NeuroImage: Clinical 29 (2021) 102559

Contents lists available at ScienceDirect

NeuroImage: Clinical

journal homepage: www.elsevier.com/locate/ynicl

Real-time fMRI neurofeedback amygdala training may influence kynurenine pathway metabolism in major depressive disorder

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ARTICLE INFO

Keywords:
fMRI neurofeedback
Amygdala
Blood markers
Inflammation
Kynurenine pathway
Depression

ABSTRACT

Real-time fMRI neurofeedback (rtfMRI-nf) left amygdala (LA) training is a promising intervention for major depressive disorder (MDD). We have previously proposed that rtfMRI-nf LA training may reverse depression-associated regional impairments in neuroplasticity and restore information flow within emotion-regulating neural circuits. Inflammatory cytokines as well as the neuroactive metabolites of an immunoregulatory pathway, i.e. the kynurenine pathway (KP), have previously been implicated in neuroplasticity. Therefore, in this proof-of-principle study, we investigated the association between rtfMRI-nf LA training and circulating inflammatory mediators and KP metabolites. Based on our previous work, the primary variable of interest was the ratio of the NMDA-receptor antagonist, kynurenic acid to the NMDA receptor agonist, quinolinic acid (KynA/QA), a putative neuroprotective index. We tested two main hypotheses. i. Whether rtfMRI-nf acutely modulates KynA/QA, and ii. whether baseline KynA/QA predicts response to rtfMRI-nf.

Twenty-nine unmedicated participants who met DSM-5 criteria for MDD based on the Mini-International Neuropsychiatric Interview and had current depressive symptoms (Montgomery-Åsberg Depression Rating Scale (MADRS) score > 6) completed two rtfMRI-nf sessions to upregulate LA activity (Visit1 and 2), as well as a follow-up (Visit3) without rtfMRI-nf. All visits occurred at two-week intervals. At all three visits, the MADRS was administered to participants and serum samples for the quantification of inflammatory cytokines and KP metabolites were obtained. First, the longitudinal changes in the MADRS score and immune markers were tested by linear mixed effect model analysis. Further, utilizing a linear regression model, we investigated the relationship between rtfMRI-nf performance and immune markers. After two sessions of rtfMRI-nf, MADRS scores were significantly reduced ($t[58] = -4.07$, $p = 0.009$, $d = 0.56$). Thirteen participants showed a $\geq$ 25% reduction in the MADRS score (the partial responder group). There was a significant effect of visit ($F[2,58] = 3.17$, $p = 0.05$) for the neuroprotective index, KynA to 3-hydroxykynurenine (3-HK), that was driven by a significant increase in KynA/3-HK between Visit1 and Visit3 ($t[58] = 2.50$, $p = 0.03$, $d = 0.38$). A higher baseline level of KynA/QA ($\beta = 5.23$, $p = 0.06$; rho $= 0.49$, $p = 0.02$) was associated with greater ability to upregulate the LA. Finally, for exploratory purposes correlation analyses were performed between the partial responder and the non-responder groups as well as in the whole sample including all KP metabolites and cytokines. In the partial responder group, greater ability to upregulate the LA was correlated with an increase in KynA/QA after rtfMRI-nf (rho $= 0.75$, $p = 0.03$). The results are consistent with the possibility that rtfMRI-nf decreases metabolism down the so-called neurotoxic branch of the KP. Nevertheless, non-specific effects cannot be ruled out due to the lack of a sham control. Future, controlled studies are needed to determine whether the increase in KynA/3HK and KynA/QA is specific to rtfMRI-nf or whether it is a non-specific correlate of the resolution of depressive symptoms. Similarly,

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https://doi.org/10.1016/j.nicl.2021.102559
Received 17 June 2020; Received in revised form 30 October 2020; Accepted 9 January 2021
Available online 19 January 2021

replication studies are needed to determine whether KynA/QA has clinical utility as a treatment response biomarker.

1. Introduction

Major depressive disorder (MDD) is a devastating neuropsychiatric disease, resulting in severe social and economic burdens (Lopez and Murray, 1998; Nestler et al., 2002; Pincus and Pettit, 2001). However, the neurobiological mechanisms underlying the pathophysiology of MDD remain unclear. Although effective treatments are available, approximately 30% of MDD patients do not respond to psychotherapy or antidepressant medications (Rush et al., 2006). Alternative approaches such as electroconvulsive therapy (ECT) have greater efficacy, especially for treatment-resistant MDD, but also have significant side effects (Datto, 2000). There is thus an urgent need for novel treatments. Neuroscience-informed non-invasive brain neuromodulation approaches for ameliorating key MDD abnormalities may be one such treatment, with real-time functional magnetic resonance imaging neurofeedback (rtfMRI-nf) serving as a promising example.

RtfMRI-nf can be used as brain-based therapies by providing individuals with information about their brain activity and offering them an opportunity to learn how to control their brain activity (Cox et al., 1995; deCharms et al., 2004; Weiskopf et al., 2007). In previous studies, we leveraged recent advances in rtfMRI-nf to provide MDD individuals with training to enhance their capacity to upregulate left amygdala (LA) activity in response to the recall of positive autobiographical memories (Young et al., 2017, 2014; Yuan et al., 2014; Zotev et al., 2016, 2011). In a previous randomized, sham-controlled clinical trial, we showed that this rtfMRI-nf training is an effective method of reducing cognitive bias and depressive symptoms in MDD (Young et al., 2018, 2017).

Although these data supporting the clinical efficacy of rtfMRI-nf have revealed system level brain circuit effects and associations with electroencephalogram (EEG) signals (Zotev et al., 2016, 2013), the molecular mechanisms through which rtfMRI-nf reduces depressive symptoms are unknown. We have previously proposed that rtfMRI-nf LA training reverses depression-associated regional impairments in the brain neuroplasticity and can restore information flow within emotion-regulating neural circuits (Young et al., 2018). Thus, the molecular mechanisms underlying rtfMRI-nf may relate to neuroplasticity.

It is now well established that 'neuroplasticity,' the remodeling of brain structure and function, occurs throughout life (Holtmaat and Svoboda, 2009; Kessels and Malinow, 2009; Yoshihara et al., 2009). Imaging studies show that on average hippocampal volume is reduced in MDD (Arnone et al., 2012; Peng et al., 2016; Zhao et al., 2014) and consistent with these data, postmortem samples from depressed individuals demonstrated decreased dendritic and spine densities, and altered expression of neuroplasticity-related genes in the hippocampus and amygdala compared to control samples (Cobb et al., 2013; Maheu et al., 2013; Stockmeier et al., 2004). The hippocampus and amygdala are also particularly vulnerable to degenerative changes caused by chronic stress (Vyas et al., 2016). Environmental risk factors (e.g., stress, inflammation) can contribute to the onset, development and course of depression through disrupted neuronal function and morphology (Bao et al., 2008; Hurley and Tizabi, 2013; Ross et al., 2018; Wuwongse et al., 2010). Accumulating evidence has shown that antidepressant treatments for MDD improve mood, cognition and behavior associated with depression as well as reverse deficits in brain neuroplasticity (Duman et al., 2016; Hayley and Litteljohn, 2013; Schwieler et al., 2016).

To adapt to the changing environment, neurons rearrange synaptic connections via the modification of dendrites, dendritic spines, and axons (Citri and Malenka, 2008). These connections grow, shrink, form *de novo*, and are eliminated in a highly dynamic manner that allows for experience-dependent optimization of neuronal circuits (Forrest et al., 2018; Yang et al., 2009). One important way that this occurs is through long-term potentiation (LTP) or long-term depression (LTD). LTP and LTD refer to the activity-dependent enhancement or weakening of synaptic connections via a complex process mediated by N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor signaling (Diering and Huganir, 2018; Lisman, 2017; Volianskis et al., 2015). The immune system affects synaptic plasticity through several pathways (Lynch, 2015; O'Reilly and Tom, 2020; Rizzo et al., 2018; Savitz, 2020; Yirmiya and Goshen, 2011): 1) cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF) alter the expression of glutamate and gamma-aminobutyric acid (GABA) receptor subunits, 2) astrocytes and microglia interact to control the release and reuptake of glutamate, 3) microglia prune synapses that are tagged with "eat me" signals (i.e. complement proteins) by leveraging the same molecular machinery used by immune cells to "eat" microorganisms, and 4) changes in the kynurenine pathway (KP) metabolism may alter the balance of NMDA receptor signaling.

Regarding the KP, tryptophan (TRP) can be metabolized into either kynurenine (KYN) or serotonin. KYN, in turn, is principally metabolized along two physiologically-separated branches via either microglia/macrophages or astrocytes to produce the neuroactive compounds quinolinic acid (QA) and kynurenic acid (KynA) (Fig. 1). Astrocytes mainly produce KynA, an NMDA receptor antagonist that is often considered to be neuroprotective, while activated microglia or macrophages metabolize KYN into the free-radical generator 3-hydroxykynurenine (3-HK) and ultimately QA, an NMDA receptor agonist that can cause neurotoxicity via several different mechanisms (Guillemin, 2012; Savitz, 2020). Under inflammatory conditions, pro-inflammatory cytokines such as interferon gamma (IFNg) and TNF upregulate the enzymes, indoleamine 2,3 dioxygenase (IDO) and kynurenine monooxygenase (KMO) thus favoring the breakdown of TRP into KYN, and KYN into 3-HK (and subsequently QA), respectively. The link between inflammation and depression may explain why we and others have consistently found either decreases in KynA or increases in QA in depression (Bay-Richter et al., 2015; Myint et al., 2007; Savitz et al., 2015b; Wurfel et al., 2017). This change in the balance of the KP may alter glutamatergic transmission because of the opposing effects of KynA and QA on the NMDA receptor (Miller et al., 2013; Savitz, 2020; Steiner et al., 2012). Alterations in glutamatergic neurotransmission via changes to NMDA receptors are, in turn, essential mediators of activity-dependent synaptic plasticity (Paoletti et al., 2013). Accordingly, KynA and QA may play a role in synaptic plasticity and constitute treatment response or therapeutic monitoring biomarkers for treatments that involve neuroplasticity such as rtfMRI-nf (Savitz, 2020).

This exploratory, proof-of-principle investigation aimed to examine whether rtfMRI-nf acutely modulates inflammatory cytokines and KP metabolites (Aim 1) and further whether baseline concentrations of these immune markers influence the capacity for adaptive neuroplasticity via rtfMRI-nf (Aim 2). Based on our previous work, the primary outcome of interest was KynA/QA, a putative index of neuroplasticity and neuroprotection.

2. Methods and materials

2.1. Design

MDD participants initially completed a centralized screening protocol at the Laureate Institute for Brain Research (LIBR). Thereafter, individuals who met entrance criteria for this naturalistic clinical trial of rtfMRI-nf LA provided written informed consent and received financial compensation for participation. The study was approved by the Western Institutional Review Board (IRB). Participants completed two

sessions of rtfMRI-nf training (Fig. 2; Visit1: baseline and Visit2: mid-point), as well as a follow-up (Visit3: endpoint) without rtfMRI-nf. All visits occurred at two-weeks intervals. At all three visits, the Montgomery-Åsberg Depression Rating Scale (MADRS) (Montgomery and Åsberg, 1979) was administered to participants and serum samples for the quantification of cytokines and KP metabolites were obtained.

2.2. Participants

Unmedicated MDD participants naive to rtfMRI-nf were recruited through the LIBR database. Participants aged 18 to 55 years met DSM-5 criteria for MDD based on the Mini-International Neuropsychiatric Interview 7.0.2 (MINI: Sheehan et al., 1998) and had current depressive symptoms (MADRS score $>$ 6). Exclusion criteria were as follows: pregnancy; previous exposure to neurofeedback; bodily implants of unsafe paramagnetic materials such as pace-makers and aneurysm clips; severe claustrophobia; psychosis; clinically-significant suicidal ideation; history of head injury with loss of consciousness; presence of an uncontrolled medical condition that was deemed by the investigators to interfere with the proposed study procedures or to put the study participant at undue risk; use of psychotropic medications within 6 weeks of the study (8 weeks for fluoxetine); current use of hormone-containing medications (excluding contraceptives and thyroxine); non-steroidal anti-inflammatory drugs that were deemed by the investigators to potentially confound the results of the study (e.g. $>$3 days/week); new-onset (or recent change in) cardiovascular medications, including antiarrhythmic, anti-anginal, and anticoagulant drugs; evidence of recreational drug use from urine test (except cannabis); BMI $\geq$ 40; acute infectious illness in the week prior to the study. Prior to enrolling in the study, each potential participant underwent a screening evaluation that included a medical and psychiatric assessment as well as a clinical MRI. A recruitment flow diagram is available in Supplementary Figure S1.

2.3. Immunophenotyping

Morning blood samples were drawn by venipuncture. Serum samples were collected with BD Vacutainer serum tubes, processed according to the standard BD Vacutainer protocol, and stored at −80 °C. Cytokines were measured with the Meso Scale Discovery (MSD) QuickPlex SQ 120 instrument and MSD V-PLEX assay kit. The lowest levels of quantification (LLOQ) were as follows: IL-6 = 0.04 pg/mL; TNF = 0.02 pg/mL; IL-10 = 0.01 pg/mL; C–C motif chemokine ligand 2 (CCL2) = 0.46 pg/mL. Samples were run in duplicate with mean intra-assay coefficients of variation of 6.33%, 2.84%, 5.33%, and 1.45% for IL-6, TNF, IL-10, and CCL2, respectively. Concentrations of TRP, KYN, KynA, 3-HK, picolinic acid (PA), and QA were measured blind to time point by Keystone Bioanalytical, Inc (https://www.keystonebioanalytical.com/). The serum metabolite concentrations were determined by high performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection using their standard protocols. CVs and LLOQ are shown in Supplementary Table S1. The KYN to TRP ratio (KYN/TRP) is usually considered to be a surrogate marker of IDO activity, i.e., activation of the KP. Similarly, KynA/3-HK and KynA/QA are often used as indices of the relative metabolism down the KynA branch relative to the QA (neurotoxic) branch of the KP. KynA/QA was considered as the primary outcome in this study, and other inflammatory markers were used for exploratory analyses.

2.4. Psychological assessment

The MADRS was used to measure the severity of depressive symptoms at baseline, mid-point and endpoint as a secondary outcome. Participants who experienced a 25% or greater reduction of the MADRS score at endpoint were classified as partial responders based on previous studies (Mauskopf et al., 2009; Nierenberg and DeCecco, 2001).

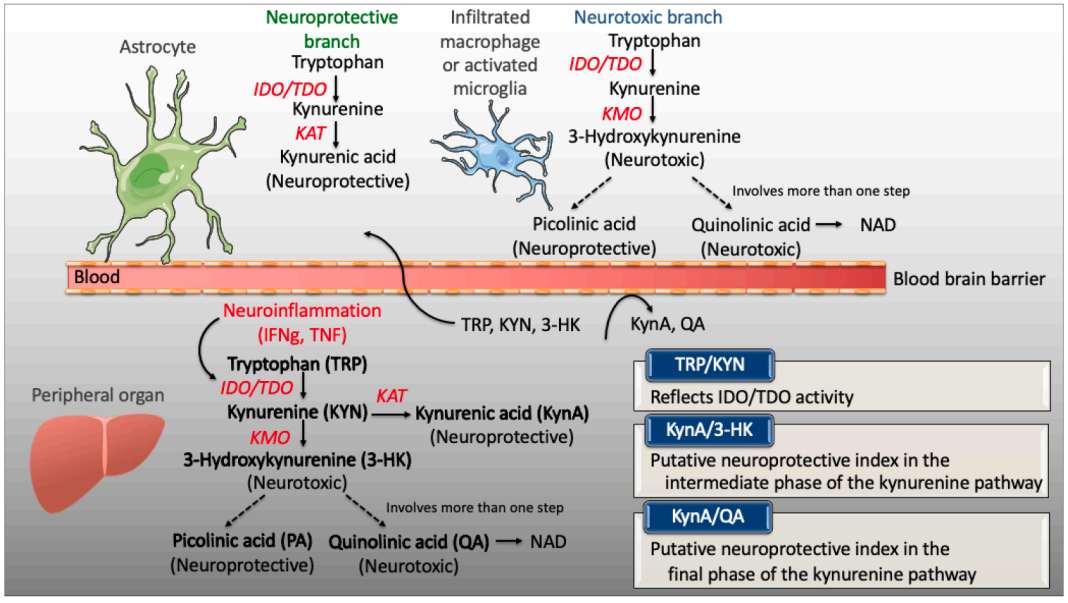

Fig. 1. The kynurenine pathway (KP) of tryptophan catabolism. Activation of the hypothalamic–pituitaryadrenal (HPA) axis can lead to the induction of tryptophan 2, 3-dioxygenase (TDO). The release of pro-inflammatory cytokines such as interferon gamma (IFNg) and tumor necrosis factor (TNF) can also induce the production of indoleamine 2, 3-dioxygenase (IDO). TDO and IDO catalyze the conversion of tryptophan (TRP) to kynurenine (KYN). KYN is then converted to either kynurenic acid (KynA) by the kynurenine aminotransferases (KATs) or 3-hydroxykynurenine (3-HK) by kynurenine monooxygenase (KMO). 3-HK is further metabolized to either picolinic acid (PA) or quinolinic acid (QA). QA is a precursor for nicotinamide adenine dinucleotide (NAD). Within the central nervous system (CNS), the KP is thought to be differentially metabolized in astrocytes or microglia. TRP uptake and metabolism in astrocytes leads to the production of the KynA and PA, which are often considered to be neuroprotective in the CNS. On the other hand, TRP uptake and metabolism in microglia leads to the production of the 3-HK and QA. QA acts as an agonist at the glutamate N-methyl-D-aspartate (NMDA) receptor and is excitotoxic and neurotoxicity at elevated concentrations. The KP metabolites measured in this study are bolded in the figure.

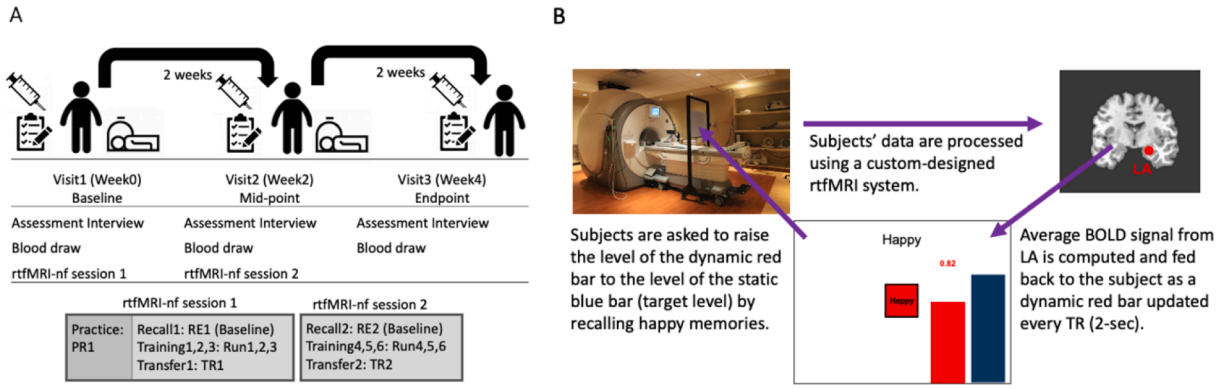

Fig. 2. Study design. **A)** rtfMRI-neurofeedback (rtfMRI-nf) visits and follow-up visit. Participants completed two sessions of rtfMRI-nf (Visit1: baseline and Visit2: mid-point), as well as a follow-up (Visit3: endpoint) without rtfMRI-nf. All visits occurred at two-weeks intervals. At all three visits, the Montgomery-Åsberg Depression Rating Scale (MADRS) was administered to participants and serum samples for the quantification of cytokines and the kynurenine pathway metabolites were obtained. **B)** rtfMRI-nf system components. During the rtfMRI-nf block ('Happy' block), participants see a screen inside the scanner. The red bar represents the actual neurofeedback signal from the left amygdala (LA), which is updated continuously by changing the height of the bar either upward or downward based on the corresponding level of blood-oxygen-level-dependent (BOLD) signal. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.5. rtfMRI-nf LA training

Our real-time fMRI system allows us to measure and display blood-oxygen-level-dependent (BOLD) signal "on the fly" and provide visual feedback in the form of a moving red bar graph depicting a subject's corresponding level of LA regional activity (Fig. 2B). Details are given in our previous studies (Young et al., 2017, 2014; Zotev et al., 2011). Briefly, each session of rtfMRI-nf LA training is comprised of five rtfMRI-nf runs: recall (RE), three training runs (Run1, 2, 3), and a transfer run (TR). Each 8-min, 40-sec run consisted of alternating 40-sec blocks of three conditions: Happy Memories, Count, and Rest. During rtfMRI-nf training, the cue 'Happy' and two-colored bars (red, blue; Fig. 2B) are displayed on the screen. The red bar represents the actual neurofeedback signal from the LA, which is updated continuously by changing the height of the bar either upward or downward based on the corresponding level of BOLD signal changes Subjects were instructed to retrieve and contemplate a positive memory while attempting to increase the level of the red bar to that of the fixed target blue bar. The participants were given a chance to practice before the first rtfMRI-nf session (practice run: PR). In the initial (RE) and the last runs (TR), participants were instructed to recall happy memories without assistance from neurofeedback signals. The initial non-neurofeedback run (RE) assessed the individual's baseline ability to upregulate the LA, and the last non-neurofeedback run (TR) tested the maintained, or transferred, ability to upregulate the LA after the neurofeedback training. The upregulation of the LA was defined as the mean % BOLD signal change for the Happy vs. Rest contrast. The individual's ability to upregulate LA activity during the training and transfer runs compared to the baseline runs was defined as a neurofeedback performance, i.e., the baseline recall run (RE) subtracted from the sum of the three training runs and transfer runs: (Run1 + Run2 + Run3 + TR1) - RE1 at Visit1 and (Run4 + Run5 + Run6 + TR2) - RE2 at Visit2 (Fig. 2A).

2.6. MRI Imaging

Imaging was performed on a 3 T MR750 Discovery (GE Healthcare) MRI scanner. BOLD fMRI data were acquired using a T2*-weighted gradient echo-planar sequence with sensitivity encoding (ge-EPI SENSE) with the following parameters: TR/TE = 2000/30 ms, acquisition matrix = 96 × 96, FOV/slice thickness = 240/2.9 mm, flip angle = 90°, voxel size 2.5 × 2.5 × 2.9 mm^3, 34 axial slices, SENSE acceleration R = 2, sampling bandwidth = 250 kHz. For anatomical reference, T1-weighted MRI structural images were acquired with a magnetization-prepared rapid gradient-echo (MPRAGE) sequence with the following parameters: TR/TE = 5/2 ms, acquisition matrix = 256 × 256, FOV/slice thickness = 240/1.2 mm, flip angle = 8°, 0.94 × 0.94 × 1.2 mm^3 voxel volume, 124 axial slices, SENSE acceleration R = 2, delay/inversion time TD/TI = 1400/725 ms, sampling bandwidth = 31.25 kHz, scan time = 4 min 59 s.

2.7. Off-line image analysis and index of neurofeedback performance

Analysis of Functional NeuroImages package (AFNI; http://afni.nimh.nih.gov) (Cox, 1996) was employed for the off-line image preprocessing. The first three TRs were discarded from the analysis. The process included despike, RETROICOR (Glover et al., 2000) and respiration volume per time (RVT) correction (Birn et al., 2008), slice-timing and motion corrections, nonlinear warping to the MNI template brain with resampling to 2 mm^3 voxels using the ANTs, spatial smoothing with 6 mm-FWHM Gaussian kernel, and scaling signal to percent change relative to the mean in each voxel. General linear model (GLM) analysis was used for evaluating the brain response in each run. The design matrix included regressors of the two task block regressors ('Happy' and 'Count') modeled with a box-car function convolved with hemodynamic response function (HRF), noise regressors of three principal components of the ventricle signal, local white matter average signal (ANATICOR) (Jo et al., 2010), 12 motion parameters (three shift and three rotation parameters with their temporal derivatives), and low-frequency fluctuation (4th-order Legendre polynomial model). Any time point with large motion (>0.30 mm frame-wise displacement (FD)) was censored within the regression (Power et al., 2015). The beta coefficient of the Happy block regressor was extracted, and then converted to percent signal changes for 'Happy' versus 'Rest' contrast to estimate brain activation of the LA during the 'Happy' block (rtfMRI-nf period). The longitudinal change of the task-related LA activity was tested by linear mixed effect model analysis (LME, *lme4* package) (Bates et al., 2015) in R version 3.5.3. The LME model included fixed effects of time, i.e., experimental runs (RE1, Run1-3, TR1, RE2, Run4-6, TR2), group (partial responders and non-responders), time by group interaction, age, sex, LA BOLD signal at PR1 (individual's ability to upregulate the LA during the practice run), and the random effect of the subject on intercept.

2.8. Statistical analyses

Serum cytokines and KP metabolites had non-Gaussian distributions and were log-transformed for the statistical analyses. Utilizing within-

subject analyses we investigated the antidepressant effect and immunological changes after rtfMRI-nf (Aim 1). The longitudinal changes in the MADRS score and immune markers were tested by linear mixed effect model analysis (LME, *lme4* package) (Bates et al., 2015) in R version 3.5.3, respectively. The LME model included the fixed effects of time (Visit1 [baseline], Visit2 [mid-point], Visit3 [endpoint]), age, sex, and the random effect of the subject on intercept. For the immune markers, group (partial responders and non-responders) and time by group interaction were also included as fixed effects. A post-hoc analysis of time effect was applied using Dunnett's multiple comparison method with Visit1 (baseline) as a reference point by *lsmeans* package (Lenth, 2016). Utilizing a linear regression model, we investigated the relationship between rtfMRI-nf performance and immune markers (Aim 2). We performed a linear regression analysis predicting rtfMRI-nf performance based on the baseline level of KynA/QA. Further, to evaluate the potential modulatory effect of KynA/QA, a linear regression analysis was performed predicting changes in the MADRS score based on rtfMRI-nf performance, changes in KynA/QA, and the interaction of these variables. Variables used in interaction terms were centered. In the case of significant results, a Huber robust regression analysis with MM-estimation (Huber, 1973) which provides a more conservative estimate of correlational associations by minimizing the effect of outliers, was also performed (*MASS* package) (Venables and Ripley, 2002) in R. Finally, for exploratory purposes further correlation analyses were performed between the partial responder group and the non-responder group as well as in the whole sample including all KP metabolites and cytokines. Because of the relatively small sample size, we used Spearman's rho (r) for the correlation analyses. For the primary outcome variable, KynA/QA, results with a p-value < 0.05 were considered statistically significant and a p-value < 0.1 were considered to be trending significant. For all other immune markers we performed a False Discovery Rate (FDR)-correction for multiple comparisons.

3. Results

3.1. Demographic data

Table 1 shows participant demographic information. Other background information related to clinical measurements is summarized in Supplementary Table S2. The changes in the LA BOLD signal during the rtfMRI-nf sessions are shown in Supplementary Figure S2.

3.2. Immune markers

The inter-correlations between cytokines and the KP metabolites at baseline are shown in Supplementary Table S3. KYN, QA and 3-HK were positively correlated with TNF while KynA/3-HK was inversely correlated with IL-6, but positively correlated with CCL2. There was no significant difference between partial responders and non-responders in the concentration of cytokines and the KP metabolites at baseline.

3.3. Anti-depressant effect of rtfMRI-nf

There was a significant main effect of visit (F [2, 58] = 5.12, p = 0.009, Supplementary Figure S3). Post-hoc analysis showed that a significant reduction in the MADRS score between Visit1 and Visit2 (t [58] = −2.55, p = 0.03, d = 0.48), and also between Visit1 and Visit3 (t [58] = −4.07, p = 0.009, d = 0.56). There was no significant association between rtfMRI-nf performance and change in the MADRS score in the whole sample (rho = −0.11, p = 0.60); however, better neurofeedback performance was inversely associated with the change in the MADRS score in the partial responder group although this association was at trend level (rho = −0.53, p = 0.09, Supplementary Figure S4). No significant association between rtfMRI-nf performance and MADRS score change was found in the non-responder group (rho = 0.01, p = 0.98).

Table 1
Demographic data.

	Partial responders	Non-responders		
	N = 13	N = 16	statistics	p-value
Age in years[a]	29.46 (7.46)	29.25 (9.50)	t (27) = 0.06	0.95
Male:Female	5:8	7:9	$\chi^2(1)$ = 0.08	0.77
MADRS[a]	23.38 (5.23)	23.13 (6.93)	t (27) = 0.11	0.91
Depression episode				
Single episode: Recurrent	5:8	3:13	$\chi^2(1)$ = 1.40	0.24
Treatment history[b]				
Antidepressant medications	8 (67%)	8 (50%)	$\chi^2(1)$ = 0.49	0.48
Psychotherapy/ counseling	6 (53%)	9 (60%)	$\chi^2(1)$ = 0.27	0.60
Comorbidity				
GAD	4 (31%)	11 (69%)	$\chi^2(1)$ = 4.14	**0.04**
Panic Disorder	3 (23%)	5 (31%)	$\chi^2(1)$ = 0.24	0.62
SAD	4 (31%)	10 (63%)	$\chi^2(1)$ = 2.89	0.09
PTSD	7 (54%)	2 (13%)	$\chi^2(1)$ = 5.73	**0.02**
SUD	3 (23%)	1 (6%)	$\chi^2(1)$ = 1.71	0.19
CTQ[a,c]	51.33 (21.68)	57.50 (18.83)	t (19) = −0.66	0.52
Emotional abuse[a,c]	12.44 (6.67)	14.42 (6.32)	t (19) = −1.40	0.18
Emotional neglect[a,c]	11.89 (4.15)	14.83 (4.79)	t (19) = 0.27	0.79
Physical abuse[a,c]	9.67 (4.45)	9.08 (4.94)	t (19) = −1.44	0.17
Physical neglect[a,c]	8.44 (2.59)	11.17 (4.90)	t (19) = 0.32	0.76
Sexual abuse[a,c]	8.89 (7.37)	8.00 (4.90)	t (19) = −0.66	0.52
Education[d]				
12th grade, nodeploma	1 (8%)	0 (0%)	$\chi^2(1)$ = 1.12	0.29
GED or equivalent	2 (15%)	1 (7%)	$\chi^2(1)$ = 0.46	0.50
High school graduate	2 (15%)	2 (14%)	$\chi^2(1)$ = 0.01	0.93
Some college, no degree	6 (46%)	7 (50%)	$\chi^2(1)$ = 0.04	0.84
Associate degree	1 (8%)	3 (21%)	$\chi^2(1)$ = 1.01	0.32
Bachelors degree	1 (8%)	1 (7%)	$\chi^2(1)$ = 0.003	0.96
Race/Ethinicity[d]				
Black/African American	1 (8%)	1 (7%)	$\chi^2(1)$ = 0.003	0.96
Indian (American)	2 (15%)	0 (0%)	$\chi^2(1)$ = 2.33	0.13
Other	1 (8%)	1 (7%)	$\chi^2(1)$ = 0.003	0.88
White	9 (69%)	12 (86%)	$\chi^2(1)$ = 1.06	0.30
Hispanic	2 (15%)	1 (7%)	$\chi^2(1)$ = 0.46	0.50

Partial responders: participants who achieved 25% or greater reduction in the MADRS score at endpoint; non-responders: participants who did not achieve 25% or greater reduction in the MADRS score. a: mean (standardized deviation), b: n = 1 data was not available for the partial responders group and the non-responders group, respectively, c: n = 4 data were not available for the partial responders group and the non-responders group, respectively, d: n = 2 data were not available for the non-responders group. GAD = generalized anxiety disorder, SAD = social anxiety disorder, PTSD = post traumatic stress disorder, CTQ =

Childhood Trauma Questionnaire (Bernstein and Fink, 1998). Bolded values: $p < 0.05$ (uncorrected for multiple comparisons).

3.4. Changes in inflammatory cytokines and KP metabolites over the course of the trial

Fig. 3A shows the changes in KynA/QA as well as the KP metabolites across all visits and Fig. 3B shows the changes in inflammatory cytokines. There was no significant main effect of visit or group by visit interaction effect for the primary outcome, KynA/QA (visit: F [2, 58] = 1.85, $p = 0.17$; interaction: F [2, 58] = 0.45, $p = 0.63$; increase in KynA/QA between Visit1 and Visit3: t [58] = 1.85, $p = 0.12$, $d = 0.34$). Further exploratory analyses revealed that, among the KP metabolites, KynA/3-HK showed a significant effect of visit (F [2, 58] = 3.17, $p = 0.05$) driven by a significant increase in KynA/3-HK between Visit1 and Visit3 (t [58] = 2.50, $p = 0.03$, $d = 0.38$). There were no other statistically significant results.

3.5. Relationship between baseline concentrations of immune markers and neurofeedback performance

A linear regression analysis was performed to determine whether the baseline level of KynA/QA could predict rtfMRI-nf performance. The regression equation trended significant (F[1,24] = 7.04, $p = 0.06$) with an R^2 of 0.14, and the baseline level of KynA/QA nominally predicted rtfMRI-nf performance ($\beta = 5.23$, $p = 0.06$, Fig. 4). A Huber robust regression analysis revealed that the baseline level of KynA/QA significantly predicted rtfMRI-nf performance ($\beta = 5.48$, $p = 0.03$, Fig. 4) by minimizing the effect of potential outliers. There were no other statistically significant baseline immune markers predicting rtfMRI-nf performance.

Higher baseline KynA/QA (rho = 0.49, $p = 0.02$) was correlated with

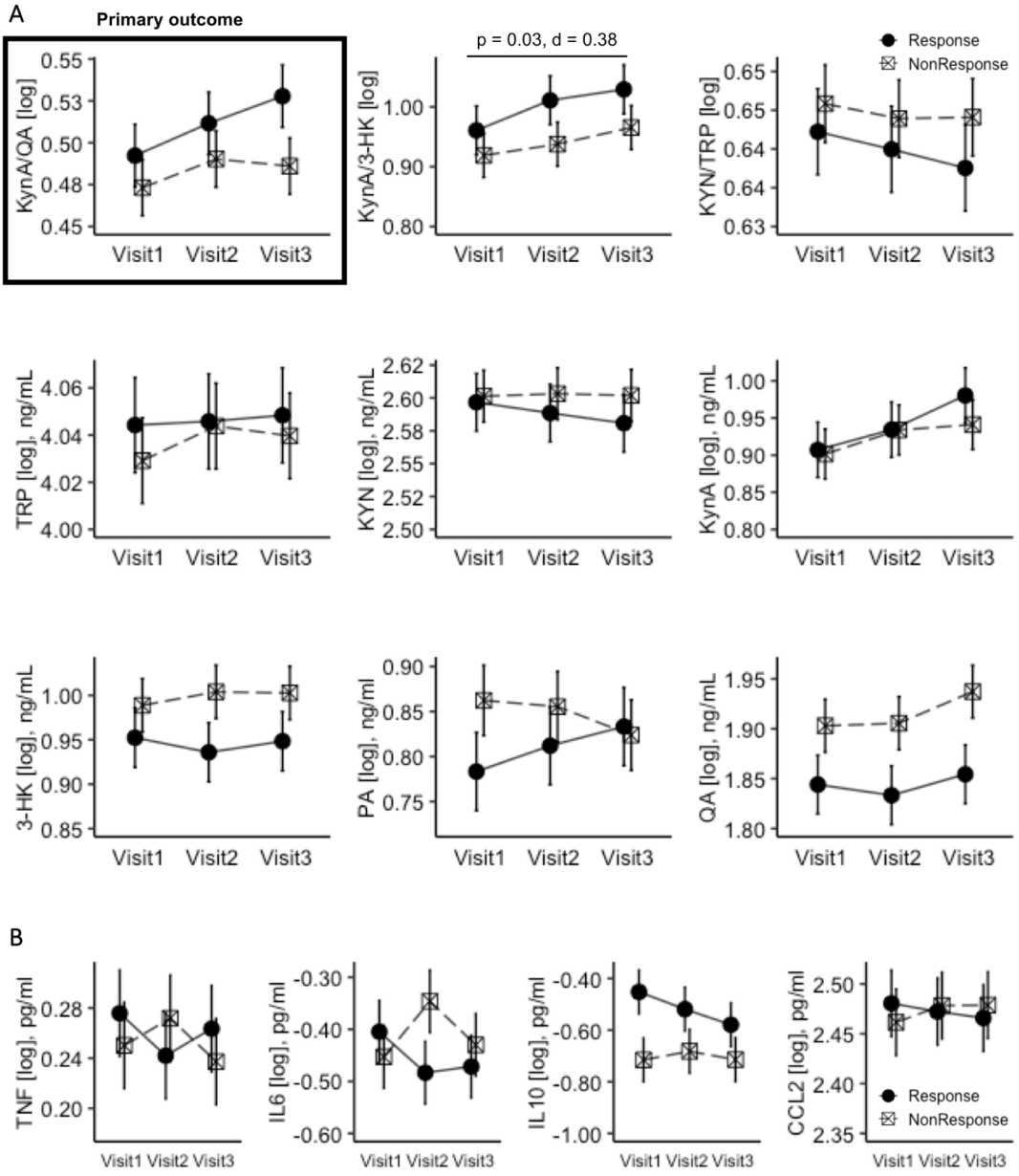

Fig. 3. Changes in immune markers over time. **A)** Changes in the KP metabolites between the partial responder and non-responder groups over the course of the trial. **B)** Changes in inflammatory cytokines between the partial responder group (Response) and the non-responder group (NonResponse) over the trial (Visit1: baseline, Visit2: mid-point, Visit3: endpoint). The error bars represent the standard error of the mean. Abbreviations: tryptophan: TRP, kynurenine: KYN, kynurenic acid: KynA, 3-hydroxykynurenine: 3-HK, picolinic acid: PA, quinolinic acid: QA, tumor necrosis factor: TNF, interleukin: IL, C–C motif chemokine ligand 2: CCL2.

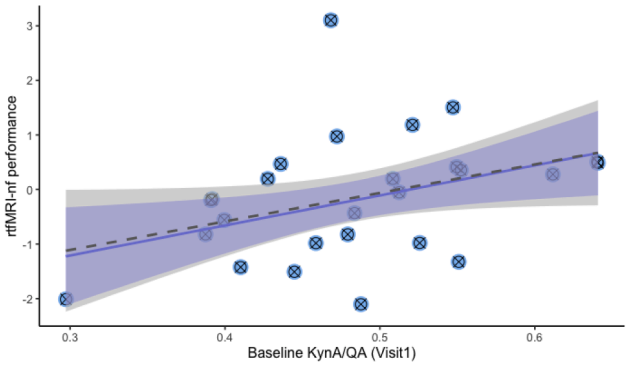

Fig. 4. Scatter plot with line of best fit showing the correlation between baseline serum level of KynA/QA and rtfMRI-nf performance (N = 26; N = 3 data were not available due to the MRI technical issues during the last transfer run). A dashed black line represents a best fit of a linear regression analysis and a blue line represents a best fit of a robust regression analysis. Shading around the lines represents the 95% confidence interval.

greater rtfMRI-nf performance. Higher baseline concentrations of CCL2 (rho = 0.50, $p = 0.04$, uncorrected), KynA (rho = 0.43, $p = 0.04$, uncorrected), and KynA/3-HK (rho = 0.46, $p = 0.03$, uncorrected) were also correlated with better rtfMRI-nf performance but these results did not remain significant after correction for multiple comparisons (Supplementary Table S4).

3.6. Relationship between changes in immune markers, neurofeedback performance and changes in depression scores

A linear regression analysis was performed to predict changes in the MADRS score based on rtfMRI-nf performance, changes in KynA/QA, and the interaction of these variables. The regression equation was not significant ($F[3, 22] = 0.83$, $p = 0.49$) with an R^2 of 0.10. We further investigated (1) the correlations between changes in immune markers and rtfMRI-nf performance, and (2) the correlations between changes in immune markers and changes in the MADRS score for exploratory purposes.

3.6.1. Correlation between changes in immune markers and neurofeedback performance

In all participants, better rtfMRI-nf performance was nominally correlated with the magnitude of change in KynA/QA between Visit1 and Visit3 (rho = 0.40, $p = 0.07$, Fig. 5A). When considering partial responders only, neurofeedback performance was significantly correlated with the magnitude of change in KynA/QA (rho = 0.75, $p = 0.03$, Fig. 5A). No statistically significant relationship was observed in the non-responder group. Regarding the cytokines, better neurofeedback performance was correlated with the magnitude of change in TNF (rho = 0.52, $p = 0.04$, uncorrected) and CCL2 (rho = 0.56, $p = 0.02$, uncorrected) across all subjects although these results were no longer significant after controlling for multiple comparisons (Supplementary Table S5).

3.6.2. Correlation between changes in immune markers and depression scores

There were no significant associations between changes in the immune markers and changes in the MADRS score between Visit1 and Visit3 across all participants (Supplementary Table S6). Nevertheless, the magnitude of change in KynA/QA between Visit1 and Visit3 was inversely but not significantly correlated with the magnitude of change in MADRS scores in the partial responder group (rho = −0.65, $p = 0.06$, Fig. 5B). In the non-responder group, the magnitude of changes in KYN/TRP (rho = −0.63, $p = 0.03$, uncorrected) and KynA/3-HK (rho = 0.65, $p = 0.02$, uncorrected) were correlated with the magnitude of change in MADRS scores, but these results did not remain significant after correction for multiple comparisons.

4. Discussion

This experimental medicine proof-of-concept study examined changes in inflammatory cytokines and KP metabolites pre-vs-post rtfMRI-nf in individuals with MDD. There were two principal findings. First, serum KynA/3-HK was significantly increased after two sessions of rtfMRI-nf (Aim 1, Fig. 3), raising the possibility that the therapeutic effects of rtfMRI-nf may be related to a shift in KP metabolism away from the so-called neurotoxic branch of the pathway. This result should be interpreted with caution since KynA/3HK was not our primary outcome and the result was no longer significant after FDR-correction. Nevertheless, because 3HK is a precursor of QA, KynA/3HK was as expected significantly correlated with KynA/QA (rho = 0.82, Supplementary Table S3). Further, a perusal of Fig. 3 shows a (non-significant) increase in KynA/QA over time.

Additional exploratory analyses demonstrated that within the partial responder group, a greater upregulation of the LA was positively correlated with a shift in the balance of neuroprotective versus neurotoxic KP metabolites (i.e., KynA/QA) over the course of the trial (Supplementary Table S5). Further, the magnitude of the rtfMRI-nf-induced increase in KynA/QA was associated at trend level with the reduction in MADRS scores in the partial responder group (Supplementary Table S6). These results are consistent with studies of the KP in the context of other treatment modalities. Serum KynA/QA was shown to increase after three ECT treatments performed over two weeks although in this case the effect was driven by a decrease in QA rather than an increase in KynA (Schwieler et al., 2016). However, in another ECT study, depressed patients showed increases in both KynA and KynA/3-HK after

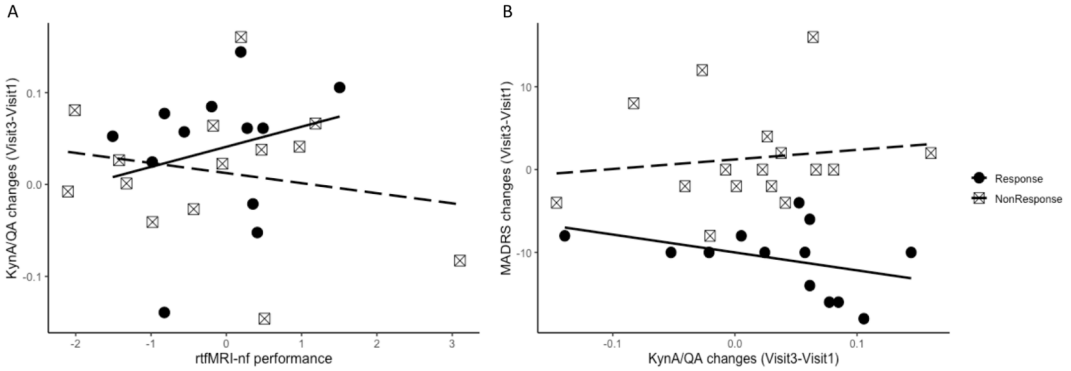

Fig. 5. Scatter plots with fitted lines. **A)** rtfMRI-nf performance and changes in KynA/QA (Visit3-Visit1). **B)** Changes in KynA/QA (Visit3-Visit1) and changes in the MADRS score (Visit3-Visit1). 'Response' indicates individuals in the partial responder group, and 'NonResponse' indicates individuals in the non-responder group.

twice-weekly treatment for an average of three weeks (Guloksuz et al., 2015). Similarly, exercise, which has anti-depressant effects (Harvey et al., 2018), was found to increase plasma concentrations of KynA as well as KynA/QA (Schlittler et al., 2016). Our results are also partially consistent with two studies that reported that response to ketamine is associated with an increase in KynA. In one study, ketamine responders displayed increased KynA as well as KynA/KYN from 24 h after the first infusion until at least two weeks after the initiation of treatment (Zhou et al., 2018) while in the second study, ketamine treatment increased KynA concentrations and decreased QA/KYN concentrations within three days of infusion in patients with treatment-resistant bipolar disorder (Kadriu et al., 2019). The potential ability of rtfMRI-nf to increase metabolism down the KynA pathway is also consistent with our previous reports of a positive correlation between hippocampal and amygdalar volume and KynA/QA (Savitz et al., 2015a, 2015b).

The above results raise the possibility that rtfMRI-nf can "re-balance" the KP, thereby protecting against inflammation-induced dendritic remodeling and neurodegeneration by modulating NMDA receptor signaling via the competing effects of KynA and QA. The specific pathway through which rtfMRI-nf-induced modulation of central KP metabolism putatively alters peripheral KP metabolism is unclear since comparatively little is known about how the brain regulates the immune system. What is known is that the hypothalamic–pituitaryadrenal (HPA) axis regulates immunity via the secretion of hormones, the para/sympathetic nervous system alters immunity via "hard-wired" connections between the vagus and other sensory nerves and the lymph nodes, and the meningeal lymphatic system delivers immune cells and immune-related signals to the periphery (Schiller et al., 2020). Conceivably, a rtfMRI-nf-mediated restoration of hippocampal-amygdalar function has "top-down" effects on peripheral immunity via one or more of these pathways. Whether this putative mechanistic pathway is specific to rtfMRI-nf or common to diverse anti-depressant therapies remains unknown.

The second principal finding in this study was that higher baseline KynA/QA predicted better ability to upregulate the LA over the course of the trial (Aim 2, Fig. 4). The effect was driven by KynA, i.e., higher baseline concentrations of KynA and KynA/3-HK were also predictive of ability to upregulate the LA during positive autobiographical memory recall (Supplementary Table S4). This result raises the possibility that the relative balance between neuroprotective and neurotoxic KP metabolites (e.g., KynA/QA) may serve as a marker of neuroplastic potential and accordingly, may predict who is likely to respond to rtfMRI-nf. Interestingly, higher baseline serum concentrations of CCL2 were also associated with better rtfMRI-nf performance (Supplementary Table S4) although this result should be interpreted with caution since it was no longer significant after FDR-correction. Further, unlike many of the KP metabolites which are capable of crossing the blood brain barrier (BBB) (Fig. 1), serum and cerebrospinal fluid (CSF) concentrations of CCL2 do not appear to be significantly correlated (Yoshio et al., 2016). CCL2 is a pleiotropic chemokine best known for its role in chemotaxis, i.e. the recruitment macrophages, granulocytes, lymphocytes, and natural killer cells to sites of inflammation in tissues, including the brain (Gschwandtner et al., 2019). Why higher levels of CCL2 would be associated with greater ability to upregulate the LA is unclear. CCL2 is expressed in several different cell types including astrocytes (Barna et al., 1994), which may explain why it was positively correlated with KynA/3-HK (Supplementary Table S3). In addition to its role in augmenting the inflammatory response, CCL2 can exert immunoregulatory or immunosuppressive effects by generating regulatory dendritic cells (DC_{reg}) and regulatory T-cells (T_{reg}) (Gschwandtner et al., 2019).

This pilot study has several limitations. First, because of the lack of a sham control group, we are unable to identify a direct causal relationship between rtfMRI-nf, immune responses, and depressive symptoms. It is possible that rtfMRI-nf exerts neuroprotective effects which leads to an improvement in depressive symptoms. Alternatively, psychosocial changes related to the rtfMRI-nf intervention or non-intervention-related factors (e.g., effect of exposure to recalling positive memories during rtfMRI-nf, subject expectations, natural recovery, etc.) could also impact depressive symptoms, and in turn alter the KP. Second, although we included possible confounding variables (i.e., age, sex, and baseline score of interest) as covariates for the correlation analyses, it is plausible that peripheral inflammatory markers were associated with other factors. For example, BMI, smoking history, menstrual cycle, and oral contraceptive use (Meier et al., 2018) may influence inflammatory markers, which could partially confound the current results. Also, other factors may affect both severity of depression and inflammatory markers (e.g., the number or duration of depressive episodes, past history of medication). Those variables were not controlled in this study due to the lack of adequate power. A future study needs to control these variables to exclude potential confounding effects. Third, the extent to which peripheral concentrations of cytokines and KP metabolites reflect brain levels of these markers is uncertain. Nevertheless, KYN and TRP are known to cross the BBB and there is some evidence that QA also has this capacity (Heyes and Morrison, 1997). Accordingly, a recent paper reported significant correlations between KYN/TRP ($r = 0.77$) and QA ($r = 0.55$) concentrations in the plasma and CSF of depressed patients (Haroon et al., 2020). However, even if it turns out that the serum concentrations of KP metabolites are not reflective of central nervous system (CNS) measurements, serum assays could be of great practical value if they predict therapeutic outcome. Fourth, because of the relatively small sample size and the exploratory nature of the correlation analyses, there is a possibility of both type I and type II errors. Thus, the results of correlation analyses should be treated with caution. Fifth, for ethical reasons, we did not include MDD participants demonstrating suicidal behavior or severe suicidal ideation, a phenotype that has been strongly linked to increased QA and reduced KynA (Bay-Richter et al., 2015). Finally, use of anti-depressant medication was an exclusion criterion, which although a significant methodological strength, does limit the generalizability of our results.

5. Conclusion

The present study examined the relationship between inflammatory markers, rtfMRI-nf, and clinical changes in MDD volunteers. This pilot study provided preliminary evidence that rtfMRI-nf-induced increases in neuroprotective kynurenines co-occur with improvements in mood and second, that the baseline serum concentration of KynA/QA may predict response to rtfMRI-nf LA emotional training. Additional controlled clinical trials with larger sample sizes are necessary to confirm a mechanistic role for KP metabolites in the therapeutic response to rtfMRI-nf and to evaluate the clinical utility of KynA/QA as a treatment response biomarker.

7. Credit author statement

J.B. and J.S. conceived and developed the study design; J.S. and J.B. acquired the financial support for the project leading to this publication; J.B., V.Z. and M.M developed rtfMRI-nf system and left amygdala training protocol; J.B, V.Z. and M.M. developed real-time data acquisition and analysis infrastructure; V.Z., M.M. and J.B. developed analysis of framework for MRI data; T.K.T developed data acquisition and analysis procedures for blood samples; A.T., J.L.S and N.E. performed experiments; A.T. analyzed the data; A.T., J.B. and J.S. wrote the original draft paper; V.Z., M.M., O.A.Z., M.P.P., J.B. and J.S. provided guidance on analyses and critical review of the paper; all authors provided comments on the manuscript.

Funding

The study was supported by the William K. Warren Foundation, the National Institute of Mental Health (R21MH113871), and the National Institute of General Medical Sciences (P20GM121312).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Julie Arterbury, Bill Alden, Julie DiCarlo, and Greg Hammond for helping with MRI and EEG-fMRI scanning and Brenda Davis in the laboratory of TKT for performing the cytokine assays.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nicl.2021.102559.

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