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Stroke Patients after repetitive Transcranial Magnetic Stimulation (rTMS) – Alterations of Tryptophan Metabolites in the Serum

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) as a new non-invasive painless procedure has been tested for augmentation of motor performance and reduction of spasticity in post-stroke patients. Stroke patients (N = 34) were recruited for rTMS treatment and brain activity analysis (EEG) was performed before and after rTMS. The effect of rTMS was evaluated using National Institute of Health Stroke Scale (NIHSS), Barthel – Index and Ashworth Scale. The content of L-tryptophan (L-TRP), L-kynurenine (L-KYN), kynurenic acid (KYNA) and anthranilic acid (ANA) was determined in the serum of stroke patients before the 1st, after the 5th and after 10th rTMS application using high performance liquid chromatography. In a separate experiment, L-TRP metabolites were determined in the serum of an independent group of stroke patients (N=47) and control subjects (N = 6). The content of L-TRP and L-TRP metabolites in the serum of stroke patients was significantly increased, L-TRY was 121% of CO; L-KYN 161% of CO, $p < 0.015$; ANA 280 % of CO, $p < 0.001$; KYNA 243% of CO, $p < 0.001$, compared to control subjects. Similar changes were found in stroke patients recruited for rTMS. After the 10th rTMS treatment L-KYN and ANA levels increased moderately but significantly in the serum and it was L-KYN 107%, $p < 0.01$; ANA 110%, $p = 0.055$, versus the value before 1st rTMS, respectively. The ratios L-KYN/TRP and ANA/KYNA increased moderately but significantly after the 10th rTMS. Creatin kinase and prolactin levels were in normal range during rTMS. Stroke patients treated with rTMS have shown a significant enhancement of motor performance and moderate reduction of spasticity. The alteration of ANA/KYNA ratio after rTMS might be of significance with respect to the clinical improvement of patients. The present study gives favour for rTMS as a means for neurorehabilitation of patients after stroke. Notable, the management of therapies following rTMS are of importance for an improvement of hand and fingers activities, as observed within this study.

Keywords: Repetitive transcranial magnetic stimulation; Finger dexterity; AMPS; Occupational therapy; Serum; Tryptophan; L-kynurenine; Kynurenic acid; Anthranilic acid; Stroke; Dementia

Introduction

In 1985 Barker and co-authors introduced transcranial magnetic stimulation (TMS) as a noninvasive and safe brain stimulation technique [1]. Applying a single magnetic stimulus over the motor cortex can induce motor evoked responses in the contralateral limb muscles [2] and this approach became a valuable tool for cortical mapping and assessment of the functional integrity of the motor system [2]. In the last years the development of stimulators significantly progressed, specially discharging at high frequencies up to 100 Hz and the application of TMS expanded into the areas of behavioral and cognitive functions assessment, as well [3,4]. Depending on stimulation parameters i.e. frequency, rate, and duration, application of repetitive stimuli to cortical regions can enhance or decrease the excitability of the affected brain structures [2,5,6]. TMS can be delivered via single-pulse, double-pulse, paired-pulse and low or high frequency repetitive pulses (rTMS) [2,5-10].

rTMS is the method currently under investigation for the use as a treatment modality for stroke patients with spastic movement disorders mainly due to its ability to modulate the excitability in the motor cortex over longer time periods (compared to other types of TMS) [11-17]. It also can enhance some cognitive processes, regulate activity in specific brain regions and provide information about the roles of different cortical regions in behavioral performance. The use of rTMS can also enhance neuroplasticity during motor training. The role of various stimulation parameters is constantly under evaluation and it seems to be important for the clinical outcome of rTMS [11-17]. The use of rTMS in the high-frequency range (>20 Hz) has been

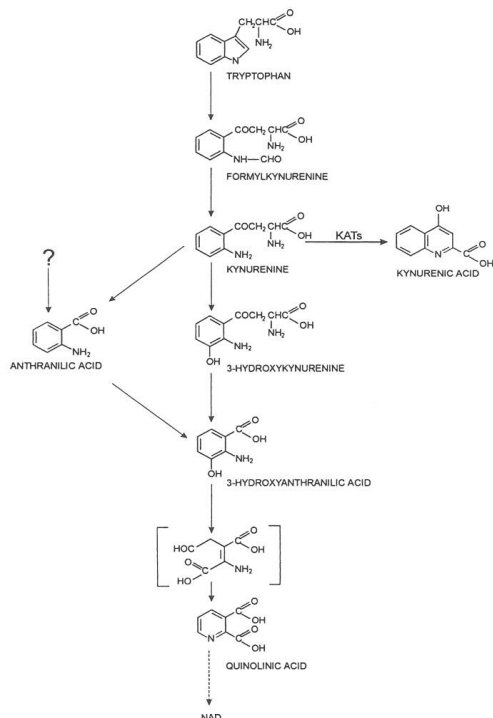
associated in some cases with seizure induction [18], whereas lower frequency rates of rTMS are potentially advantageous for therapeutic effects. Peripheral organs and central nerve tissues are excessively involved in L-TRP metabolisms along the kynurenine pathway [19,20], (Graph 1.). One of the metabolites of L-KYN metabolism is KYNA, well-known as endogenous antagonist of the glutamate ionotropic excitatory amino acid receptors [21,22] and of the nicotine cholinergic subtype α -7 receptor [23]. KYNA is altered significantly in various neuropsychiatric and immunologic disorders and in the aging process [24-28]. Notably, an enhancement of KYNA in the CNS has been suggested as a cause for impairment of memory and cognition [29, 30]. ANA, another metabolite of L-KYN, has been suggested to be involved in the 3-hydroxy-antranilic acid (3-OH-ANA) synthesis [31] but the precise mechanism is not clear yet. Alterations of L-TRP metabolite levels found immediately after stroke [32,33] indicate a significant activation of metabolism along the kynurenine pathway.

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Graph 1: The L-Tryptophan metabolism along kynurenine pathway

Under physiological as well as under pathological condition the blood brain barrier (BBB) and brain-CSF barrier play an important role in the distribution of tryptophan metabolites in the human body [34].

Aim of the present study was the investigation of L-TRP metabolism in the serum of stroke patients in the later period after the stroke event, between 6 months and 5 years, by measuring the content of L-TRP, L-KYN, ANA and KYNA. Then, in independent experiment changes of L-TRP metabolites in the serum before the 1st, after the 5th and after the 10th rTMS application in post-stroke patients were analysed. As a marker for epileptic seizure events creatin kinase (CK) [35] and prolactin [36] levels were measured. In addition, also for safety reasons the measurement of brain activities by means of EEG was applied before and after rTMS application. Results on L-TRP metabolites and the therapeutic effect with respect to an enhancement of motor performance and a reduction of spasticity were evaluated. A part of the data was published in an abstract form [37].

Material and Methods

Chemicals

L-TRP, L-KYN, KYNA, ANA, 3-hydroxy-kynurenine (3-OH-KYN) and 3-OH-ANA were purchased from Sigma. All other chemicals used were of the highest commercially available purity.

Subjects

Out of a larger series of stroke patients 61 patients (44 male and 17 female) at the age of 72.4 ± 1.3 years with recovery period from 6 month to 5 years after stroke, were involved in this study. 45 stroke patients (33 male and 12 female) at the age of 74.2 ± 1.4 years, were recruited for treatment with rTMS according to the clinical protocol, then blood collection before and after rTMS treatment was performed.

16 stroke patients of 61 were recruited only for blood collection. Blood collected from these patients and from stroke patients recruited for rTMS (the 1st blood before rTMS), served for L-TRP metabolite investigations of a stroke patients group (STROKE). Stroke patients received anticoagulant or platelet anti-aggregant treatment, as well as cholesterol lowering and antihypertensive medication.

Among a larger series of headache patients 6 individuals (2 male, 4 female, 62.2 ± 7.0 years old) were selected as normal subjects for this study (CO). Cranial computer tomography (CT) and magnetic resonance tomography (MRT) and further clinical investigations, which included electroencephalography (EEG) and transcranial Doppler-sonography, were in the normal range.

For neurochemical analyses samples of serum were collected immediately in 1 ml aliquots and stored at -40°C until analysed. Serum was coded to make anonymous and the study was carried out according to Lower Austrian Ethical Regulations.

Methods

Measurement of CK and Prolactin

Determination of prolactin and CK was carried out using routine laboratory methods [36,38]. CK and prolactin were analysed using Cobas 6000 (Roche®). CK was analysed by UV-detection, prolactin by Electro-chemiluminescence immunoassay (ECLIA).

Neuroradiological investigations

Routine clinical investigations of MRT and EEG were carried out.

Neuronavigated repetitive transcranial magnetic stimulation (rTMS)

For the purpose of neuronavigation of the stimulation coil respectively the exact stimulus application point T_1 weighted 3D MRI images of the patient's brain were used in every case. On the basis of these images, the exact and individually best location for the rTMS application could be marked (primary motor cortex). For stimulation, a magstim® Rapid² stimulator with figure-8 coil was used. Neuronavigation was computer-assisted byBrainsight™ (Rogue Research Inc.) which allows an exact array of stimuli per day. Stroke patients recruited for rTMS treatment received on ten following days the rTMS. Location, intensity and number of pulses were adjusted individually to the threshold evaluation according to the study protocol and application of rTMS was performed according to the ethical regulations of the government of Lower Austria. A group of patients with Sham- rTMS was not recommended from the ethical committee in this study but was recommended to perform EEG for safe reasons before the 1st, after 5th and 10th rTMS.

In all patients the affected primary motor cortex was stimulated with 120% of cortical motoric threshold of the unaffected hemisphere at rest. Frequency of stimulation was set at 3 Hz. Ten trains of ten seconds duration (i.e. 30 stimuli per train) were applied with 50 seconds interval. Motoric response was monitored at the musculus abductor pollicis brevis on both sides. For safety reasons, EEG investigations were performed before and after the first, fifth and tenth rTMS treatment. Blood samples were taken before the 1st, after the 5th and after the 10th rTMS treatment. Serum was stored at -40°C until analyses.

Evaluation of the therapeutic effects of rTMS

For evaluation of the clinical outcome, the patients' abilities were assessed before and after rTMS therapy by National Institute of Health

Stoke Scale (NIHSS) and Barthel – Index [39]. Moreover spasticity was evaluated by modified Ashworth Scale before and after the 10th rTMS application.

Occupational Therapy

After rTMS a facultative enhancement in activities of daily living in stroke patients was further investigated and occupational therapy was applied after rTMS. Used assessments tools beside, Barthel Index were assessment of motor and process skills (AMPS), Box and block test and Jamar Dynamometer. In one group of stroke patients (N=6) occupational therapy was applied immediately after rTMS and in other group (N=6) at least one hour brake after rTMS and the effects were evaluated. This part of the study was performed as a master thesis work [40].

Measurement of L-TRP and L-TRP metabolites

Sample preparation: Briefly, samples of serum 200 µl were mixed with 14 µl of 50% trichloroacetic acid and 0.2 M HCl (vol/vol) and centrifuged (20 min, 14,000 rpm) and obtained supernatant was divided and immediately used for the measurement of L-tryptophan and tryptophan metabolites, and for purification of kynurenic acid followed by determination, respectively.

HPLC method for tryptophan metabolites detection: L-TRP, L-KYN, 3-OH-KYN, ANA, and 3-OH-ANA were measured by isocratic HPLC with fluorescence and UV detection as introduced by Baran et al., with modification [41]. Briefly, the HPLC system consisted of the following: Merck Hitachi LaChrom Pump L-7100, Autosampler L-7200, Fluorescence Detector L 7485, UV Detector L-7400 and a Merck Hitachi D-7500 Integrator. The HPLC-method utilized a mobile phase of 42 mM ammonium acetate 7 mM sodium hydrogen phosphate, 7 mM sodium acetate, 11 mM ammonium hydroxide, 59 mM acetic acid, 1.380 mM 1-octanesulfonic acid, 74 µM sodium disulfide (pH=4.8) pumped through a Chromolith™ Performance RP-18e, 100-4,6 mm column (Merck KGaA Darmstadt Germany) at a flow rate of 0.7 ml/min. The injection volume was 50 µl. The fluorescence detector was set at an excitation wavelength of 299 nm and an emission wavelength of 420 nm. The retention time of 3-OH-ANA, ANA, L-TRP was approximately 4.2, 10.4, 13.6 min respectively, with a sensitivity of 80, 800 and 150 fmol per injection, respectively (signal: noise ratio = 5). Using the same HPLC condition and UV detector set at 366 nm wavelength 3-OH-KYN and L-KYN was eluted with a retention time of 2.3 and 3.3 min, and with a sensitivity of 700 and 500 fmol per injection, respectively (signal: noise ratio = 5).

Measurement of KYNA: Measurement of KYNA was performed according to Swartz et al. [42] with modification as described by Baran and Kepplinger [41]. Briefly, the serum samples were mixed with 0.2 M HCl (vol/vol) and centrifuged for 20 min, at 14,000 rpm. The supernatant obtained was applied to a Dowex 50W cation exchange column pre-washed with 0.1 M HCl. Subsequently, the column was washed with 1 ml 0.1 M HCl and 1 ml distilled water, and KYNA was eluted with 2 ml distilled water [43] and was quantitated by a high performance liquid chromatography (HPLC) system coupled with fluorescence detection. The HPLC system consisted of the following: Merck Hitachi Elite La Chrom Pump L-2130, Autosampler L-2200, Fluorescence Detector L-2485 and a data processor Windows® XP Professional HP. The mobile phase consisted of 50 mM sodium acetate, 250 mM zinc acetate, an 4 % acetonitril, pH 6.15, and was pumped through a 10 cm X 0.4 cm column (HR-80, C-18, Particle size 3 µM, In Chrom, Austria) at flow rate of 0.9 ml/min. The fluorescence detector was set at an excitation wavelength of 340 nm and an emission

wavelength of 398 nm. The injection volume was 50 µl. The retention time of KYNA was approximately 6 min, with a sensitivity of 25 fmol per injection (signal: noise ratio = 5).

Data analyses: All data are given as means ± S.E.M. For statistical analyses, the one-way ANOVA analyse of variance and a Student's t-test was applied, respectively. Each sample was determined in doubly or three plicate. Asterisks indicate a significant difference: **p* < 0.05; ***p* < 0.01; ****p* < 0.001 vs. corresponding control.

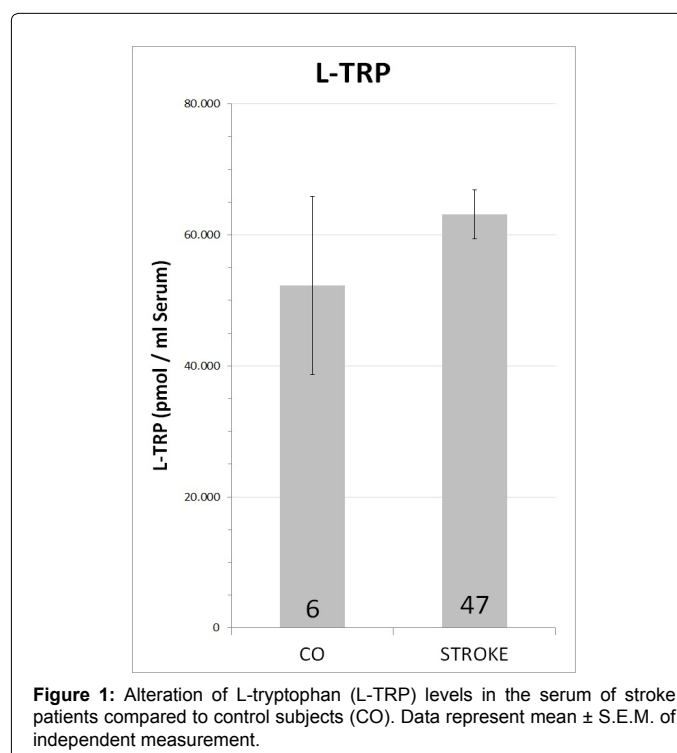
Results

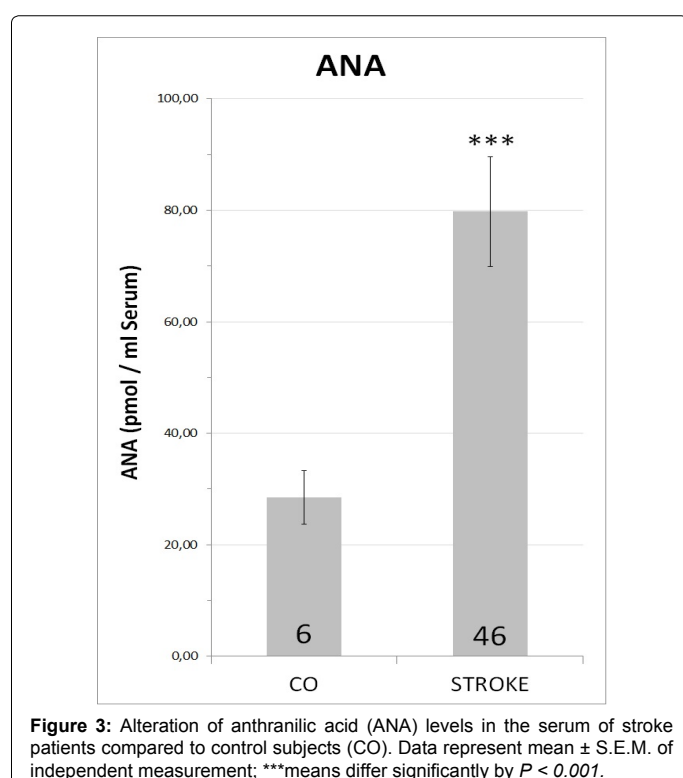
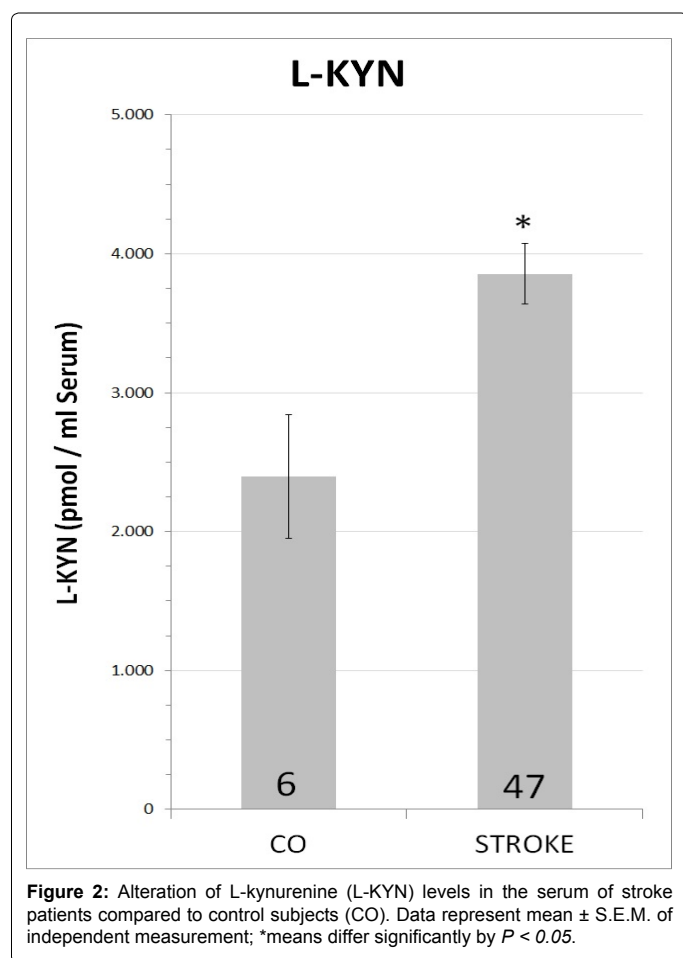
Tryptophan metabolite in the serum of control subjects

Determination of L-TRP, L-KYN, KYNA and ANA in the serum of human control subjects (N = 6) revealed following concentration: L-TRP was 52,306.7 ± 13,590.5 [pmol/ml serum]; L-KYN was 2,395.4 ± 445.2 [pmol/ml serum]; KYNA was 39.93 ± 2.35 [pmol/ml serum]; ANA was 28.48 ± 4.78 [pmol/ml serum]. Ratios of L-TRP metabolites: L-KYN/L-TRP was 0.051 ± 0.007; KYNA/ L-KYN was 0.020 ± 0.005; ANA/L-KYN was 0.015 ± 0.005; ANA /KYNA was 0.742 ± 0.151 (N=6, respectively). Under measurement condition the concentration of 3-OH-KYN and 3-OH-ANA were not detectable in the serum.

Alterations in tryptophan metabolism after stroke

L-TRP levels increased moderately (121 % of CO) in the serum of stroke patients, comparing to control (CO), (Figure 1). L-KYN level was increased significantly (161 % of CO; *p* = 0.015) in the serum of stroke patients (Figure 2). Also the levels of ANA and KYNA were significantly increased (280 % of CO, *p* < 0.001; and 243 % of CO; *p* < 0.001; respectively) in the serum of stroke patients, comparing to control (Figures 3 and Figure 4). Ratios of L-TRP metabolite after stroke: L-KYN/L-TRP was 0.063 ± 0.003 (124 % of CO); KYNA/L-KYN was 0.027 ± 0.002 (133 % of CO); ANA/L-KYN was 0.023 ± 0.003 (149 % of CO); ANA /KYNA was 0.886 ± 0.120 (119 % of CO).





Effect of rTMS: influence of rTMS on therapeutic output

Among 45 stroke patients recruited for rTMS 34 patients finished the rTMS stimulation program and these patients experienced positive effects. In nine patients the treatment with rTMS was interrupted because of increased activities of generalized sharp waves found by EEG and these patients were no further applied to rTMS and were excluded from this study. Two patients due to a high number of other therapies wished to be excluded from this study.

Investigation of NIHSS as well as Barthel-Index showed significant improvement in patients' abilities after 10th rTMS applications (Table 1). NIHSS before rTMS was 4.18 ± 0.39 and after 10th rTMS application was 2.97 ± 0.38 ; $p < 0.05$. Barthel-Index before rTMS was 58.48 ± 4.85 and after 10th rTMS was 74.38 ± 3.9 ; $p < 0.01$. Tonus evaluation following modified Ashworth Scale indicated a reduction of spasticity after rTMS, however the effect was not statistically significant. Ashworth Scale before rTMS was 1.6 ± 0.2 and after rTMS was 1.4 ± 0.2 ; $p = 0.4$.

Influence of rTMS on finger and hand activities

Stroke patients receiving occupational therapy immediately after rTMS showed a significant increase of finger dexterity and amelioration in the Barthel Index [40].

Influence of rTMS on tryptophan metabolites in stroke patients

L-Tryptophan (L-TRP): A tendency of lowering of L-TRP levels in the serum after 5th and 10th rTMS applications could be observed (Figure 5). One-way ANOVA analysis of variance between 3 groups revealed the means of L-TRP not statistically different in the serum ($F = 0.1469$, $P = 0.8634$, Figure 5).

L-Kynurenine (L-KYN): L-KYN increased moderately but significantly in the serum after 10th rTMS application (107 % vs. before 1st; $p = 0.01$), (Figure 6). One-way ANOVA analysis of variance between 3 groups revealed the means of L-KYN levels not statistically different in the serum ($F = 0.5571$, $P = 0.5746$, Figure 6).

Anthranilic acid (ANA): A moderate increasing of ANA levels was found in the serum of stroke patients after 10th rTMS applications (110 % vs. before 1st; $p = 0.055$), (Figure 7). One-way ANOVA analysis of variance between 3 groups revealed the means of ANA levels not statistically different in the serum ($F = 0.1237$, $P = 0.8837$, Figure 7).

Kynurenic acid (KYNA): KYNA levels were unaffected in the serum of stroke patients after 10th rTMS application (Figure 8). One-way ANOVA analysis of variance between 3 groups revealed the means of KYNA levels not statistically different in the serum ($F = 0.0183$, $P = 0.9818$, Figure 8).

Ratios of L-TRP metabolite

Ratio L-KYN/L-TRP: The ratio between L-KYN and L-TRP increased significantly after 10th rTMS applications (110 % vs. before

	before 1 st rTMS	after 10 th rTMS
NIHSS	4.18 ± 0.39	2.97 ± 0.38 *
Barthel-Index	58.48 ± 4.85	74.38 ± 3.95 **
Ashworth Scale	1.6 ± 0.2	1.4 ± 0.2

Table 1: Alterations in ability scores i.e. NIHSS, Barthel-Index and Ashworth Scale in stroke patients before rTMS (before 1st rTMS) and after rTMS (after 10th rTMS) treatment. Data represent mean \pm S.E.M. of independent measurement; *means differ significantly by $P < 0.05$; **means differ significantly by $P < 0.01$.

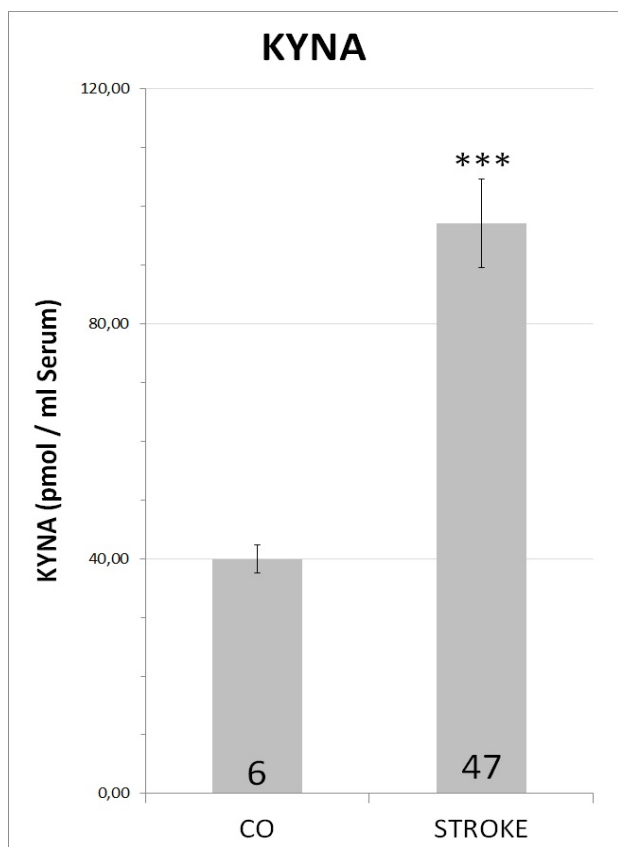


Figure 4: Alteration of kynurenic acid (KYNA) levels in the serum of stroke patients compared to control subjects (CO). Data represent mean \pm S.E.M. of independent measurement; ***means differ significantly by $P < 0.001$.

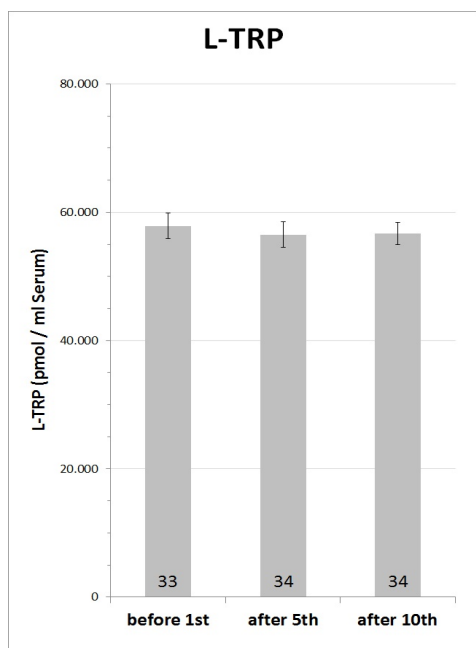


Figure 5: L-tryptophan (L-TRP) levels in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean \pm S.E.M. of independent measurement.

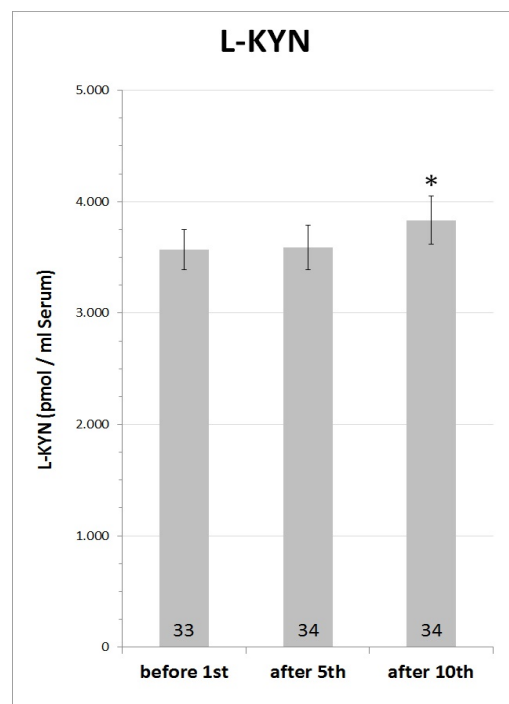


Figure 6: L-kynurenine (L-KYN) levels in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean \pm S.E.M. of independent measurement; *means differ significantly by $P < 0.05$ vs. before 1st.

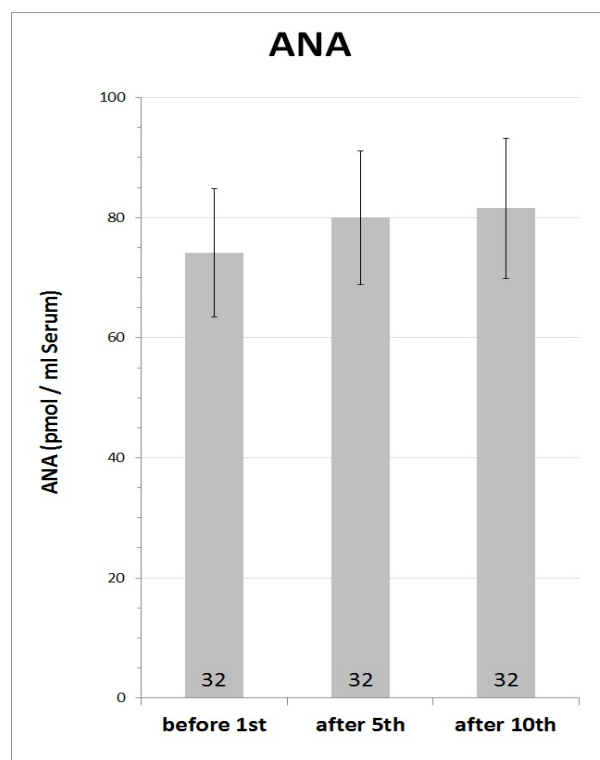


Figure 7: Anthranilic acid (ANA) levels in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean \pm S.E.M. of independent measurement.

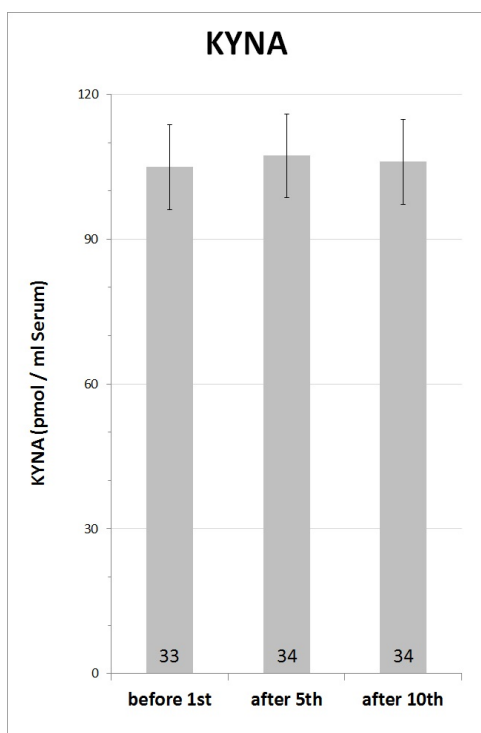


Figure 8: Kynurenic acid (KYNA) levels in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean \pm S.E.M. of independent measurement.

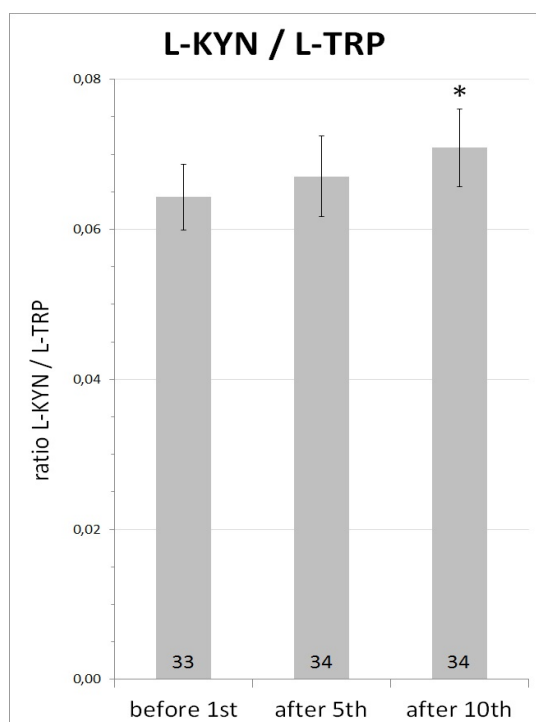


Figure 9: Ratio between L-kynurenine and L-tryptophan (L-KYN/TRP) in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean \pm S.E.M. of independent measurement; *means differ significantly by $P < 0.05$ vs. before 1st.

1st; $p = 0.04$) (Figure 9). The effect was visible after the 5th and after the 10th rTMS applications, as well. One-way ANOVA analysis of variance between 3 groups revealed the means of ratio L-KYN/L-TRP in the serum not statistically different ($F = 0.4263$, $P = 0.6541$, Figure 9).

Ratio ANA/L-KYN: The ratio between ANA and L-KYN was not influenced significantly after 10th rTMS. It showed a tendency of increasing after 5th but without progression of increasing after 10th rTMS (Figure 10). One-way ANOVA analysis of variance between 3 groups revealed the means of ANA/L-KYN ratio not statistically different in the serum ($F = 0.0368$, $P = 0.9639$, Figure 10).

Ratio KYNA/L-KYN: The ratio between KYNA and L-KYN was not influenced significantly after 10th rTMS applications. After 5th rTMS applications the ratio showed tendency of increasing but after 10th rTMS applications the ratio was moderately lowered (96 % of before 1st), (Figure 11). One-way ANOVA analysis of variance between 3 groups revealed the means of KYNA/L-KYN ratio not statistically different in the serum ($F = 0.1281$, $P = 0.8799$, Figure 11).

Ratio ANA/KYNA: Ratio between ANA and KYNA increased significantly and progressively after 10th rTMS application (123 % vs. before 1st; $p < 0.05$), (Figure 12). One-way ANOVA analysis of variance between 3 groups revealed the means of ANA/KYNA ratio not statistically different in the serum ($F = 0.2688$, $P = 0.7649$, Figure 12).

Influence of rTMS on CK and Prolactin in stroke patients

Creatine kinase (CK) CK levels in stroke patients were in the normal range of control (Figures 13 and 14). However, among the male and female stroke patients, a tendency of increased CK levels can be seen in patients of “71 - 89 years old” group comparing to patients of “52-70 years old”. Obtained data are comparable with previously published (Table 2) [35,44].

After rTMS no significant change of CK levels in the serum of male and female stroke patients was found. One-way ANOVA analysis of variance showed not significant differences (male: $F = 0.1438$, $P = 0.8662$, Figure 13; female: $F = 0.6694$, $P = 0.5267$, Figure 14).

Prolactin

Prolactin levels in male and female stroke patients are in the normal range (Figure 15 and 16) and data are comparable to published study [36,45,46]. However, among male stroke patients a tendency of increased prolactin levels can be seen in group of “71 - 89 years old” comparing to “52-70 years old” ($P < 0.001$; Figure 15). In female stroke patients of “71 - 89 years old” prolactin levels were markedly lowered, but not significantly ($P < 0.066$) comparing to “52-70 years old” group.

After rTMS prolactin levels were not affected in male stroke patient, one-way ANOVA analysis of variance between 3 groups revealed not statistical difference in the serum ($F = 0.0424$, $P = 0.9584$, Figure 15). Whereas in female stroke patients with age from 52 to 70 years prolactin levels after 10th rTMS applications were even lowered

		Male	Female
CK	Stroke	73.89 \pm 9.84 (U/l)	49.83 \pm 7.00 (U/l)
	Normal range	39 – 308 U/l	26 – 192 U/l
Prolactin	Stroke	11.63 \pm 1.08 (ng/ml)	13.29 \pm 2.4 (ng/ml)
	Normal range	4.04 – 15.2 (ng/ml)	4.79 – 23.3 (ng/ml)

Table 2: CK and Prolactin levels in the serum of male and female stroke patients. Data represent mean \pm S.E.M. of independent measurement.

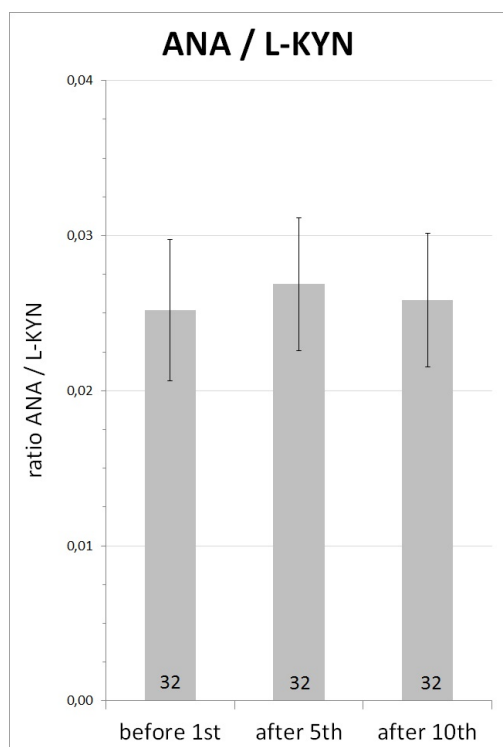


Figure 10: Ratio between anthranilic acid and L-kynurenine (ANA/L-KYN) in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean ± S.E.M. of independent measurement.

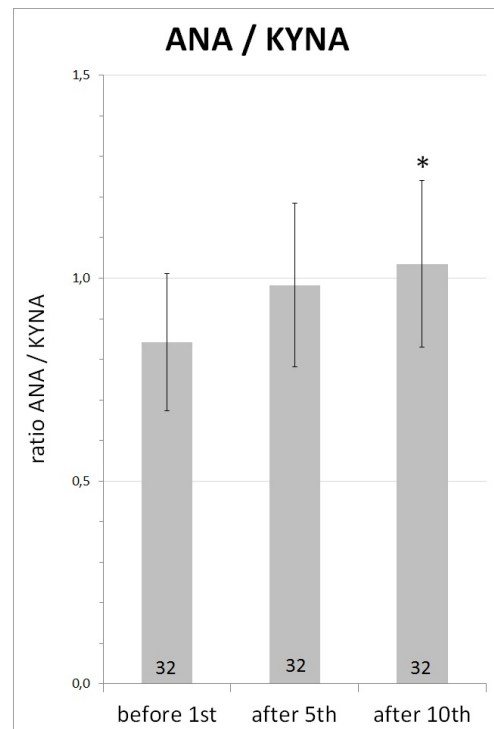


Figure 12: Ratio between anthranilic acid and kynurenic acid (ANA/KYNA) in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean ± S.E.M. of independent measurement; * means differ significantly by $P < 0.05$ vs. before 1st.

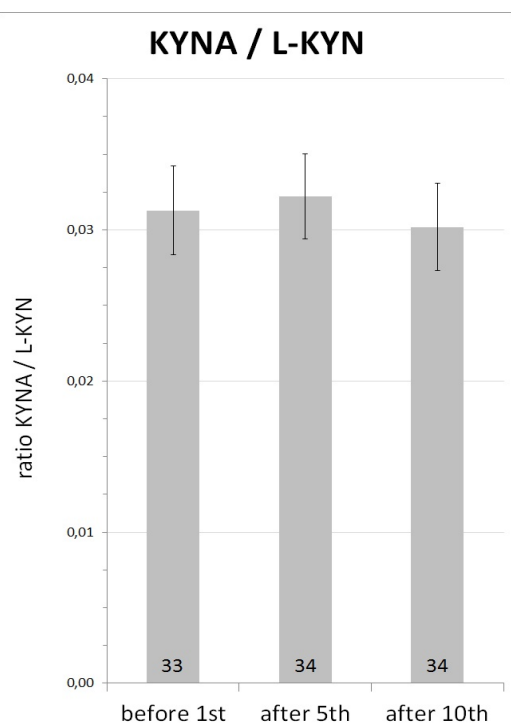


Figure 11: Ratio between kynurenic acid and L-kynurenine (KYNA/L-KYN) levels in the serum of stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean ± S.E.M. of independent measurement.

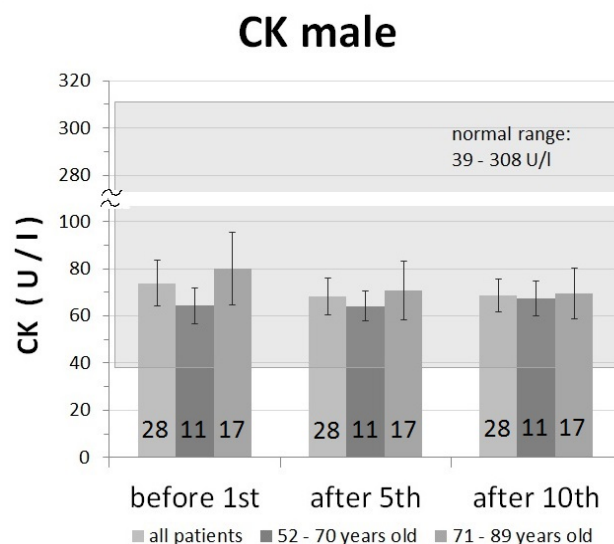
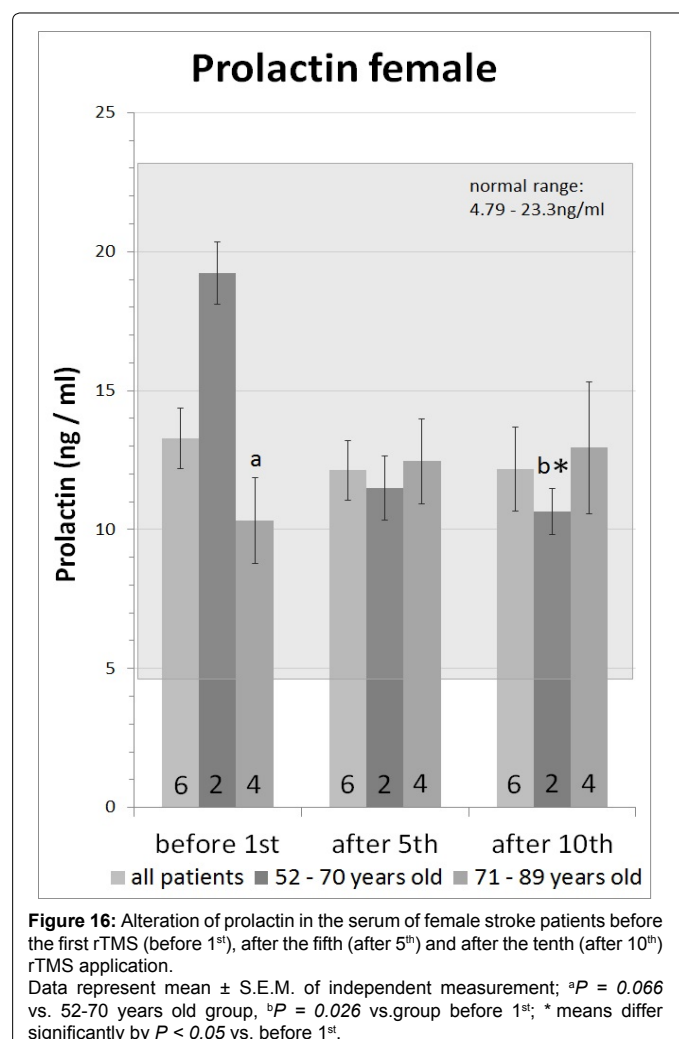
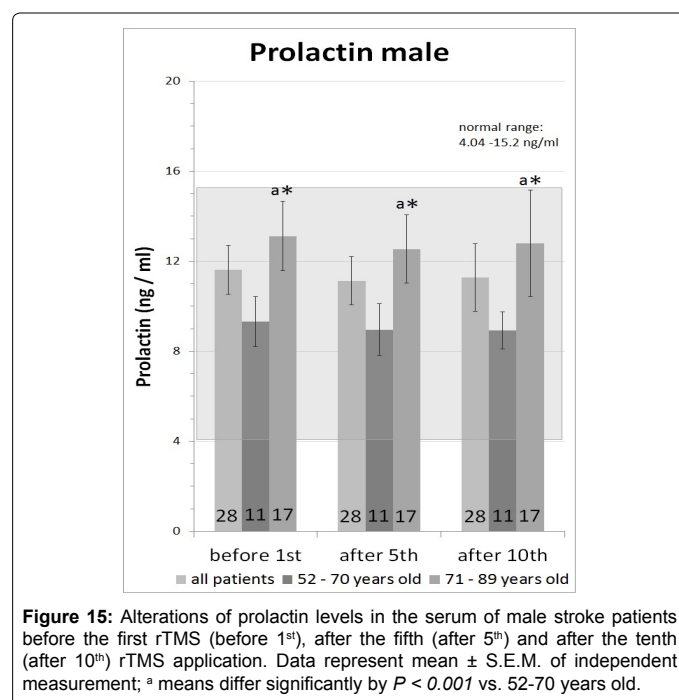
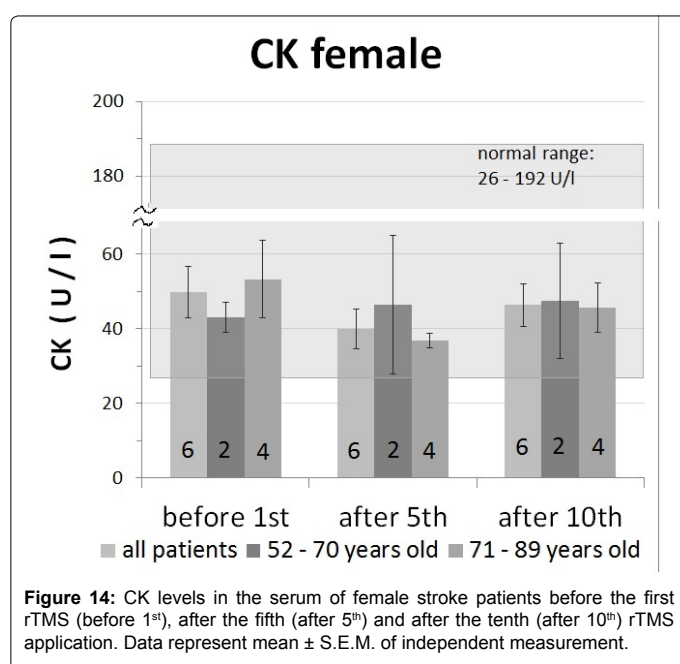


Figure 13: Alterations of CK levels in the serum of male stroke patients before the first rTMS (before 1st), after the fifth (after 5th) and after the tenth (after 10th) rTMS application. Data represent mean ± S.E.M. of independent measurement.

significantly ($P < 0.05$; Figure 16), but these values were still within the normal range. One-way ANOVA analysis of variance between 3 groups revealed the means of prolactin levels not statistically different in the serum ($F = 0.1128$, $P = 0.8941$).

Discussion

Our study found that tryptophan metabolism is markedly activated in the serum of stroke patients, even at a later period after the stroke incidence. For the first time we demonstrate a marked enhancement of ANA (260 % of CO) followed by KYNA (160 % CO), L-KYN (161 % of CO) and L-TRP in the serum of stroke patients from 6 months to 5 years after stroke. Furthermore, the marked increase of ANA/L-KYN ratio and to a lesser degree the KYNA/L-KYN ratio suggests profound activation of ANA formation compared to KYNA and this might have a notable functional meaning. Darlington and co-authors described changes of L-TRP metabolism during acute period after stroke [32] and suggested that the development or occurrence of the infarct is characterized by a significantly raised L-KYN/L-TRP ratio at 24 hours after stroke event [32]. Our study demonstrates that this increase of the L-KYN/L-TRP ratio still is present years after the stroke event suggesting permanent activation of L-TRP metabolism. Darlington and co-authors also suggested that a decreased ratio of 3-OH-ANA/ANA is related with lethality [32]. In the present study the determination of 3-OH-ANA and 3-OH-KYN was not performed but the increase of ANA in the serum in stroke patients found in our study and the lowered 3-OH-ANA/ANA ratio after stroke [32] suggests long lasting activation of ANA synthesis. Our data do not support the proposed idea of a correlation between lethality and increased ANA levels, since due to an enhancement of ANA levels synthesizing quinolinic acid would compensate the enlarged inhibitory effect caused by increase of KYNA levels. This interpretation of finding correlates with ANA changes found after rTMS therapy, too. We believe that a high KYNA content is a more predictable marker for lethality. Also Darlington and co-workers measured marked elevation of KYNA content in the serum of patients who died within 21 days, whereas in others stroke patients only moderate KYNA changes were observed [32]. Our preliminary data on KYNA levels in the CSF show marked elevation of KYNA in the acute period of stroke, as well [47]. Revealed correlations between various parameters of the stroke event and kynurenine metabolites suggest their functional involvement and importance. An interruption of blood supply and the subsequent deprivation of oxygen and glucose



cause neuronal dysfunction, necrosis of neuronal tissue and even death and proportional increase of KYNA are visible. For example, in the asphyxia model we found marked elevation of KYNA levels in the brain, whereas 3-OH-KYN which is the bio precursor for 3-OH-ANA was only moderately altered [48]. Notably, increase of KYNA was significantly dependent on the duration of time of asphyxia [48]. An enhancement of KYNA levels in the serum and CNS (central nervous system) was found in the experimental temporal lobe epilepsy model [49] or in the asphyxia model [48], then during inflammatory conditions in an Picornea encephalo myocarditis virus (EVMC) infection model [50] and in HIV-1 infection conditions [26,27] or even in DOWN syndrome [51].

It is also important to mention that increased KYNA levels might have an impact on the impairment of memory and cognition in stroke patients, as well as in post-stroke depression. Movement disturbance up to immobility in stroke patients could be in part responsible for the increase of KYNA levels in the serum, since on the other hand exercise by means of stochastic resonance therapy (SRT) significantly lowered KYNA levels in the serum of healthy subjects [52]. An importance of exercise for the improvement of chronic stroke patients has been already described [53]. A neurorehabilitation program applying rTMS to stroke patients yielded also promising data in improving the recovery of sensorimotor and cognitive functions [54,55]. In good correlated with other recently published studies [13-16] also our study reveals the improvement of motor ability in stroke patients after rTMS. Augmentation of motor performance and a moderate reduction of spasticity could be observed, too, and this is in line with previously published observations [11]. Interestingly, we found that the effect of rTMS had a significant impact on the output of occupational therapy if these trials were performed immediately after rTMS. An increase of finger dexterity and amelioration of Barthel Index was found. Importantly, in patients with high nursing needs less service was required if the occupational therapy was performed right way after rTMS [40]. These are notable and important observations for optimizing the therapy management.

Our data demonstrate for the first time that rTMS affected L-TRP metabolism in the serum of stroke patients significantly. Whereas L-TRP levels lowered moderately in the serum after 10th rTMS, L-KYN content was significantly increased, suggesting an activation of enzyme (s) responsible for L-TRP degradation. Notable, the value of L-KYN/L-TRP ratio increased after 5th and significantly after 10th rTMS indicating the ability of rTMS to influence dose dependently degradation of L-TRP at least in the serum. Also changes of the amino acid transporter action due to rTMS for L-TRP or for some L-TRP metabolites between brain and periphery could take place due to rTMS. Interestingly, there are some trials applying the rTMS approach with 100 Hz to promote selectively drug transport into organs including the brain [56]. While ANA/L-KYN and KYNA/L-KYN ratio values altered moderately but not significantly, the ANA/KYNA ratio values were dose dependently and significantly increased after 10th rTMS treatment. An increase of the ANA/KYNA ratio value due to rTMS is a notable finding and might be important as an event with neuromodulatory abilities. An enhancement of ANA/KYNA ratio values could be involved in the improvement of motor performance too, but this finding needs to be confirmed. Besides that, a study including a sham-rTMS group would be of values in order to confirm the positive effect of rTMS application in stroke patients.

Several studies documented the effect of rTMS on plasma levels of a variety of hormones including cortisol, prolactin and

thyroid stimulating hormone [57-59]. Results from these studies are inconclusive but indicate that rTMS might significantly affect neuroendocrine functions. The reasons of disperse results are probably due to differences of values among different age and sex distribution. According to prolactin levels the values are in very broad range. Indeed, differences in prolactin we could find with respect to the age and sex in stroke patients, although data are still in control range [45,46]. Interestingly, in female stroke patient with an age from 52-70 years the prolactin levels were significantly lowered after 10th rTMS. It is likely that rTMS indirectly, due to release of dopamine [60], blocks the secretion of prolactin. CK levels also differ between female and male and a moderate increase with age could be seen but no effect of rTMS on CK levels was found.

Summary

Improvement of daily activities of living and of motor performance was revealed after rTMS and notably, a significant amelioration of finger dexterity and increase of Barthel Index values could be achieved when occupational therapy immediately followed rTMS treatment. Interestingly, L-TRP metabolism was significantly activated in the serum of stroke patients, up to five years after the stroke event. Furthermore, rTMS further activated L-TRP metabolism and progress of significant changes of the ANA/KYNA ratio values in post-stroke patients has been found after rTMS, too, at least in the serum. These findings might suggest induction of endogenous compensatory mechanism, which might be of significance for motoric improvement of stroke patients

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Ethical Considerations

The procedures of the research proposal from Berthold Kepplinger have been approved by Lower Austrian Ethical Regulations. Research was based on voluntary participation and oral and/or written informed consent with the institution Neurological Department (head at the time period of investigation: Prim. MD. Berthold Kepplinger, MSc) Landeskrankenhaus Mauer-Amstetten and patients.

Conflict of Interest

The authors declare no conflict of interest. The idea for the article was conceived by Berthold Kepplinger and Halina Baran. The experiments were designed by Berthold Kepplinger, Sabine Eigner and Halina Baran. The experiments were performed by Sabine Eigner, Berthold Kepplinger, Brenda Sednitsky-Semler, Petra Berger and Pavol Kalina. The data were analyzed by Brenda Sednitsky-Semler, Sabine Eigner, Berthold Kepplinger, Petra Berger, Pavol Kalina, Halina Baran.

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