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# EEG-Neurofeedback Training and Prolidase in Anxiety Disorders: An Exploratory Study

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**Abstract**

**Objective:** Prolidase is an enzyme that releases proline and is vital in extracellular matrix (ECM) remodeling, fueling white matter dynamics. Serum prolidase activity (SPA) is elevated in various neuropsychiatric conditions and may influence cognitive functions. Aim of the study was to explore the relation of SPA to neuropsychological functioning and its response to treatment in anxiety disorders. **Methods:** Twenty demographic-matched patients with anxiety were recruited. Six patients were given EEG-neurofeedback training (EEG-NFT), eight were treated pharmacologically (treatment as usual; TAU) with EEG-NFT, and six patients were treated only pharmacologically (TAU group). Beck Anxiety Inventory (BAI) and Beck Depression Inventory (BDI) were used to assess anxiety and comorbid depression, respectively. **Results:** Symptom reduction was seen in all groups. SPA decreased considerably in EEG-NFT group. Mental speed and spatial working memory negatively correlated with SPA in EEG-NFT group. Focused attention, sustained attention, verbal working memory, and spatial construction ability negatively correlated with SPA in EEG-NFT+TAU group. Mental speed in TAU group was also inversely proportional to SPA. **Conclusion:** Inverse correlation between SPA and neuropsychological functions in EEG-NFT group is suggestive of prolidase-mediated microstructural changes in white matter, which may have an influence on cognitive enhancement in anxiety disorders (AD).

***Keywords***: anxiety disorders; prolidase; proline; EEG-neurofeedback training; neurocognitive functions; executive functions

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## Introduction

Anxiety disorders (AD) are the most prevalent mental disorders worldwide (Stein et al., 2017). They are highly comorbid with other neuropsychiatric disorders. AD are a highly debilitating condition, posing significant disease burden and impacting quality of life (Sagar et al., 2020).

There is a dearth of definite biomarkers for AD owing to their invasive nature (cerebrospinal fluid) or cost effectiveness (neuroimaging methods). A need for peripheral biomarkers, which are economical, minimally-invasive and easy to obtain, is perceived (Vismara et al., 2020). Lately, serum prolidase activity has been studied in neuropsychiatric disorders such as schizophrenia (Güneş et al., 2016), bipolar disorder (Selek et al., 2011), major depressive disorder (Kokacya et al., 2014; Verma et al., 2017), Alzheimer's dementia (Krishna et al., 2020), and generalized anxiety disorder (GAD; Ercan et al., 2017) and is found to be elevated in comparison to controls.

Prolidase is an enzyme that cleaves iminodipeptides containing glycyl-proline and hydroxyproline at the carboxy terminal. During collagen metabolism, prolidase splits iminodipeptides, produces proline as

an end product, and makes byproducts available for reuptake and recycling for protein synthesis, cell matrix remodeling, and cell growth (Namiduru, 2016). Extracellular matrix (ECM) is prominently made of collagen of which 20% consists of proline and hydroxyproline (Ramshaw et al., 1998). Increase in proline has been shown to decrease antioxidants in brain of rats, thus inducing oxidative stress (Delwing, Bavaresco, Chiarani, et al., 2003a; Delwing, Bavaresco, Wannmacher, et al., 2003b; Delwing, 2005). Proline is considered as an amino acid and its abundance in the brain gives it the status of a neurotransmitter (Lajtha & Toth, 1974). Defective ECM caused by altered serum prolidase activity (SPA) has shown to cause disorganized cytoarchitecture, defect of meninges, and compromised membranal integrity leading to mental deficiency (Insolia et al., 2020) and thus possibly causing impairments in cognitive functioning. Prolidase deficiency is a rare genetic disorder in which high levels of proline containing iminodipeptides in brain are seen to be associated with mental retardation (Namiduru, 2016). Proline is also implicated in modulation, that is, release and reuptake, of glutamate (Delwing et al., 2007) and activates NMDA receptors and thus depolarizing neurons (Ault et al., 1987). Glutamate is an excitatory neurotransmitter and excess of it leads to excitoxicity causing seizures or mental retardation and nerve cell death (Cohen & Nadler, 1997). It plays a role in neurodevelopment, cognition, learning, and memory in humans (Nasir et al., 2020). Hence, it is important to explore if SPA is associated with neuropsychological functioning due to its direct or indirect role through increased oxidative stress, inflammation, and ECM remodeling.

First line of treatment for AD has been pharmacotherapy (mostly selective serotonin reuptake inhibitors [SSRIs]) and cognitive behavior therapy (CBT; Garakani et al., 2020). However, a considerable number of patients do not respond to either of the treatments and that gives rise to the need of looking newer modalities of treatment. EEG-neurofeedback training (EEG-NFT) is a promising state-of-the-art treatment for AD (Micoulaud-Franchi et al., 2021). It enables a person to alter their cortical electrical activity through real-time feedback. Alpha-theta training has been effectively used to treat AD (Abdian et al., 2021; Hammond, 2005; Moore, 2000). EEG-NFT works on the electrophysiology of brain by synchronizing random electrical activity through training specific electrical waves repeatedly over a period of time. Electrical impulses to axons are known to induce myelination (Demerens et al., 1996; Ishibashi et al., 2006) which is a putative mechanism for training-induced white matter tract changes in the brain (Takeuchi et al., 2010). Electrocortical activity is associated with changes in the subcortical structures, and training brain waves through EEG-NFT is known to increase the cortical excitability and inducing neuroplasticity (Ros et al., 2010). Though, the cellular mechanism of these remain open to be explored.

We hypothesize that prolidase-mediated ECM alterations may influence synaptic plasticity in AD. With treatment, SPA may decrease in AD patients, thus impacting the brain regions involved in AD. As a consequence, cognitive functions are expected to improve with symptom reduction too. To our knowledge, this is the first study to explore serum prolidase levels in response to treatment in AD as assessed by clinical evaluations and neuropsychological functions.

## Methods

### Subjects

Patients of all genders between 18–45 years who were diagnosed with AD (i.e., social anxiety disorder, panic disorder with and without agoraphobia, or GAD) were recruited from a tertiary hospital in Southern India. Clinical diagnosis was made through clinical interview by a consultant psychiatrist based on ICD-10 criteria. Patients with presence or history of any neurosurgical, neurological, and severe mental illness; and those who were undergoing psychotherapy were excluded. Only right-handed people were included to maintain uniformity. The study was approved by the Institute Ethics Committee, NIMHANS. Informed consent was obtained from each patient before recruiting.

### Clinical Assessments

Beck Anxiety Inventory (BAI) was administered to assess the severity of anxiety and patients with a score of >16 (moderate-severe anxiety) were recruited. Beck Depression Inventory (BDI) was administered to assess severity of depression.

### Cognitive Assessments

Subtests from NIMHANS neuropsychology battery (Rao et al., 2004) were administered. Attention, executive functions, and memory were examined. Digit Symbol Substitution Test (DSST) for mental speed, Digit Vigilance Test (DVT) for sustained attention, Color Trails-1 (CT-1) for focused attention, and CT-2 for mental flexibility and response inhibition were used. *N*-back and spatial span from Wechsler Memory Scale-III (WMS-III) were used for verbal and visual working memory, respectively.

Controlled Oral Word Association Test (COWAT) was used to assess fluency. Rey's Auditory Verbal Learning Test (AVLT) and Complex Figure Test (CFT) were used to assess verbal and visual memory, respectively.

### Blood Sample Analysis

Five ml of random blood was drawn through venipuncture by a trained staff nurse in the morning. It was left at room temperature for an hour for clotting to occur. The serum was then separated on the same day by spinning the sample in a centrifuge at a speed of 5000 rpm for 15 min. Once separated, serum was transferred to a new set of tubes and was stored at −80$^{o}$ celsius until batch analysis. Spectrophotometric method was used to measure the proline released through SPA (Chinard, 1952; Myara et al., 1982). SPA is reported in U/ml.

## Treatment

### EEG-NFT

20 sessions of 30 min each were provided to patients on alternate days by a trained clinical psychologist. The training was given at O1 and O2 location to enhance alpha and theta waves; that is, a reward in terms of points (auditory) would be given if the thresholds for alpha and theta to increase were met. The thresholds were set on auto mode; that is, an average of the last three min of alpha and theta activity was taken and the next threshold was set at that limit. The training was conducted on Atlantis Module (produced by BrainMaster Technologies, Inc., Oakwood Village, Ohio).

### Treatment as Usual (TAU)

Patients who were on psychotropic medications and no other treatment modality formed the treatment as usual group. Patients in both groups were stabilized on medication for at least three weeks before conducting any assessments and EEG-NFT.

### Statistical Analysis

IBM SPSS version 28 for Windows was used for statistical analysis. Mean and standard deviations of socio-demographic variables, clinical symptoms and SPA at baseline are presented. Wilcoxon Signed Rank test was used to measure the pre to post change in all variables within groups. Effect sizes are determined by dividing the *Z* scores by square root of observations of the respective variables. Spearman's rank correlation was used to find correlations between SPA, clinical, and cognitive variables.

## Results

In this exploratory study, age, sex, and educational status matched subjects were included in the order of six patients in the EEG-NFT group (five males and one female) with a mean age of 29.33 ± 4.59, eight patients in the EEG-NFT+TAU group (six males and two females) with a mean age of 26.13 ± 5.16, and six patients in TAU group (five males and one female) with a mean age of 33.83 ± 9.84 years. All the recruited subjects' BAI, BDI, and cognitive indices scores were statistically similar across groups at baseline (Table 1).

Upon treatment, neuropsychological functions improved in all groups. Significant improvement is seen in anxiety and comorbid depressive symptoms in all the groups after treatment. Highest improvement is seen in EEG-NFT group, followed by EEG-NFT+TAU group (See Table 2).

**Table 1**
*Sociodemographic and Clinical Variables at Baseline*

| Variables | EEG-NFT | EEG-NFT+TAU | TAU | *Sig.* |
|---|---|---|---|---|
| Age in years | 29.33 ± 4.59 | 26.13 ± 5.16 | 33.83 ± 9.84 | 0.226 |
| Duration of illness in years | 2.67 ± 1.50 | 2.38 ± 2.07 | 4.17 ± 3.37 | 0.285 |
| Education in years | 17.00 ± 4.64 | 16.25 ± 1.98 | 12.50 ± 6.05 | 0.414 |
| Beck Anxiety Inventory | 26.00 ± 3.84 | 39.88 ± 11.56 | 31.00 ± 6.57 | 0.074 |
| Beck Depression Inventory | 22.83 ± 12.70 | 30.88 ± 8.72 | 21.83 ± 10.62 | 0.257 |
| Serum prolidase activity U/ml | 8.65 ± 1.48 | 8.01 ± 3.02 | 7.38 ± 2.66 | 0.729 |

**Table 2**
*Wilcoxon Signed Rank Test to Assess the Change with Treatment in Variables in Different Groups*

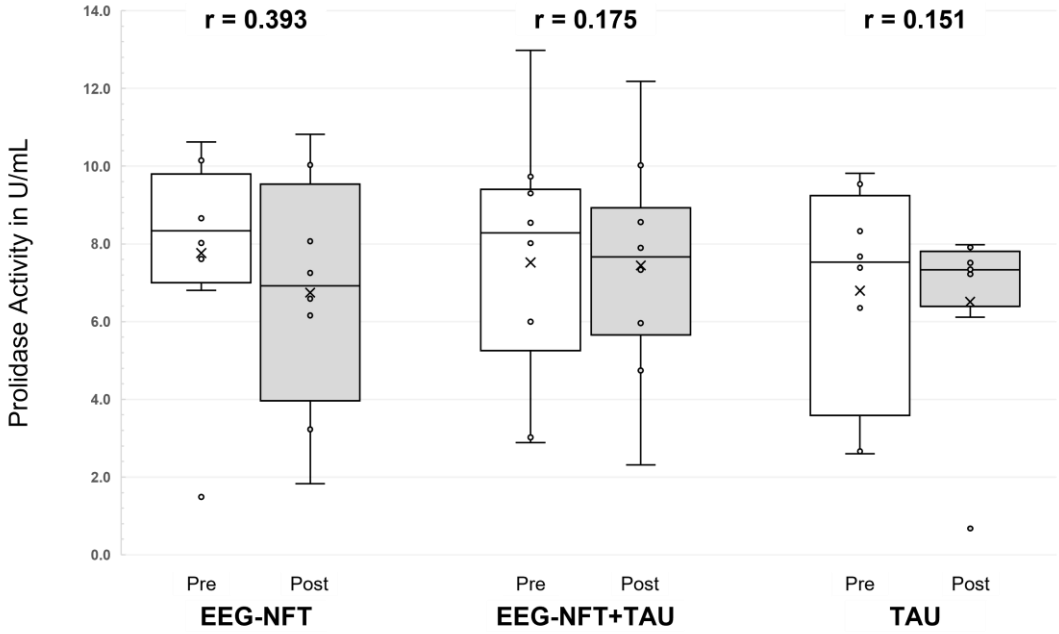
| | Groups | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | EEG-NFT | | | EEG-NFT+TAU | | | TAU | | |
| Measures | *Z* | *r* | *Sig.* (2-tailed) | *Z* | *r* | *Sig.* (2-tailed) | *Z* | *r* | *Sig.* (2-tailed) |
| Beck's Anxiety Inventory | −2.207 | −0.64** | 0.03# | −2.524 | −0.63** | 0.01# | −2.214 | −0.64** | 0.03# |
| Beck Depression Inventory | −2.201 | −0.64** | 0.03# | −2.100 | −0.53** | 0.04# | −2.201 | −0.64** | 0.03# |
| DSST | −2.207 | −0.64** | 0.03# | −0.980 | −0.25 | 0.33 | 0.000 | 0.00 | 1.00 |
| Color Trails-1 | −1.572 | −0.45* | 0.12 | −0.350 | −0.09 | 0.73 | 0.000 | 0.00 | 1.00 |
| Color Trails-2 | −1.153 | −0.33* | 0.25 | −1.051 | −0.26 | 0.29 | −1.826 | −0.53** | 0.07# |
| Digit vigilance total time | −0.943 | −0.27 | 0.35 | −1.680 | −0.42* | 0.09 | −1.826 | −0.53** | 0.07# |
| Digit vigilance - errors | −1.461 | −0.42* | 0.14 | −1.620 | −0.41* | 0.11 | −0.365 | −0.11 | 0.72 |
| COWAT | −2.032 | −0.59** | 0.04# | −1.556 | −0.39* | 0.12 | −0.272 | −0.08 | 0.79 |
| *N*-Back 2 hits | −1.511 | −0.44* | 0.13 | −2.060 | −0.52** | 0.04# | −0.272 | −0.08 | 0.79 |
| *N*-Back 2 errors | −1.473 | −0.43* | 0.14 | −2.401 | −0.60** | 0.02# | −1.095 | −0.32* | 0.27 |
| Spatial span forward | −1.289 | −0.37* | 0.20 | −0.986 | −0.25 | 0.32 | −0.921 | −0.27 | 0.36 |
| Spatial span backward | −0.756 | −0.22 | 0.45 | −1.354 | −0.34* | 0.18 | −0.184 | −0.05 | 0.85 |
| Spatial span total | −1.633 | −0.47* | 0.10 | −0.341 | −0.09 | 0.73 | −0.535 | −0.15 | 0.59 |
| AVLT total | −1.572 | −0.45* | 0.12 | −2.371 | −0.59** | 0.02# | −1.826 | −0.53** | 0.07# |
| AVLT immediate recall | −2.070 | −0.60** | 0.04# | −2.041 | −0.51** | 0.04# | −1.604 | −0.46* | 0.11 |
| AVLT delayed recall | −1.089 | −0.31* | 0.28 | −2.032 | −0.51** | 0.04# | −1.633 | −0.47* | 0.10 |
| CFT COPY | −1.604 | −0.46* | 0.11 | −1.604 | −0.40* | 0.11 | −0.816 | −0.24 | 0.41 |
| CFT immediate recall | −1.166 | −0.34* | 0.24 | −2.328 | −0.58** | 0.02# | −1.289 | −0.37* | 0.20 |
| CFT delayed recall | −1.572 | −0.45* | 0.12 | −2.176 | −0.54** | 0.03# | −0.730 | −0.21 | 0.47 |
| Serum prolidase activity | −1.363 | −0.39* | 0.17 | −0.700 | −0.18 | 0.48 | −0.524 | −0.15 | 0.60 |

# Significant at 0.05 level, * Moderate effect size < − .30, ** Large effect size < −.50. DSST- Digit Symbol Substitution Test, COWAT- Controlled Word Association Test, AVLT- Rey's Auditory Verbal Learning Test; CFT- Complex Figure Test.

With respect to prolidase activity, a considerable reduction in the EEG-NFT group and marginal reduction in the other two groups noted (Figure 1). A positive correlation was found between prolidase and comorbid depression at the baseline in the EEG-NFT group $r(4) = 0.943$, $p = .005$. This is in agreement with previous studies conducted in major depressive disorder (Kokacya et al., 2014; Verma et al., 2017). However, no significant correlation was found between symptom reduction and decreased prolidase activity (Table 3).

Interestingly, when the cognitive scores were closely scrutinized, mental speed $r(4) = -0.899$, $p = .015$ and spatial working memory $r(4) = -0.899$, $p = .015$ negatively correlated with prolidase in EEG-NFT group. Focused attention $r(6) = -0.714$, $p = .047$; sustained attention $r(6) = 0.708$, $p = .05$; mental flexibility and set-shifting ability, verbal working memory *N*-back hits $r(6) = -0.778$, $p = .023$ and *N*-back errors $r(6) = 0.876$, $p = .004$; and spatial construction ability $r(6) = -0.791$, $p = .019$ negatively correlated with prolidase in EEG-NFT+TAU group. Mental speed $r(4) = -0.812$, $p = .05$ in TAU group is also negatively correlated with prolidase (See Table 3).

**Figure 1.** *Change in SPA Across Groups After Treatment.*




**Note.** Mean (marked as x) and median values are presented of EEG-NFT group (*n* = 6), EEG-NFT+TAU group (*n* = 8) and TAU group (*n* = 6). Wilcoxon Signed Rank test was used to analyze and effect size was calculated using $r = Z/\sqrt{N}$.

**Table 3**

*Correlations Between SPA and Neuropsychological Scores Presented as Spearman's Rho*

| | | Correlations | | |
|---|---|---|---|---|
| | | **EEG-NFT** | **EEG-NFT+TAU** | **TAU** |
| DSST | Correlation Coefficient | −0.899* | −0.429 | −0.812* |
| | *p*-value | 0.015 | 0.289 | 0.050 |
| CT-1 | Correlation Coefficient | −0.143 | −0.714* | −0.174 |
| | *p*-value | 0.787 | 0.047 | 0.742 |
| DVT-e | Correlation Coefficient | −0.493 | 0.708* | −0.290 |
| | *p*-value | 0.321 | 0.050 | 0.577 |
| NB2-H | Correlation Coefficient | 0.058 | −0.778* | 0.698 |
| | *p*-value | 0.521 | 0.023 | 0.123 |
| NB2-E | Correlation Coefficient | 0.118 | 0.876** | −0.696 |
| | *p*-value | 0.824 | 0.004 | 0.125 |
| SS-fw | Correlation Coefficient | −0.899* | 0.061 | −0.058 |
| | *p*-value | 0.015 | 0.885 | 0.913 |
| CFT-COPY | Correlation Coefficient | −0.638 | −0.791* | −0.334 |
| | *p*-value | 0.173 | 0.019 | 0.518 |

** correlation is significant at 0.01 level (2-tailed), * correlation is significant at 0.05 level (2-tailed). DSST - Digit Symbol Substitution Test, CT-1 - Color Trails-1, DVT-e - Digit Vigilance Test errors, NB2-H - Verbal *N*-Back-2 hits, NB2-E - Verbal *N*-Back-2 errors, SS-fw - Spatial span - forward, CFT-COPY - Complex Figure Test copy.

## Discussion

The role of SPA is now being explored in neuropsychiatric disorders. These conditions are associated with neuropsychological deficits. SPA is observed to be elevated in common mental disorders like depression and GAD. Present study is an exploration of the effect of EEG-NFT for anxiety on prolidase activity and its relation to cognitive functions. We had hypothesized, with decrease in SPA, clinical symptoms and cognitive functions will improve.

Deficits in spatial working memory and spatial long-term memory is seen in an inherited disorder of amino acids called hyperprolinemia type II which is characterized by excessive proline (Bavaresco et al., 2005). Higher proline levels are shown to destroy cells in hippocampus, which is an important region for memory consolidation and retention (Nadler et al., 1988). Surprisingly, no correlations between SPA and short-term and long-term memory were found. However, mental speed and spatial working memory negatively correlated with prolidase in EEG-NFT group. Focused attention, sustained attention, verbal working memory, and spatial construction ability negatively correlated with prolidase in EEG-NFT+TAU group too. Mental speed in TAU group is also negatively correlated with prolidase. The tests assessing these functions are not susceptible to practice effects, and we can safely attribute the improvement to the treatment. The aforementioned cognitive functions are the building blocks for executive functions and targeting these have shown to be effective for higher cognition as well. As seen on performance of Color Trails-2, patients' mental flexibility and set-shifting ability and response inhibition improved along with attention and verbal working memory. These cognitive functions are a part of executive functions and are largely governed by fronto-parietal networks (Coull et al., 1996; Figueroa-Vargas et al., 2020; Scolari et al., 2015). The present intervention-neurofeedback training, has shown to bring about microstructural changes in white matter pathways connecting the frontal and parietal regions (Ghaziri et al., 2013). The ECM provides a support framework to the synapses and thus the subcortical regions and is essential for neuroplasticity (Burnside & Bradbury, 2014). Prolidase plays an important role in ECM remodeling. Findings from the present study imply that NFT-induced, possibly prolidase-mediated microstructural changes in white matter have therapeutic influence in AD. It warrants further large-scale studies to confirm these observations.

## Limitations and Future Directions

The limitation of the study was small sample size. Hence, the findings should be interpreted with caution. However, we found that prolidase activity responds to existing treatment modalities. We also saw a reduction in prolidase of a moderate effect size through EEG-NFT. The mechanisms of these remain to be explored. It was interesting to see the reduction in SPA also correlated with multiple neuropsychological functions independently of the clinical symptoms. This is indicative of the role of SPA in strengthening the ECM and sustaining the cognitive networks and pathways. We speculate that the enhanced synapses through EEG-NFT are supported by the ECM through SPA and thus reinforce the cognitive enhancement. These are preliminary findings and further controlled studies with a bigger sample are required for establishing the role of SPA in AD.

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