grams (131-136). We propose to use a dose of 6 grams. This literature review does not demonstrate any significant side effects other than some minor transient effects on cognition, mood and sleep. On the contrary, there have also been improvements observed in memory and attention, and worsening of memory, attention, executive dysfunction and psychomotor speed generally occurred only at very high concentrations of TRYP (118). Similar is true to mood; improvements have been seen in anger, depression, vigor and alertness but high concentrations of TRYP have shown minor worsening in these domains in a few studies (118). Abnormal sleep behaviors have been improved in many studies; however, sleepiness is a reported side effect (118). Occasional side effects have been reported at higher TRYP doses (generally >6 grams daily), which include tremor, nausea, and dizziness (137). Mild dyspepsia and diaphoresis have occurred in people ingesting TRYP. The dose used in this study is lower than that used in most published studies. By giving the water and TRYP mixture slowly we will mitigate dyspeptic reactions. A very recent study (138) reported on the side effects of up to 5 gram/day of TRYP in healthy volunteers and found no changes in laboratory measures, food intake, body weight or mood side effects. In schizophrenia, we anticipate only mild, transient effects on cognition. In 1988 and 1989, there was an outbreak of eosinophilia-myalgia syndrome (EMS) that was traced to a single

manufacturer in Canada, leading to a ban in the US for 12 years; however, this is no longer considered a risk, and the ban was lifted in the US in 2001 (139).

One potential serious side effect is the serotonin syndrome, which could occur when TRYP is administered in combination with other serotonergic agents. The occurrence of the serotonin syndrome with TRYP is rare and generally only occurs after routine use of TRYP. The serotonin syndrome includes the following symptoms: delirium, myoclonus, hyperthermia and coma. We will include these items in the SEC as potential side effects to ensure our ability to detect their occurrence.

In summary, side effects with TRYP are mild, even in studies that have used higher doses for longer periods of time than will be done in the current study (118).

Adequacy of Protection Against Risks

Informed consent:

Schizophrenia-Related Disorders: A partial HIPPA waiver will be obtained to permit the identification of potential participants through chart review. In addition, participants will be referred by the person's treatment team for consideration of study participation. No potential SRD participant will be approached for recruitment without approval of a primary clinician, who will determine suitability of the person for the protocol. A chart review will be completed for all potential SRD participants to reduce the likelihood that they will be found ineligible after participating in more extensive assessment. The study recruiter will verify with the primary clinician that a potential participant is sufficiently stabile to consider participation and has capacity to provide consent. This is done prior to the study recruiter approaching a potential participant. The study recruiter will be introduced to the person and provide a brief overview of the project. Research staff members are trained to recognize symptoms of severe mental illness and cognitive impairment that could undermine an individual's ability to provide informed consent.

All Participants: Interested people will be provided study information and an informed consent form that contains all pertinent details of participation and includes the following: a brief explanation of the purpose of the research and a brief explanation of the requirements of the participant, including: a) willingness to be randomly assigned to either intervention, b) completing a series of interviews about symptoms, c) completing assessment tasks, and d) being available for follow-up assessments. The consent form will include an explanation of the risks and benefits of participation; assurances of confidentiality; and an explanation that participation is entirely voluntary, the decision to participate will in no way influence or restrict services at participating sites, and the participant is free to withdraw at any time with no negative consequences. Potential participants will be informed that the drug used in this study is not known to be effective for any indication under investigation. As some potential participants will have poor reading skills, the consent form will be read aloud to all participants in tandem with their own silent reading of the document. The individual securing consent will review any points about which the participant is unclear, and the participant will be invited to ask questions as needed.

Our research staff is carefully trained in strategies for interacting with people with severe mental illness, including speaking slowly and clearly, stopping to summarize frequently, and providing time for questions. They are all supervised by senior staff members. All participants who express willingness to provide consent will be queried about the consent form in order to ensure that they have adequate understanding of the study requirements. This questioning is performed systematically, and research staff members document that this review has been completed. After reading the consent, and before obtaining a signature, a brief questionnaire is

administered to verify that the participant is competent to provide consent and has demonstrated comprehension of the consent document. This questionnaire is attached to the informed consent form and is completed immediately after explaining the informed consent form and before obtaining the participant's signature on the form. If the participant does not understand the consent form, the recruiter will try to explain points of confusion, and administer the questionnaire again. Those failing to answer the questions adequately will not be recruited into the study. The recruiter will also make a clinical judgment and not recruit participants who appear unable to grasp key aspects of the procedure. This approach, which requires a proactive demonstration on the part of the participant that they understand what is being requested, has been used extensively at the MPRC. Included participants must also be judged competent to consent by the Evaluation to Sign Consent (ESC) questionnaire, and provide voluntary informed consent. Per University of Maryland School of Medicine IRB regulations, a copy of the signed consent form is given to the participant, a copy is placed in the person's medical record, and the original is kept in the laboratory. Research assistants obtaining informed consent will be experienced clinicians. They will receive detailed and standardized training as to how to obtain informed consent from people with serious mental illnesses. They will be observed obtaining informed consent from a study participant by senior staff prior to being allowed to recruit on their own.

Protections Against Risk:
We will make every attempt to minimize all study-related risks. Sexually active women of childbearing age will agree to the use of medically approved birth control, which includes condoms, oral contraceptives, diaphragms, or intrauterine device for 2 weeks after the study. Pregnant and lactating females will be excluded. We will test for pregnancy at each visit. In addition, the Brief Stay Unit (BSU) is a psychiatric and medical unit with an ACLS-certified nurse and physician. The BSU is stocked with an emergency medication supply and crash cart and has direct line to the adjacent hospital for emergency support. All BSU staff are trained in behavioral and medical emergencies. Because we hypothesize that the kynurenine pathway dysregulation may be present in schizophrenia and KYNA level increases may be associated with the pathophysiology of the illness, we anticipate slight worsening in neurocognition in the schizophrenia group. However we do not anticipate worsening in clinical symptoms. We will be carefully monitoring for side effects and distress in a well-staffed medical unit with psychiatric and medical coverage. We have a full backup emergency plan and available medications for medical and psychiatric emergencies. In order to provide objective criteria for distress and symptom worsening and study discontinuation, we have developed the following criteria:

- Participants will be withdrawn from any remaining study assessments if at any time there is significant distress reported by the schizophrenia patient or control.
- To be objective in psychiatric symptoms, we will apply a modified battery of psychiatric assessments after TRYP administration and prior to imaging. This modified assessment allows us to specifically monitor psychotic symptoms, hostile behavior or suicidality.
- Thus, we will look out for an increase in 3 or more points from the premedication BPRS in positive symptoms, somatic concern, or hostility on the BPRS, a score of a 3 or more on the suicidality item on the Calgary Depression Scale (item 8), or a two or more point increase on the CGI. If a participant scores ≥ 6 and this is at least a two point increase from baseline, the study procedures will be discontinued.
- At any time, however, a participant can be discontinued from the study.

Throughout the challenge, we will closely monitor for side effects, tolerability and any signs or symptoms of serotonin syndrome. Heart rate, blood pressure, temperature, diaphoresis, and

muscle twitching will be monitored continually. With regards to imaging, prior to entry to the study, participants are carefully screened for medical illness and to ensure that they will be safe in the MR scanner. All participants are screened for contra-indications for magnetic resonance, namely severe claustrophobia, pregnancy, cardiac pacemaker, orthodontics, or other non-MR compatible ferromagnetic implant. In addition, access to the scan room is strictly controlled to ensure that no ferromagnetic materials are introduced. The scanner to be used in the study is FDA approved and operates under radiofrequency power monitoring software at all times. All scan procedures at magnetic field strengths of 1.5T and 3T are FDA approved and are not classified as investigational devices. Some participants may become claustrophobic, bored or restless during the testing. The participants have the option of terminating the testing at any time without penalty if they so choose. The risks of psychological discomfort will be minimized by encouraging participants to let the examiner know if they are feeling any discomfort, and by continuous monitoring by experienced operators for participant fatigue, inattention, or annoyance. Scanning will be halted if the participant so requests, or if the examiner feels that the participant is becoming uncomfortable. The participant will be encouraged to report any adverse symptoms during the MRI. The MRI can be stopped by the participant either by verbalizing the request to stop (which can be heard through the microphone in the MRI) or by pushing a “panic button” provided to him/her. A potential concern with MRI studies is so-called radio-frequency heating due to radiofrequency power deposition in the participant. The FDA has set strict and very conservative guidelines to guard against this risk. Such power deposition increases with the square of the magnetic field strength. To be 100% sure of compliance with the FDA guidelines, power deposition is monitored continuously by the software of the manufacturers, and scans are not possible if the power deposition exceeds guidelines.

Confidentiality

All records of the research will be kept in locked files with subjects identified by code only. A separate file will hold the code key. Subjects will not be personally identified in any publications or reports of the study. All biological specimens collected for research purposes will be identified at the laboratory by code only. Whenever possible, existing clinical laboratory data will be used as a source of information for the study, which will minimize testing done on subjects. Any data used will be recopied to research files with the subject identified by code only. Computerized records of data are kept in password-protected computers in locked rooms. Appropriate firewalls and protections of computerized data are maintained to ensure that entry by those other than research personnel is not possible.

Potential Benefits of the Proposed Research to the Participants and Others There are few potential benefits to participating in the study. Participants will have a series of medical and laboratory workup prior to study start that may provide new or helpful information on their physical and mental health. Mild and transient changes in neurocognition are possible or even expected. We feel the importance of the knowledge gained outweighs the risks of the study.

Importance of the Knowledge to be Gained:
We will be able to examine, for the first time, the connection between the kynurenine pathway, cognitive function and neuroimaging in people with schizophrenia. In addition, we will examine the role of stress hormones and immune function in the control of kynurenine pathway metabolism. This important information could be the first clinical study to link this pathway to symptoms and outcomes of people with schizophrenia.

Data and Safety Monitoring Plan:
A Data Safety Monitoring Board (DSMB) is already established for monitoring studies at the MPRC and will be used for this study. The DSMB is comprised of two psychiatrists, a pharmacist, a statistician, and a community representative. The psychiatrist will be an expert in the clinical treatment of people with schizophrenia. The DSMB will be charged with the following responsibilities: 1) to establish a regular meeting schedule; 2) to review the protocol; 3) to review the consent form; 4) to monitor the occurrence of side effects/adverse events, and serious adverse events throughout the course of the study; and 5) to review with investigators, the study data management system; and 6) to establish stop rules for the study as a whole. The DSMB will review prior to study enrollment and then receive annual side effect/adverse event updates. In addition, all serious adverse events (SAEs) will be reported to the DSMB, PIs, and the University of Maryland School of Medicine IRB. The PI will receive all SAE reports within 24 hours of their occurrence. If the incidence of any side effect/adverse event is 25% or more or any SAE occurs in excess in either treatment group, the DSMB will notify the PI. The PI and the DSMB will determine whether possible protocol modifications are required to minimize the further occurrence of such events. Unexpected adverse events will be reported in accord with NIH and Federal requirements. Non-serious and expected adverse events will be reported annually to the IRBs. The PI is required to attend the DSMB meeting annually and is asked to give a review of the past year with a particular emphasis on safety, side effects, and enrollment. The data would remain blinded unless risks to participants justify unblinding. To safeguard confidentiality, data are presented in aggregate or are identified only by an ID number.

6. Clinical Trials.Gov Requirements:
The study will be registered on ClinicalTrials.gov prior to the enrollment of any participants.

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