$\alpha 4\beta 2^*$ Nicotinic Cholinergic Receptor Target Engagement in Parkinson Disease

Running Head: Varenicline in Parkinson Disease

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Abstract:

Objective: Attentional function deficits secondary to degeneration of brain cholinergic systems are significant contributors to gait-balance deficits in Parkinson disease (PD). To assess whether $\alpha4\beta2^*$ nicotinic acetylcholine receptor (nAChR) stimulation improves attention and gait-balance function, we performed a target engagement study of the $\alpha4\beta2^*$ nAChR partial agonist varenicline.

Methods: Non-demented PD participants with cholinergic deficits were identified with [^{18}F]fluoroethoxybenzamicol positron emission tomography (PET). $\alpha4\beta2^*$ nAChR occupancy after subacute oral varenicline treatment was measured with [^{18}F]flubatine PET. With a dose selected from the receptor occupancy experiment, varenicline effects on gait, balance, and cognition were assessed in a double-masked placebo-controlled crossover study. Primary endpoints were normal pace gait speed and a measure of postural stability.

Results: Varenicline, 0.25 mg per day, 0.25 mg b.i.d., 0.5 mg b.i.d., and 1.0 mg b.i.d., produced 60% - 70% receptor occupancy, with 0.5 mg po b.i.d chosen for the crossover study. Thirty-three (of thirty-four) participants, completed the crossover study with excellent tolerability. Varenicline had no impact on the postural stability measure and resulted in slower normal pace gait speed. Varenicline reduced distraction effects under dual task gait conditions and improved performance on a sustained attention test. In 28 participants in whom treatment compliance was confirmed by plasma varenicline measurements, we obtained identical conclusions.

Interpretation: Varenicline occupied a significant fraction of $\alpha4\beta2^*$ nicotinic acetylcholine receptors, was tolerated well, enhanced attentional function, and improved dual task gait

performance. $\alpha4\beta2^*$ nicotinic agonists may be useful in mitigating gait and balance disorders in PD.

Introduction:

Dopamine replacement therapy (DRT)-refractory gait and balance disorders are among the most morbid aspects of Parkinson disease (PD). Gait deficits, including postural instability and freezing, worsen with disease progression and substantially increase fall risk. Falls are a significant source of morbidity in PD patients, with a relatively high rate of serious falls leading to fractures and hospitalizations, precipitation of nursing home placement, and increased mortality associated with falls.[1,2,3,4]

The DRT-refractory nature of gait and postural deficits in PD indicates involvement of non-dopaminergic systems. Considerable evidence suggests that DRT-resistant gait and balance disorders are associated with degeneration of central nervous system (CNS) cholinergic projection systems.[5-13] Fall risk in PD is likely increased by the conjunction of striatal dopaminergic denervation and degeneration of cholinergic neurons of the basal forebrain corticopetal complex (BFCC) and pedunculopontine-laterodorsal tegmental complex (PPN-LDT). The best defined role of the BFCC is in attention with suggestions that PPN-LDT cholinergic neurons play a role in alertness.[12,14,15] Preclinical model experiments indicate that an intact BFCC system can compensate for motor deficits secondary to striatal dopaminergic denervation. As BFCC neurons are lost, gait-balance dysfunction may increase markedly as BFCC cholinergic deficits unmask the full impacts of striatal dopaminergic deficits.[12,16] This model is consistent with results of dual task paradigm experiments, in PD and control

participants, indicating that impaired attention is associated with worsening gait-balance functions and increased fall risk.[17]

Cholinergic neurotransmission is mediated by both G-protein coupled receptors and ionotropic nicotinic receptors (nAChRs). The predominant CNS nicotinic receptor is the $\alpha4\beta2^*$ (*potential other subunits) nAChR.[18] Stimulation of cortical $\alpha4\beta2^*$ receptors plays an important role in attention.[12] This is likely the mechanism by which nicotine enhances attention. In the setting of BFCC projection degeneration, pharmacologic stimulation of $\alpha4\beta2^*$ nAChRs might improve attention and mitigate gait-balance deficits.

Varenicline (VCN) is a potent (K_i = 0.4 nM) $\alpha4\beta2^*$ nAChR partial agonist (efficacy = 45%) used widely for tobacco abuse cessation.[19,20] VCN has an excellent safety record and favorable pharmacokinetic features.[20-24] To explore the potential of VCN to improve DRT-resistant gait-balance deficits, we performed a target engagement study of VCN in PD participants with neocortical cholinergic deficits. We assessed target engagement along 2 dimensions; VCN binding to brain $\alpha4\beta2^*$ nAChRs, and VCN effects on laboratory-based measures of gait, balance, and cognitive functions.

Another goal was to explore measures for assessing target engagement. While there is abundant literature characterizing gait, balance, and fall risk in PD and in normal aging, there are no laboratory-based measures predicting intervention outcomes. In the absence of measures with predictive validity, measures linked to pathophysiologic mechanisms are more likely to be adequate indices of target engagement. We studied both conventional gait-balance and cognitive outcome measures, and measures more specifically linked to disrupted attentional and cholinergic functions.

Materials and Methods:

Regulatory Compliance: Informed consent was obtained from all participants according to the Declaration of Helsinki. This study was approved by the University of Michigan Medical School Institutional Review Board. An Investigational New Drug application waiver for varenicline study was obtained from the Food & Drug Administration. This study was registered at ClinicalTrials.gov (NCT04403399, NCT02933372).

Participant Selection: PD participants were recruited from a larger cohort characterized with [^{18}F]fluoroethoxybenzovesamicol positron emission tomography ([^{18}F]FEOBV PET).[9] The vesicular acetylcholine transporter ligand [^{18}F]FEOBV was used to determine the magnitudes of cortical cholinergic terminal deficits. All participants met the International Parkinson and Movement Disorder Society clinical diagnostic criteria for PD. All underwent [^{11}C]dihydrotetrabenazine PET to confirm the presence of nigrostriatal dopaminergic terminal deficits. No enrolled participant was dementia or was using drugs or supplements with cholinergic properties, or using tobacco products. Because of anecdotal reports of worsening mood disorders, adverse ethanol interactions, and myocardial infarctions, we excluded individuals with an active mood disorder (Geriatic Depression Scale >5 and evidence of recent, worsening mood), alcohol use disorder (Alcohol Use Disorder Identification Test score >7 for those over 65 years; >8 for those 65 years and younger), and active cardiovascular disease. Participants were counseled to avoid alcoholic beverages during study participation. Only participants with cortical cholinergic deficits were enrolled. In PD, the occipital cortex has highest vulnerability for cholinergic transporter losses compared to other brain regions.[25] Hypocholinergic status was defined as falling within the lower tertile of occipital cortical [^{18}F]FEOBV binding in normal older adults. Participants were maintained on stable DRT

regimens throughout these experiments. To ensure that there was not a marked difference between VCN interaction with $\alpha4\beta2^*$ nAChRs in PD participant and control brains, we performed a more limited dose-response experiment in normal participants. Age-matched control participants without clinical evidence of parkinsonism or other neurologic disorders, and not using any cholinergic agents or tobacco products, were studied.

VCN Occupancy of $\alpha4\beta2^*$ nAChRs Study: VCN occupancy of $\alpha4\beta2^*$ nAChRs was assessed with ascending doses of VCN and the selective $\alpha4\beta2^*$ nAChR PET ligand [^{18}F]Flubatine.[26] Participants were treated with oral VCN for 10 days with ascending dose schedules. Higher dosing cohorts were begun after the previous dosing cohort completed its scheduled treatment and follow-up. Doses chosen were 0.25 mg per day, 0.25 mg b.i.d., 0.5 mg b.i.d., and 1.0 mg b.i.d. The clinically used VCN dose is 1.0 mg b.i.d. Participants received an initial 0.25 mg dose following confirmation of eligibility and baseline evaluations and were monitored for 4 hours. In participants scheduled for higher VCN doses, the total daily dose was escalated over the next 2 days, followed by 8 days of stable daily VCN dose. $\alpha4\beta2^*$ nAChR agonists may induce nAChR expression.[27] The conventional strategy of imaging participants before and at the end of a drug exposure period might result in underestimation of receptor occupancy. Consequently, we imaged participants at the end of their drug exposure periods and again after 5 days (~5 half-lives) of washout from drug exposure. [^{18}F]Flubatine was synthesized as described previously.[28] Participants were scanned on a Biograph TruePoint Model 1094, using a dynamic acquisition of 18 frames over 90 minutes (four x 0.5 min; three x 1 min; two x 2.5 min; two x 5 min; seven x 10 min). $\alpha4\beta2^*$ nAChRs occupancy was estimated by comparing [^{18}F]Flubatine standardized uptake values (SUVs) on and off VCN. SUVs were calculated as (B-D)/(B-ND), where B is the SUV of the "baseline" scan (off VCN), and D is the

SUV of the "drug" scan (on VCN), and ND is estimated of the non-displaceable SUV. ND is calculated from the x-intercept of a regression of (B-D) on D.

Crossover Study: Following selection of a study dose from the $\alpha4\beta2^*$ nAChR occupancy experiment (**Results** below), we completed a double-masked, placebo-controlled crossover study to assess VCN effects on measures of gait, balance, and cognition (**Figure 1**). Participants completing the initial receptor occupancy study were eligible to enroll in this experiment. Participants were randomized 1:1 to one of two treatment sequences: placebo followed by VCN 0.5 mg b.i.d., or VCN followed by placebo. A statistician prepared the randomization list using permuted blocks with random block sizes. The list with randomization number and treatment allocation was sent to the research pharmacy and a blinded list of randomization numbers was sent to the study coordinator. After patient consent was completed and eligibility confirmed, the coordinator assigned the next randomization number to the participant, and sent a prescription with participant ID and randomization number to the research pharmacist who dispensed the appropriate study medication. To mask drug, VCN pills or placebo were encapsulated in gelatin sheaths. Participants received an initial 0.25 mg dose or equivalent placebo following baseline evaluations and were monitored for 4 hours after initial study medication administration with total daily dose or equivalent placebo escalated over the next 2 days. Treatment periods were 3 weeks in duration and interrupted by a 3 week washout period. Participants underwent a standard evaluation at baseline, at the end of the first treatment period, at end of the washout period-beginning of the second treatment period, and at the end of the second treatment period (**Figure 1**). Outcome measures at the end of the VCN and placebo treatment periods were compared to assess VCN effects. The standard evaluation was a battery of motor, cognitive, and behavioral measures (see below). We *a priori* selected a measure of gait performance, normal

pace gait speed, and a measure of postural stability, Mancini et al.'s JERK, as co-primary endpoints.[29,30] JERK is the time based derivative of lower trunk accelerations during standing spontaneous sway. JERK was chosen because it tracks postural instability in PD.[30] Normal pace gait speed was chosen as prior studies indicated that neocortical cholinergic denervation is associated with slower gait speed in PD.[8] We hypothesized that VCN treated participants would ambulate faster and that VCN treatment would reduce JERK. Gait analysis was performed on an 8 meter GAITRite pressure sensitive walkway (CIR Systems, Inc.) and standard parameters were analyzed using ProtoKinetics Movement Analysis Software (GAITRite version 5.09C; ProtoKinetics, LLC). Gait assessments were repeated with a dual-task protocol in which participants counted backwards by three starting at a random number (under 100) provided by the examiner. Postural stability was assessed with the Ambulatory Parkinson's Disease Monitoring (APDM) wearable sensor system (APDM Wearable Technologies, Inc.) using the iSWAY protocol, with participants standing on a foam pad with eyes open and eyes closed. Standard postural measures, including JERK, were assessed and calculated using the manufacturer's software (Mobility Lab Version 1). We used the APDM system's iTUG (Timed Up and Go) protocol to collect additional exploratory data. For cognition, we used a general cognitive measure, the Montreal Cognitive Assessment (MoCA) and selected tests to examine major cognitive domains but focusing on attention and executive function.

Motor Assessments: Movement Disorder Society Unified Parkinson's Disease Rating Scale, part III (MD-UPDRSIII; "on" state); MDS-UPDRSIII postural instability and gait subscore (PIGD) subscale score (sum of items 3.1, 3.9-3.13); Gait Speed (normal pace); Gait Speed (fast pace); Gait Speed (normal pace - dual task); Gait Speed (fast pace – dual task); Postural stability measures – mean sway velocity, JERK, root mean square sway distance (RMS). To assess the

effects of attentional loading, normal pace and fast pace gait were performed under dual task conditions. Dual task conditions typically lead to slower gait speed. Differences in gait speed between dual task and no dual task conditions are a measure of the attentional burden imposed by the dual task. To assess the effects of VCN on this aspect of gait performance, we compared the differences between no dual task and dual task gait speed between VCN and placebo treatment periods.

Cognitive Assessments: MoCA; Wechsler Adult Intelligence Scale-III Digit Symbol modalities test; California Verbal Learning Test (CVLT) short term memory test; CVLT long term memory test; CVLT recognition test; Delis-Kaplan Executive Function System (D-KEFS) Stroop III; D-KEFS sorting total; D-KEFS verbal fluency letters total; D-KEFS verbal fluency animals; D-KEFS Trail Making Test 4; Judgment of Line Orientation (JOLO) test. We assessed attentional function with a Sustained Attention Test (SAT), established to reflect CNS cholinergic systems function in humans (Lustig *et al.*, 2013; Berry *et al.*, 2014; Kim *et al.*, 2017).[31,32,33] The SAT is performed with 2 conditions; without and with a distractor (dSAT). SAT and dSAT results are reported as the vigilance index, a measure that corrects estimates of accurate detection with penalties for false detections and not confounded by errors of omission.[34]

Behavioral Assessments: Geriatric Depression Score (GDS) and Columbia-Suicide Severity Rating Scale (C-SSRS).

Treatment Compliance Monitoring: To assess compliance, we measured plasma VCN levels at the ends of treatment periods. VCN concentrations were assessed by the University of Michigan College of Pharmacy Pharmacokinetics Core. Plasma samples were deproteinated with acetonitrile, extracts centrifuged at 3500 RPM for 10 minutes, and supernatants used for liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Calibration curve

with VCN concentrations from 2.5 ng/ml 250 ng/ml was highly linear (r=0.999). Assay accuracy and precision were evaluated at 5 ng/ml, 10 ng/ml, and 200 ng/ml (N=3). Accuracy was 106% or less and precision was 10% relative standard deviation or less.

Statistical Plan: The sample size (planned initially at four participants per dosing group) for the VCN-α4β2* nAChR occupancy study was based on logistical considerations. For the Crossover study, we calculated that 33 participants would provide at least 80% power to detect within-patient treatment differences of 0.122 m/s in gait speed and -0.131 m^2/s^5 for JERK, assuming within-participant correlation of $\geq$ 0.64 and $\geq$ 0.72, respectively, using a paired t-test and a two-sided Type I error of 0.025 (Bonferoni adjustment for co-primary endpoints). This approach is conservative given our analysis method uses mixed effects models. Estimates for treatment differences were based on Bohnen *et al.* for normal pace gait speed and Mancini *et al.* for JERK.[8,29]

We conducted exploratory analyses to examine the distributions of outcomes under each treatment, as well as individual and mean profiles over time. Graphical approaches such as boxplots and scatterplots with linear or non-linear (e.g., loess) methods were used, allowing identification of outliers, linearity, and correlation of measurements within participant and across time. Log transformations were applied when outcome data did not appear normally distributed.

Descriptive statistics for efficacy and safety outcomes were provided for each dosing cohort in the VCN-α4β2* nAChR occupancy study. For the Crossover study, linear mixed models containing treatment sequence, treatment period, treatment group, and dependent-variable baseline value, with participant within treatment sequence as a random effect, were used for analysis of continuous outcomes. To compare differences between VCN and placebo, a test for

carryover based on the sequence effect was conducted using patient with sequence as the error term. Results are presented as least squares (LS) mean and standard error (SE). The co-primary endpoints were tested at the 2-sided, 0.025, significance level. All other tests were based on a 2-sided significance level of 0.05; no adjustments for additional multiple comparisons.

Primary and secondary efficacy endpoints were analyzed in all randomized participants (intention-to-treat [ITT] population). Secondary continuous endpoints were analyzed similarly to the primary endpoints. Categorical analyses were based on Gart's test.[35]

Safety endpoints were analyzed in all randomized participants who received at least one dose of study medication. We included adverse events that occurred in the washout period with the treatment given in period One.

Results:

Participants: Characteristics of the fifteen PD participants enrolled for the initial VCN-$\alpha4\beta2^*$ nAChR occupancy study are described in **Table 1a.** Ten participants completed this phase of the study; two participants received 0.25 mg VCN per day, three participants 0.25 mg bid VCN per day, three participants 0.5 mg bid per day, and two participants 1.0 mg bid per day. To confirm that $\alpha4\beta2^*$ nAChR – VCN interactions were not grossly different in PD compared to normal brain, an additional ten control participants were studied with ascending doses of VCN and [^{18}F]Flubatine PET in a protocol identical to that used for PD. Data from one control participant were excluded because of suspected covert tobacco abuse. Four participants received 0.25 mg per day, two participants 0.25 mg po b.i.d. per day, and three participants 0.5 mg b.i.d. per day. Characteristics of participants for the VCN-$\alpha4\beta2^*$ nAChR occupancy studies are described in **Table 1a.** Characteristics for the Crossover study participants are shown in **Table 1b**.

VCN-α4β2* nAChR occupancy study: VCN displacement of thalamic [^{18}F]Flubatine binding to α4β2* nAChRs is reported, the region with the highest [^{18}F]Flubatine binding (**Figure 2**). Analysis of other regions gave very similar results (data not shown). The lowest daily dose of VCN, 0.25 mg per day, produced significant receptor occupancy. There was little evidence of a dose-response relationship with all VCN doses producing 60-70% occupancy of α4β2* nAChRs. Results in control participants were very similar (data not shown). As 0.5 mg p.o. b.i.d. produced approximately the same α4β2* nAChR occupancy as 1.0 mg p.o. b.i.d., 0.5 mg p.o. b.i.d. was chosen as the dose for the Crossover study.

Crossover Study:

Intention-to-Treat and Treatment Compliant Participants Analyses: The primary analyses of the Crossover study used the ITT population. Secondary analyses were conducted using identical statistical methods in participants who were compliant with study treatment. We excluded participants without evidence of significant increases in VCN plasma levels between placebo and VCN treatment periods to define the treatment compliant participants. Safety analyses are based on all participants.

Safety: We enrolled 34 PD participants. There was 1 drop-out for reasons unrelated to the study (withdrawn 4 days into period 2 while on VCN). Among the 34 participants, there were 56 adverse events (serious and non-serious) in 22 (65%) participants (**Table 2**). There were more adverse events in the VCN periods than placebo periods: 17 participants on VCN experienced 35 AEs, while 8 participants on placebo experienced 18 AEs. These were largely expected adverse events such as nausea and insomnia. There were 2 serious adverse events; one in each treatment period and neither related to VCN treatment. Two participants required dose reductions to 0.5 mg p.o. per day and 1 participant to 0.25 p.o. mg per day – 0.25 mg p.o. b.i.d.

for study completion. As the VCN-$\alpha4\beta2^{*}$ nAChR occupancy experiment demonstrated substantial nAChR occupancy at the lowest VCN dose, these participants are included in all analyses.

Motor Function Measures – ITT Population: Of the primary outcome measures, there was no statistically significant difference in JERK performance between VCN and placebo treatment periods (**Table 3**). For normal pace gait speed, VCN treatment was associated with statistically significant gait slowing, a result opposite to the hypothesized effect (**Table 3**). Analysis of secondary/exploratory measures returned disparate results. MDS-UPDRSIII scores modestly worsened during the VCN treatment periods (**Table 3**). The PIGD subscore was not different between VCN and placebo periods. Other postural stability measures - mean sway velocity and RMS, were not different between placebo and VCN treatment periods. VCN significantly reduced the difference in normal pace gait speed between no dual task and dual task conditions. VCN treatment had no effect on gait speed under fast pace conditions. There was no effect on the difference between fast pace gait speed under dual task and no dual task conditions. VCN treatment had significant and predictable effects on normal pace cadence, stride length, and stride time. Consistent with lack of VCN effect on gait speed under the fast pace condition, there was no effect on fast pace cadence, stride length, or stride time. iTUG measures showed little VCN effect (data not shown).

Cognitive Measures – ITT Population: The SAT, a measure of attentional function that reflects CNS cholinergic functions, showed positive effects of VCN treatment (**Table 4**). There was little effect of VCN on dSAT performance, which was poor in both VCN treatment and placebo periods. Neither the MoCA, nor any conventional domain specific measures showed effects of VCN treatment (**Table 4**).

Behavioral Measures – ITT Population: VCN treatment had no effects on GDS (**Table 4**) or C-SSRS scores (data not shown).

Analysis of Treatment Compliant Population: We excluded the participant who dropped out prior to study completion. Four participants exhibited VCN levels below the level of quantification at the end of both treatment periods, suggesting non-compliance. All analyses of motor, cognitive, and behavioral measures were repeated for the 28 compliant participants (**Tables 5** and **6**). Results were essentially identical to those of the ITT population. For the primary endpoints, VCN was associated with slower normal pace gait speed and no effect on JERK. VCN improved SAT performance. There was a similar reduction in the difference between dual task normal pace gait performance and no dual task normal pace gait performance in the compliant population, however, this was not statistically significant in this smaller sample (p=0.06; **Table 5**). The magnitude of the mean VCN effect on the difference between dual task normal pace gait speed and no dual task normal pace gait speed in the compliant population was very similar to that seen in the ITT population (**Tables 3** & **5**).

VCN Levels: At the end of the placebo period, VCN levels were below the level of quantification for all participants, and mean VCN levels were 13.94 ng/ml (SD 7.91) at the end of the varenicline treatment period. VCN concentrations at the end of the treatment period are consistent with previously reported pharmacokinetic data.[22]

Discussion:

We assessed target engagement in complementary experiments. In our dose-finding experiment, we employed [^{18}F]flubatine PET to establish that relatively low daily VCN produces significant $\alpha4\beta2^*$ nAChR occupancy. There was no evidence of a dose-response relationship, suggesting that low oral doses saturate $\alpha4\beta2^*$ nAChRs. This inference is consistent with limited data

suggesting that low oral VCN doses are effective for smoking cessation.[36] We established that there is no gross difference in VCN-$\alpha4\beta2^*$ nAChR interactions in PD and control CNS. Our normal control results are similar to those obtained by Lotfipour *et al.*, who used [^{18}F]2-fluoro-3-[2(S)-2-azetidinylmethoxy]pyridine PET to demonstrate high $\alpha4\beta2^*$ nAChR occupancy in normal participants after a single, 0.5 mg, oral VCN dose.[37] Our second experiment, using a daily dose based on receptor occupancy, was a placebo controlled, double-masked crossover study. VCN had no effect on our chosen measure of postural stability or other measures of postural stability studied. VCN was associated with slower normal pace gait speed and significantly narrowed the difference between normal pace gait speed under baseline and distracting dual task conditions. VCN had a positive effect on SAT performance, an attention measure directly linked to CNS cholinergic functions, but little effect on conventional cognitive measures. Results were almost identical in analyses of ITT and treatment compliant participants. VCN narrowing of differences in dual task normal pace gait speed and no dual task normal pace gait speed was not statistically significant in the compliant population, likely reflecting the reduced sample size of the compliant population. The mean magnitude of VCN effects on the difference between dual task and no dual task gait speed was essentially identical between the ITT and compliant populations.

Our results are similar to those of Hall *et al.*, who randomized 36 PD participants to VCN, 1 mg p.o. b.i.d., or placebo for an 8 week trial period.[37] Participants were slightly older than our participants but comparable in PD severity and cognitive status. Their primary outcome measure was Berg Balance Scale (BBS) performance. Cognitive effects of VCN were assessed with the Mini-Mental State Examination (MMSE) and the Frontal Assessment Battery (FAB). There was no VCN effect on any of these measures. There are important methodological differences

between this study and our work. Cholinergic systems are intact in many moderately advanced PD participants.[7,9] It is likely that Hall *et al.* enrolled some participants with normal cholinergic systems. We enrolled participants with cortical cholinergic deficits. This difference may be particularly important in the context of a partial agonist. In the presence of normal levels of endogenous agonists, partial agonists exhibit antagonist properties, potentially impairing cholinergic signaling. Perhaps most important is the difference in outcome measures. The BBS does not contain a dual task component. The 6 item FAB contains 2 items (4 and 5) with attentional components, but there was no measure comparable to the SAT, a specific measure of attention reflecting cholinergic functions.

Disruption of attentional functions subserved by CNS cholinergic systems is likely a mechanism mediating the effects of cholinergic system deficits on gait and balance functions. Cortical $\alpha4\beta2^{*}$ nAChR activation is a key modulator of attentional function, leading to the hypothesis that VCN would improve gait and postural control. One of our primary end points, normal pace gait speed, was chosen on the basis of prior data indicating that gait speed was related to cortical cholinergic deficits.[8] The second primary endpoint, JERK, was chosen as a measure of postural control on the basis of prior data indicating that it differentiated PD participants from controls and tracked disease progression in PD.[29,30] Prior studies did not, however, specifically link JERK changes to CNS cholinergic deficits.

In terms of the selected primary endpoints, we found no effect on the measure of postural stability, JERK. We documented a significant effect on normal pace gait speed, but opposite to our hypothesis that nAChR stimulation would increase normal pace gait speed. It is plausible, however, that slower normal pace gait speed associated with VCN treatment might reflect greater attentiveness. In a rat model of variations in BFCC function, animals with better attentional

capacity secondary to more robust BFCC function were more cautious during performance of an attentionally demanding gait task under single task conditions and less likely to fall under distracting conditions.[39] An outcome measure specifically focused on gait performance in the context of attentional function – the difference between normal pace gait speed at baseline and with a dual task distractor – showed a positive effect of VCN treatment. Similarly, Kucinski *et al*. showed that gait performance (less vulnerability to falls) was better for high-attention capacity animals.[39] While there were no discernable effects of VCN on conventional measures of cognitive function, SAT performance, which is known to reflect CNS cholinergic dysfunction in humans, including PD participants, showed a positive VCN effect. Using the same model as the Kucinski *et al.*, Paolone *et al.* demonstrated better SAT performance in animals with better BFCC function, and linked superior performance directly to $\alpha 4\beta 2^{*}$ nAChR stimulation.[40] Our results are consistent also with those of Mocking *et al.*, who showed that subacute oral VCN administration (0.5 mg/day for 3 days; then 1.0 mg/day for 4 days) in healthy participants improved working and declarative memory.[41] Coupled with our [^{18}F]flubatine PET data, we suggest that VCN narrowing of the difference between normal pace gait speed under baseline and distracting conditions, and the SAT results, constitute clinical evidence of target engagement.

We did not find any VCN effects under fast pace gait conditions. This may be because fast pace gait involves conscious focus on gait performance, strengthening attentional functions. Similarly, we did not find any VCN effect on dSAT performance, possibly due to floor effects. We did not find any effects on other postural control measures, mean sway velocity and RMS, studied. None of the postural control measures studied have been directly linked to attentional functions or cholinergic deficits and may not be appropriate outcome measures for interventions

aimed at improving attentional functions. We did note a rise in MDS-UPDRSIII scores with VCN treatment. The magnitude of this effect was modest, almost 3 points, and below the threshold of a minimally clinically important worsening in MDS-UPDRSIII scores.[42]

Our results highlight some of the difficulties involved with assessing interventions for DRT-refractory gait and balance disorders. Similar to the results of Hall *et al.*, VCN did not have effects on conventional endpoints. We did find positive effects of VCN treatment on dual task normal pace gait performance and the SAT, measures that arguably more closely reflect the cholinergic system – attentional deficits that are likely major contributors to DRT-refractory gait and balance disorders. Targeting $\alpha4\beta2^{*}$ nAChRs may be a viable approach to mitigating this morbid PD feature. We suggest also that pursuing receptor subtype pharmacology, either for nAChRs or muscarinic cholinergic receptors, is more likely to be useful than non-specific approaches such as use of acetylcholinesterase inhibitors.[43]

Future intervention studies for DRT-refractory gait-balance disorders will likely require laboratory-based measures that are both proxy measures of fall risk and permit efficient evaluation of target engagement. Our experience suggests that outcome measures tied closely to underlying pathophysiologic mechanisms will be more robust biomarkers of target engagement.

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Acquisition and Analysis of Data: RLA, MLTMM, NIB, CS, AS, CL, RAK, WTD

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Figure Legends:

Figure 1: Design of Crossover Study.

Figure 2: Varenicline Occupancy of $\alpha4\beta2^*$ nAChRs. Top panel is dose-response relationship between daily oral dose and estimated per cent receptor occupancy (mean and standard deviation). X-axis units are mg. Bottom panel is parametric images of a single participant on and off 0.5 mg po b.i.d.

Table 1A: VCN-α4β2* nAChR Occupancy Study Participant Baseline Characteristics

	PD Participant N = 15	**Control Participants N=10**
Age (Years)		
Mean (SD)	**67.3 (5.20)**	**66.4 (8.95)**
Min, Max	**52, 73**	**50, 76**
Male, n (%)	**14 (93%)**	**8 (80%)**
White, n (%)	**14 (93%)**	**10 (100%)**
Age at Diagnosis (Years)		
Mean (SD)	**61.4 (5.69)**	**Not applicable**
Min, Max	**51, 70**	
MDS-UPDRS III		
Mean (SD)	**31.9 (12.83)**	**3.5 (1.84)**
Min, Max	**9, 57**	**1, 7**
GDS		
Mean (SD)	**3.2 (3.75)**	**1.6 (2.01)**
Min, Max	**0, 12**	**0, 6**
MoCA		
Mean (SD)	**25.5 (1.73)**	**26.8 (2.30)**
Min, Max	**23, 29**	**23, 30**

Table 1B: Crossover Study Participant Baseline Characteristics

	Placebo then Varenicline N = 16	**Varenicline then Placebo N = 18**
Age (Years)		
Mean (SD)	64.2 (5.3)	68.1 (5.7)
Min, Max	52, 76	56, 78
Male, n (%)	13 (81%)	15 (83%)
White, n (%)	16 (100%)	17 (94%)
Age at Diagnosis (Years)		
Mean (SD)	57.7 (7.22)	61.3 (6.66)
Min, Max	43, 70	50, 74
MDS-UPDRS III		
Mean (SD)	30.7 (12.4)	33.2 (13.92)
Min, Max	13, 62.5	15, 58
GDS		
Mean (SD)	4.3 (4.19)	2.4 (2.06)
Min, Max	0, 15	0, 6
MoCA		
Mean (SD)	26.8 (1.97)	27.2 (2.37)
Min, Max	24, 30	23, 30

MDS-UPDRS III = Movement Disorders Society - Unified Parkinson's Disease Rating Scale, Part III motor subscale score. Range 0 to 137, with higher scores indicating worse symptoms
GDS = Geriatric Depression Scale total score. Range = 0 to 30, with higher scores indicating worse depression.
MoCA = Montreal Cognitive Assessment total score. Range = 0 to 30, with lower scores indicating worse severity.

Table 2: Crossover Study Adverse Events (AEs)

	Placebo N=34	**Varenicline N=34**
# Serious AEs	1	1
# Participants with ≥ 1 Serious AE	1	1
# Non-Serious AEs[a]	18	35
# Participants with ≥ 1 Non-Serious AE	8	17
Non-Serious AE Severity		
Mild	15	29
Moderate	3	6
Non-Serious AE Relatedness		
Related	4	16
Unrelated	14	19

[a]8 non-serious AEs occurred during washout, 6 in the placebo then varenicline arm and 2 in the varenicline then placebo arm; AEs during washout are categorized to the treatment in period 1 in this table; one subject randomized to varenicline then placebo had an unknown date of onset and thus is not included in this table (but their non-serious AE is included in summaries in the text).

Table 3: Crossover Study Motor Outcomes. Intent-to-Treat Population (N=34)

	Varenicline LS Mean (SE)	Placebo LS Mean (SE)	P-value
Primary Outcomes[a]			
Gait Speed–Normal Pace–No Dual Task (cm/s)	121.27 (1.36)	124.89 (1.36)	0.003
JERK (m^2/sec^5)	0.97 (0.20)	1.04 (0.20)	0.73
Secondary Outcomes			
MDS-UPDRS III			
Total	31.90 (1.15)	28.77 (1.13)	0.02
PIGD Subscore	4.41 (0.32)	4.89 (0.32)	0.25
Gait			
Gait Speed–Fast Pace–No Dual Task	148.92 (1.73)	151.26 (1.73)	0.17
Gait Speed–Normal Pace–Dual Task minus Gait Speed–Normal Pace–No Dual Task	-6.07 (1.11)	-9.10 (1.11)	0.02
Gait Speed–Fast Pace–Dual Task minus Gait Speed–Fast Pace–No Dual Task	-13.40 (1.37)	-13.81 (1.36)	0.76
Cadence–Normal Pace–No Dual Task	109.50 (0.58)	111.73 (0.58)	0.003
Cadence–Fast Pace–No Dual Task	120.70 (0.85)	121.97 (0.85)	0.10
Mean Stride Length–Normal Pace–No Dual Task	132.58 (1.16)	134.56 (1.16)	0.03
Mean Stride Length–Fast Pace–No Dual Task	147.96 (1.18)	148.75 (1.18)	0.47
%CV Stride Length–Normal Pace–No Dual Task	3.87 (0.23)	3.67 (0.23)	0.39
%CV Stride Length–Fast Pace–No Dual Task	3.55 (0.22)	3.41 (0.22)	0.61
Mean Stride Time–Normal Pace–No Dual Task	1.10 (0.006)	1.08 (0.006)	0.004
Mean Stride Time–Fast Pace–No Dual Task	1.00 (0.007)	0.99 (0.007)	0.12
%CV Stride Time–Normal Pace–No Dual Task	2.55 (0.58)	3.36 (0.58)	0.31
%CV Stride Time–Fast Pace–No Dual Task	2.54 (0.15)	2.63 (0.15)	0.67
Mean Sway Velocity	0.31 (0.03)	0.26 (0.03)	0.30
Sway RMS	0.15 (0.01)	0.14 (0.01)	0.43

LS = least squares; JERK = time-based derivative of lower trunk accelerations; PIGD = postural instability and gait disorder; RMS = root mean square.

Estimates (least squares or adjusted means and standard errors) and p-values obtained from linear mixed model containing treatment sequence, treatment period, treatment group, and dependent-variable baseline value, with participant within treatment sequence as a random effect. Postural measures (JERK; Mean Sway Velocity; RMS) analysis based on a model with baseline eyes open-foam pad covariate and baseline eyes closed-foam pad covariate.

[a]Nominal alpha = 0.025 owing to Bonferroni correction for two co-primary endpoints.

Table 4: Crossover Study Cognitive and Behavioral Outcomes. Intent-to-Treat Population (N=34)

	Varenicline LS Mean (SE)	Placebo LS Mean (SE)	P-value
Cognitive			
MoCA	**26.83 (0.29)**	**27.23 (0.29)**	**0.34**
WAIS-III Digit Symbol	**62.38 (1.29)**	**62.34 (1.28)**	**0.97**
CVLT STM	**12.03 (0.32)**	**12.36 (0.32)**	**0.41**
CVLT LTM	**12.44 (0.30)**	**12.80 (0.30)**	**0.07**
CVLT recognition	**15.56 (0.13)**	**15.47 (0.13)**	**0.36**
D-KEFS Stroop III (interference)	**112.52 (3.42)**	**115.31 (3.37)**	**0.54**
JOLO	**25.84 (0.48)**	**24.48 (0.48)**	**0.53**
D-KEFS Sorting Total	**84.55 (1.57)**	**85.63 (1.55)**	**0.54**
D-KEFS Verbal Fluency - Letters Total	**43.53 (1.05)**	**41.69 (1.04)**	**0.14**
D-KEFS Verbal Fluency - Animal	**18.19 (0.72)**	**18.07 (0.71)**	**0.87**
Trail Making Test TMT4	**103.19 (8.12)**	**103.38 (8.03)**	**0.98**
SAT[a]	**0.73 (0.06)**	**0.66 (0.06)**	**0.03**
dSAT[a]	**0.49 (0.06)**	**0.43 (0.06)**	**0.18**
Behavioral			
GDS	**3.56 (0.28)**	**3.31 (0.27)**	**0.38**

LS = least squares; MoCA = Montreal cognitive assessment; WAIS = Wechsler adult intelligence scale; CVLT = California verbal learning test; Delis-Kaplan executive function system; SAT = Sustained attention test; dSAT = Distractor sustained attention test; GDS = Geriatric depression scale.

Estimates (least squares or adjusted means and standard errors) and p-values obtained from linear mixed model containing treatment sequence, treatment period, treatment group, and dependent-variable baseline value, with participant within treatment sequence as a random effect.

[a] Estimates (least squares or adjusted means and standard errors) and p-values obtained from linear mixed model containing treatment sequence, treatment period, and treatment group, with participant within treatment sequence as a random effect.

Table 5: Crossover Study Motor Outcomes. Compliant Participants (n=28)

	Varenicline LS Mean (SE)	Placebo LS Mean (SE)	P-value
Primary Outcomes[a]			
Gait Speed–Normal Pace–No Dual Task (cm/s)	119.17 (1.54)	122.06 (1.54)	0.03
JERK (m^2/sec^5)	1.05 (0.27)	1.20 (0.27)	0.48
Secondary Outcomes			
MDS-UPDRS III			
Total	33.54 (1.29)	30.72 (1.27)	0.06
PIGD Subscore	4.83 (0.36)	5.40 (0.36)	0.23
Gait			
Gait Speed–Fast Pace–No Dual Task	145.67 (1.90)	146.70 (1.89)	0.59
Gait Speed–Normal Pace–Dual Task minus Gait Speed–Normal Pace–No Dual Task	-6.56 (1.28)	-9.24 (1.27)	0.06
Gait Speed–Fast Pace–Dual Task minus Gait Speed–Fast Pace–No Dual Task	-14.62 (1.44)	-14.48 (1.44)	0.93
Cadence–Normal Pace–No Dual Task	109.69 (0.66)	111.87 (0.66)	0.01
Cadence–Fast Pace–No Dual Task	121.57 (0.91)	121.93 (0.90)	0.62
Mean Stride Length–Normal Pace–No Dual Task	130.02 (1.34)	131.51 (1.33)	0.12
Mean Stride Length–Fast Pace–No Dual Task	143.63 (1.33)	144.40 (1.33)	0.57
%CV Stride Length–Normal Pace–No Dual Task	3.93 (0.26)	3.81 (0.26)	0.64
%CV Stride Length–Fast Pace–No Dual Task	3.78 (0.26)	3.54 (0.26)	0.46
Mean Stride Time–Normal Pace–No Dual Task	1.10 (0.01)	1.07 (0.01)	0.02
Mean Stride Time–Fast Pace–No Dual Task	0.99 (0.01)	0.99 (0.01)	0.67
%CV Stride Time–Normal Pace–No Dual Task	2.64 (0.68)	3.61 (0.68)	0.31
%CV Stride Time–Fast Pace–No Dual Task	2.64 (0.16)	2.64 (0.16)	0.99
Mean Sway Velocity	0.35 (0.04)	0.27 (0.04)	0.18
Sway RMS	0.16 (0.02)	0.14 (0.01)	0.50

LS = least squares; JERK = time-based derivative of lower trunk accelerations; PIGD = postural instability and gait disorder; RMS = root mean square.

Estimates (least squares or adjusted means and standard errors) and p-values obtained from linear mixed model containing treatment sequence, treatment period, treatment group, and dependent-variable baseline value, with participant within treatment sequence as a random effect.

[a]Nominal alpha = 0.025 owing to Bonferroni correction for two co-primary endpoints.

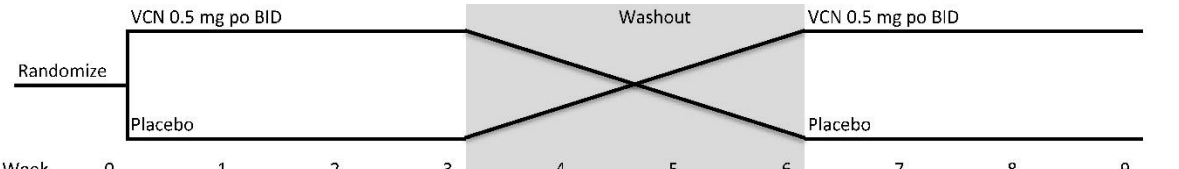
Table 6: Crossover Study Cognitive and Behavioral Outcomes. Compliant Participants (n=28)

	Varenicline LS Mean (SE)	Placebo LS Mean (SE)	P-value
Cognitive			
MoCA	**26.72 (0.34)**	**27.02 (0.33)**	**0.52**
WAIS-III Digit Symbol	**61.28 (1.41)**	**61.03 (1.40)**	**0.82**
CVLT STM	**11.60 (0.38)**	**12.07 (0.37)**	**0.30**
CVLT LTM	**12.21 (0.34)**	**12.51 (0.34)**	**0.09**
CVLT recognition	**15.50 (0.16)**	**15.36 (0.15)**	**0.22**
D-KEFS Stroop III (interference)	**115.85 (4.18)**	**119.11 (4.10)**	**0.56**
JOLO	**25.71 (0.54)**	**24.95 (0.53)**	**0.22**
D-KEFS Sorting Total	**83.71 (1.58)**	**85.19 (1.55)**	**0.48**
D-KEFS Verbal Fluency - Letters Total	**41.91 (1.21)**	**40.29 (1.19)**	**0.25**
D-KEFS Verbal Fluency - Animal	**18.20 (0.81)**	**18.28 (0.79)**	**0.92**
Trail Making Test TMT4	**110.92 (9.87)**	**112.83 (9.72)**	**0.85**
SAT[a]	**0.72 (0.05)**	**0.62 (0.04)**	**0.04**
dSAT[a]	**0.48 (0.05)**	**0.39 (0.04)**	**0.13**
Behavioral			
GDS	**3.57 (0.32)**	**3.41 (0.32)**	**0.64**

LS = least squares; MoCA = Montreal cognitive assessment; WAIS = Wechsler adult intelligence scale; CVLT = California verbal learning test; Delis-Kaplan executive function system; SAT = Sustained attention test; dSAT = Distractor sustained attention test; GDS = Geriatric depression scale.

Estimates (least squares or adjusted means and standard errors) and p-values obtained from linear mixed model containing treatment sequence, treatment period, treatment group, and dependent-variable baseline value, with participant within treatment sequence as a random effect.

[a] Estimates (least squares or adjusted means and standard errors) and p-values obtained from linear mixed model containing treatment sequence, treatment period, and treatment group, with participant within treatment sequence as a random effect.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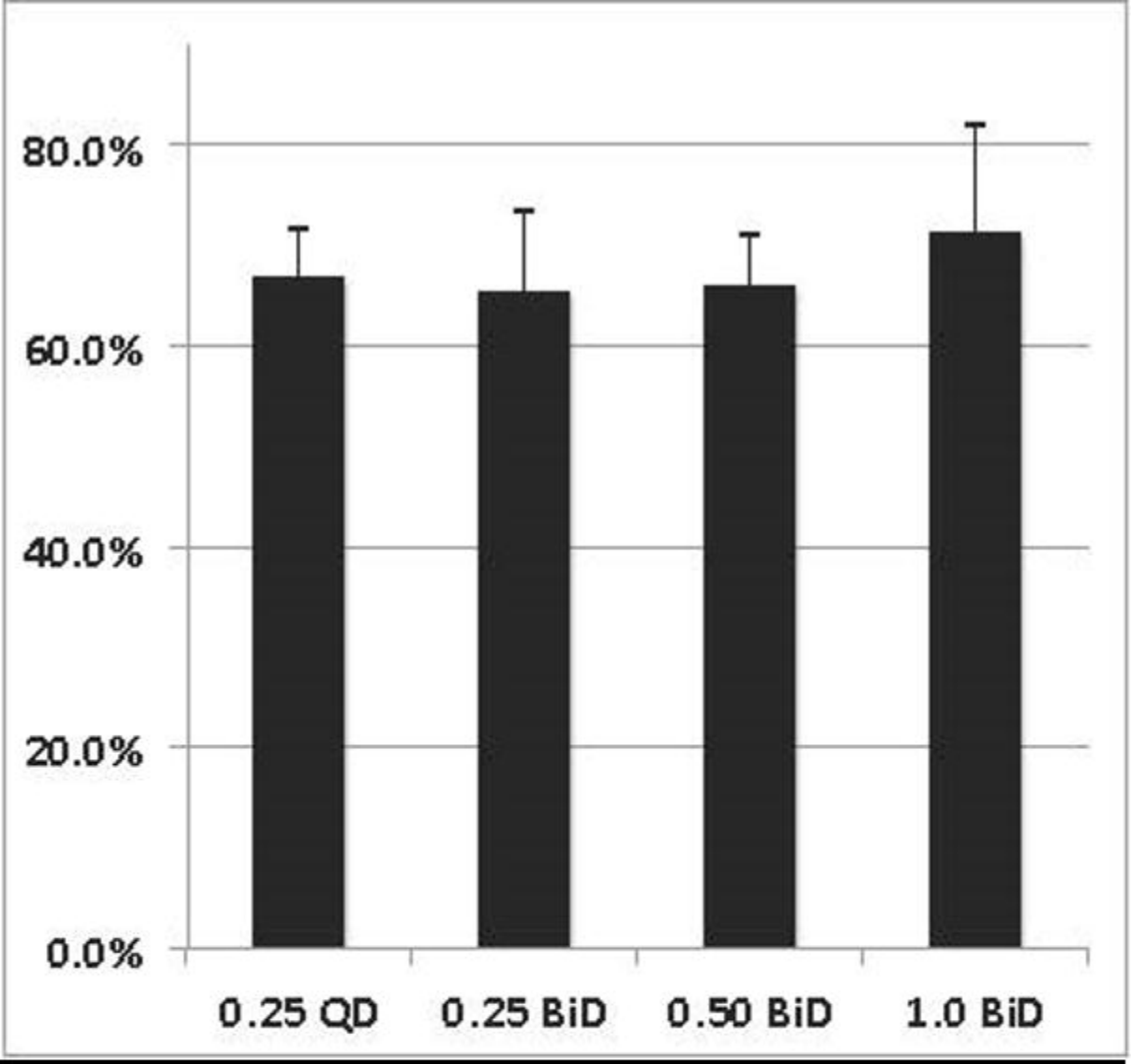
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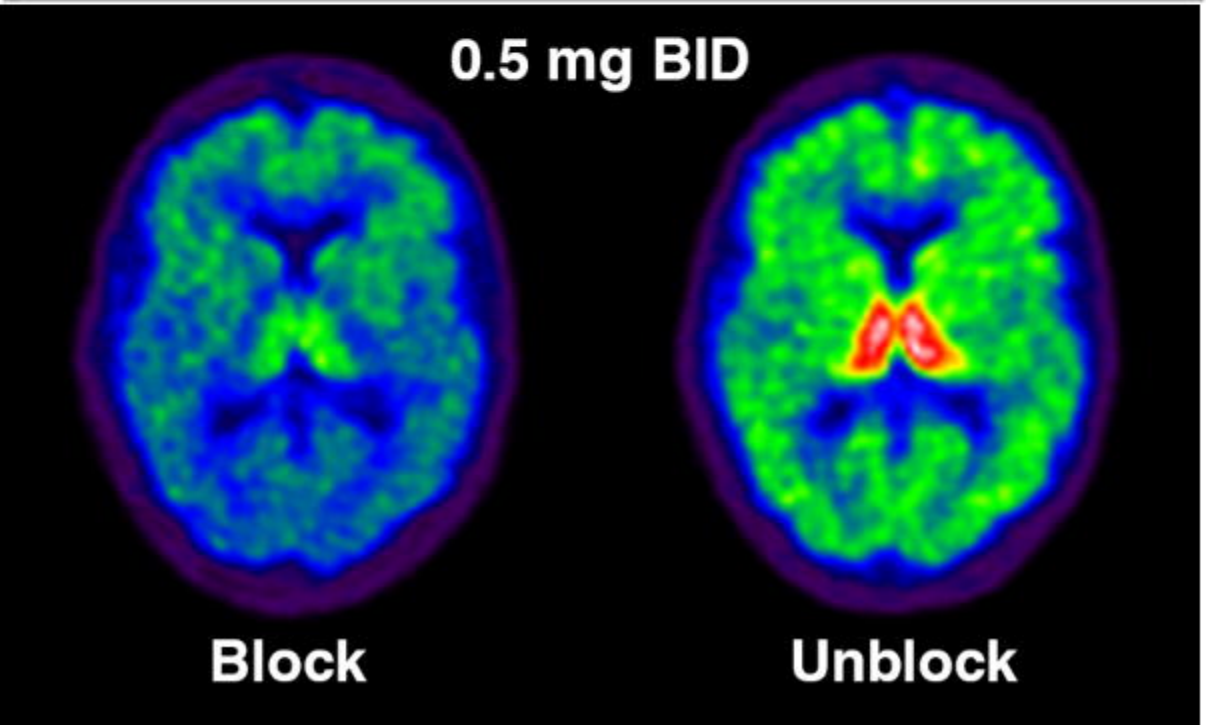
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