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Theogallin and L-theanine as active ingredients in decaffeinated green tea extract: I. electrophysiological characterization in the rat hippocampus in-vitro

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Abstract

The in-vitro hippocampus slice preparation was used to mimic a physiological situation where nervous tissue is exposed directly to the water soluble extract of green tea and some of its constituents. This investigation provides evidence that L-theanine- and theogallin-enriched decaffeinated green tea extract is able to change the physiological pattern of electrical hippocampus activity in a concentration dependent manner (EC50 3 mg L^{-1}). Of the seven fractions or single components tested (fraction containing all amino acids without L-theanine, fractions containing all amino acids plus L-theanine, glutamic acid, theogallin, its metabolites quinic acid and gallic acid, and L-theanine alone), glutamic acid produced the strongest changes in terms of increased population spike amplitude after single stimuli and increased long-term potentiation, commonly taken as representative for enhancement of spatial and time dependent memory. The presence of theogallin alone shifted the activity in the same direction. Similar results as with theogallin were obtained in the presence of quinic acid. No effect was seen with gallic acid. Opposite changes (decrease of population spike amplitude and attenuated long-term potentiation) were observed in the presence of L-theanine alone. No effects were detected during the addition of the amino acid mixture unless L-theanine was added, leading to a decrease of the responses as observed for the action of L-theanine alone. The results provide evidence for the involvement of several active principles in the action of enriched green tea extract on electrical brain activity. The overall enhancement of hippocampal pyramidal cell responses as observed for the crude extract seems to be due to the combined action of glutamic acid and theogallin (or its presumable metabolite quinic acid), whereas L-theanine seems to have an opposite effect. However, this action was not strong enough to antagonize the effects of glutamic acid and theogallin. The results are in line with the observation that the tested green tea extract improves cognition at concomitant mental relaxation in man.

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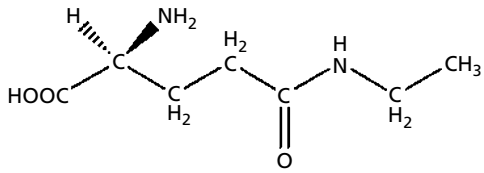
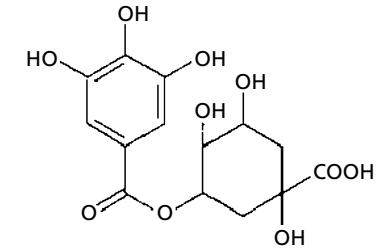
Introduction

The stimulating properties of green tea in general have been attributed to the presence of caffeine. However, green tea also contains other ingredients, known from earlier publications to be unique to green tea (i.e. Graham 1992; Kaneko et al 2006), namely theogallin (3-galloylquinic acid) and L-theanine (5-N-ethylglutamine) (Figure 1), as well as other compounds. Therefore, a decaffeinated extract enriched with theogallin and L-theanine but lacking other constituents was produced. The question arose whether these two compounds were pharmacologically active under the condition of direct exposure to the brain. Absolutely no information is available regarding the possible pharmacological action of theogallin, whereas there is evidence from in-vivo and in-vitro experiments, as well as from clinical studies in man, that L-theanine has pharmacological effects (Nathan et al 2006; Kimura et al 2007). We therefore decided to use a well-accepted in-vitro model to extend our knowledge on this newly developed green tea extract by analysing the action of it and its constituents by in-vitro (hippocampus slice) and in-vivo (Tele-Stereo-EEG, as reported in the accompanying paper) techniques.

The hippocampal slice preparation is a validated model for direct analysis of substance interaction with living neuronal tissue (Lynch & Schubert 1980; Dingledine 1984). Due to the preservation of the three-dimensional structure of the hippocampal tissue, effects on the



L-Theanine (*N*-ethyl-L-glutamine)



Theogallin (3-galloyl-quinic acid)

Figure 1 Structure of theogallin and L-theanine.

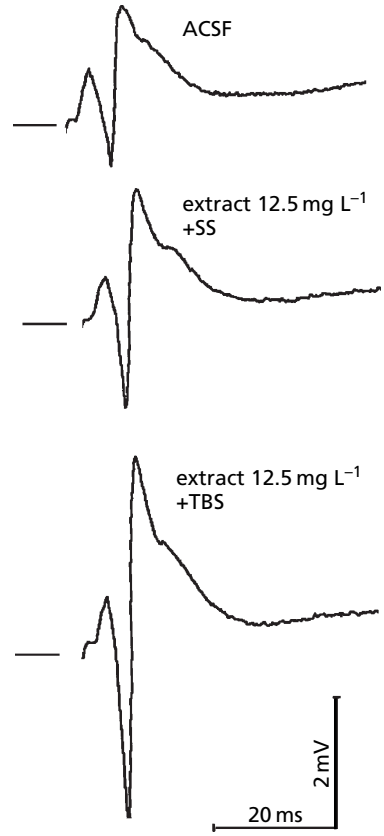


Figure 2 Depiction of examples of original signals showing the effects of pre-challenge values, the signal in the presence of green tea extract using single stimuli (SS) or theta-burst stimulation (TBS).

excitability of pyramidal cells can be studied in a unique manner. The stimulation of Schaffer Collaterals leads to release of glutamate, resulting in excitation of the postsynaptic pyramidal cells. The result of the electrical stimulation can be recorded as so-called population spike (pop-spike, Figure 2). The amplitude of the resulting population spike represents the number of recruited pyramidal cells. The advantage of the model is that not only is there the possibility of recording in-vitro during 8 h but also the excitability of the system can be modified to create pathophysiological conditions. An interesting result using this model was, for example, the ability of memantine (Dimpfel 1996), a substance used in the treatment of dementia, to increase population spike amplitude in response to single stimuli and to increase long-term potentiation. This methodology has been successfully used for the characterization of other natural compounds to detect their direct action on brain matter (Dimpfel et al 1995; Dimpfel 2006).

Besides the total extract, all ingredients were looked at in a concentration-dependent manner under the condition of single stimuli as well as under the condition of burst-pattern stimulation to induce long-term potentiation: a combination of amino acids with or without L-theanine, L-theanine alone or theogallin alone, and its presumable metabolites quinic acid and gallic acid, as well as glutamic acid.

Materials and Methods

Materials

Extract and fractions tested were diluted in artificial cerebrospinal fluid (ACSF) to give the final concentration as indicated and tested using 4 different slices.

Extract and test samples were supplied by Plantextrakt: Enriched green tea extract Lab 15920096; glutamic acid (Lab. 15920366); theogallin 97% pure (Lab. 15920398); amino acid mixture with L-theanine (Lab. 15920353); amino acid mixture (synthetic) (Lab. 15920364); L-theanine pure (Lab. 15920365); quinic acid 98% pure (charge No. S28053-485 from Sigma-Aldrich Chemie GmbH, Taufkirchen); gallic acid 98% pure (charge No. 09316PC from Sigma-Aldrich Chemie GmbH, Taufkirchen).

The extract was prepared by water extraction and subsequent removal of caffeine by liquid–liquid extraction. Enrichment of L-theanine and theogallin was performed by column chromatography. The extract was concentrated and spray dried. The composition of the extract was: water 3.52%, minerals (ash) 24.01%, protein (Nx6.25) 20.60%, amino acids (HPLC, sum) 3.52%, glutamic acid 1.18%, fat 0.10%, polyphenols (Folin) 2.50%, tea catechines (HPLC, sum) 0.10%, caffeine (HPLC) < 0.01%, L-theanine (HPLC) 9.71%, theogallin (HPLC) 4.65%.

Methods

The experiments were performed in congruence with German law on the use of animals in research. Hippocampus slices were obtained from 26 adult male Sprague–Dawley rats (Charles River Wiga, Sulzbach, Germany). Rats were kept

under a reversed day–night cycle for 2 weeks before the experiments, to allow recording of in-vitro activity from slices during the active phase of their circadian rhythm (Dimpfel et al 1994; Dimpfel 1995). Rats were exsanguinated under ether anaesthesia, the brain was removed in total and the hippocampal formation was isolated under microstereoscopic sight. The mid-section of the hippocampus was fixed to the table of a vibratom (Rhema Labortechnik, Hofheim, Germany) using a cyanoacrylate adhesive, submerged in chilled bicarbonate-buffered saline (ACSF; composition in mM: NaCl 124, KCl 5, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, glucose 10), and cut into slices of 400 μm thickness. All slices were pre-incubated for at least 1 h in Carbogen saturated ACSF (pH 7.4) in a prechamber before use (Dimpfel et al 1991).

During the experiment the slices were held and treated in a special superfusion chamber (List Electronics, Darmstadt, Germany) according to Haas et al (1979) at 35°C (Schiff and Somjen 1985). The preparation was superfused with ACSF at 180–230 mL h^{-1}. Electrical stimulation (200 μA constant current pulses of 200 μs pulse width) of the Schaffer Collaterals within the CA2 area and recording of extracellular field potentials from the pyramidal cell layer of CA1 was performed according to conventional electrophysiological methods (Dimpfel et al 1991) using the Labteam Computer system Neurotool software package (Medisyst GmbH, Linden, Germany). Examples of single recordings are shown in Figure 2. Measurements were performed at 10-min intervals to avoid potentiation mechanisms. Four stimulations, each 20 s apart, were averaged for each time point. After obtaining three stable pre-drug values the perfusion was changed to drug containing ACSF and continued for at least 30 min. The mean amplitude of the three pre-drug signals were averaged and set to 100%. All averaged post-drug changes refer to this reference value.

Long-term potentiation was induced following the methodology of Lynch & Schubert (1980), consisting of a particular theta stimulation pattern, resembling physiological activation (Larson et al 1986). This stimulation pattern has been recognized as optimal for studying long-term potentiation of synapses in the CA1 region of the hippocampus (Capocchi et al 1992).

Fractions or compounds tested were diluted in ACSF to give the final concentration as indicated and tested using 4 different slices. Experiments were performed according to German guidelines for animal experimentation.

Statistical methods

For statistical analysis the non-parametric Wilcoxon, Mann and Whitney *U*-test was applied. Critical values of *U* were taken from Milton (1964). Comparisons of amplitude differences in the presence of increasing concentrations of total extract, fractions or single compounds were made with respect to independent controls with ACSF (saline). Each comparison was based on evaluation of 4 slices. Statistical evaluation was only used to learn whether or not any concentration of the extract or one of its ingredients had a pharmacological effect in comparison to saline. Therefore no multiple testing was intended.

Results

Action of the total extract on population spike amplitudes

Compounds were superfused on fresh brain slices from the hippocampus, which were electrically stimulated to excite pyramidal cells. An example is given in Figure 2. Their activity reflects the influence of active components on the amplitude of the population spike in a concentration-dependent manner and allows the determination of EC50 values. The effect of the total extract is depicted in Figure 3. The continuous superfusion of the total extract in the slice preparation resulted in concentration-dependent increases of the amplitude of the population spike. At a concentration of 9 mg L^{-1}, the maximum effect could be observed. The EC50 value amounted to about 3 mg L^{-1}. Stimulation using the theta-burst pattern resulted in increases of the population spike amplitude up to 230% of the baseline value. This induction of long-term potentiation (LTP) could be further increased with an EC50 value of about 3 mg L^{-1} ($P < 0.01$) in the presence of the total extract.

Action of theogallin and its possible metabolites on population spike amplitudes

Exposure of the hippocampal slices to theogallin alone resulted in a concentration-dependent increase after single stimuli as well as after theta-burst stimulation (Figure 4). Its maximum after single stimuli was reached with 4 mg L^{-1}, but the EC50 value was reached already with about 0.5 mg L^{-1}. Similar action could be documented for theta-burst stimulation reaching a maximum with 2 mg L^{-1} (EC50 about 1 mg L^{-1}).

Theogallin might be metabolized in-vivo to give gallic acid and quinic acid. Whereas the presence of gallic acid did not change the amplitude of the population spike (see Figure 6), quinic acid increased the amplitude in a similar manner to the

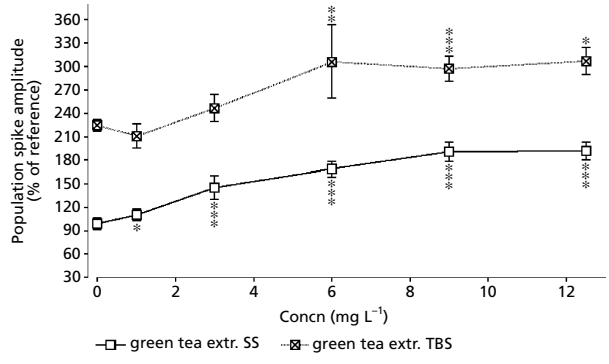


Figure 3 Freshly prepared rat hippocampal slices were superfused with different concentrations of the total extract. Amplitude of the population spike is given in percent of the baseline value (reference). Data are given as mean ± s.e.m. of n = 4 slices. *$P < 0.1$, **$P < 0.05$, ***$P < 0.01$, compared with ACSF (saline) performed under identical conditions (Wilcoxon-Mann-Whitney U-Test).

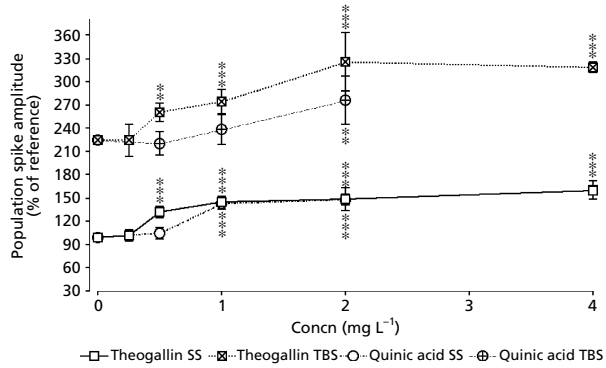


Figure 4 Freshly prepared rat hippocampal slices were superfused with different concentrations of theogallin or quinic acid (values partially superpose those of theogallin). Amplitude of the population spike is given in percent of the baseline value (reference). *$P < 0.1$, **$P < 0.05$, ***$P < 0.01$, compared with ACSF (saline) performed under identical conditions (Wilcoxon-Mann-Whitney U-Test).

mother compound, reaching a maximum at 2 mg L^{-1} after single-shock stimulation and theta-burst stimulation (Figure 4).

Action of L-theanine on population spike amplitudes

Superfusion of the preparation with very low amounts of L-theanine led to concentration-dependent decreases of the population spike amplitude after single-shock stimulation. A maximum effect was already reached with 1 mg L^{-1} giving an EC50 of about 0.5 mg L^{-1}. The same feature could be seen using theta-burst stimulation (Figure 5).

Action of amino acids on population spike amplitudes

The combination of amino acids without glutamic acid did not change the amplitude of the population spike (Figure 6).

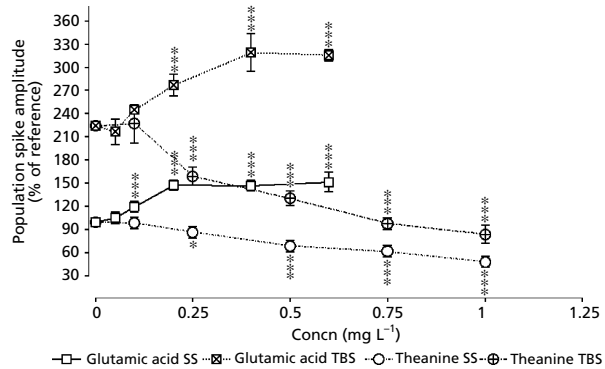


Figure 5 Freshly prepared rat hippocampal slices were superfused with different concentrations of glutamic acid or theanine. Amplitude of the population spike is given in percent of the baseline value (reference). *$P < 0.1$, **$P < 0.05$, ***$P < 0.01$, compared with ACSF (saline) performed under identical conditions (Wilcoxon-Mann-Whitney U-Test).

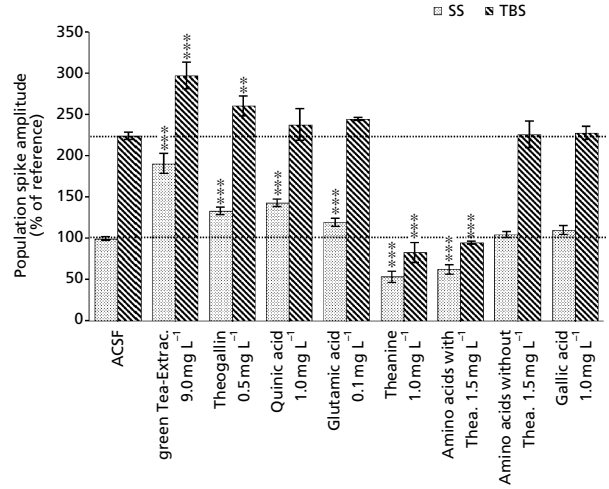


Figure 6 Overview on experiments performed with total tea extract and its different components in concentrations roughly corresponding to their content within the extract. *$P < 0.1$, **$P < 0.05$, ***$P < 0.01$, compared with ACSF (saline) performed under identical conditions (Wilcoxon-Mann-Whitney U-Test).

However, very low concentrations of glutamic acid induced a concentration-dependent increase of the amplitude of the population spike under both stimulatory conditions. Maxima were reached with 0.2 mg L^{-1} under the condition of single stimuli, with 0.4 mg L^{-1} under the condition of theta-burst stimulation (Figure 5).

Discussion

The aim of the investigation was to explore the interaction of a special extract from green tea (L-theanine- and theogallin-enriched but decaffeinated) directly with brain matter. We succeeded in detecting a profound change of hippocampal pyramidal cell activation as measured after single stimuli and induction of long-term potentiation by TBS. This change in physiological activity was concentration dependent over the range of 1–10 mg L^{-1}, a concentration which possibly is reached within the brain after oral administration of the extract to man. Since enhancement of long-term potentiation has been related to better spatial and time-dependent memory, cognitive enhancing effects should be expected in the presence of effective blood levels of the extract components, if they can pass the blood–brain barrier.

The question therefore arose as to which of the components of green tea were responsible for this action. Fractionation of the extract and chemical analysis revealed that amino acids made up a considerable part of the mass and that among them was L-theanine (about 9.7%) and glutamic acid (about 1.18% of the total extract). A compound characteristic to tea, theogallin, was also present in an amount of 4.6%. Thus, these fractions or components had to be tested separately to explain the net effect of the total extract.

No effects could be seen in the presence of an amino acid mixture excluding L-theanine and glutamic acid at concentrations present in the total extract. The exposure of

the hippocampal tissue to glutamic acid revealed a higher recruiting of pyramidal cell activity measured as increase of the population spike amplitude after stimulation of the collateral pathway. Increase of long-term potentiation was likewise expected, since we are dealing with a glutamatergic synapse where this amino acid serves as neurotransmitter. Surprisingly, theogallin had a similar action. Up to now virtually no biological action of theogallin has been reported in the literature. Therefore it would be worth studying this compound characteristic to green tea in more detail. Since theogallin is composed of gallic acid and quinic acid, these two possible metabolites were tested as well. The action of quinic acid was similar to that of theogallin, whereas no effect was seen with gallic acid. This means that either the quinic acid moiety is responsible for the action of theogallin or theogallin is metabolized during the perfusion procedure by brain matter. This is an extremely interesting result since up to now no action of theogallin and quinic acid on the central nervous system has been reported.

The concentration-dependent attenuation of pyramidal cell activity by L-theanine is a new finding which might have very important consequences. Since L-theanine crosses the blood–brain barrier using a leucine-preferring transport system (Yokogoshi et al 1998), effects on brain activity in general can be expected. On the other hand, L-theanine is metabolized to ethylamine and glutamic acid (Unno et al 1999). However, glutamic acid has opposite effects on the activity of the pyramidal cells. This means that the effect of L-theanine on the brain might be due to the ethylamine moiety. Direct administration of L-theanine into the brain (lateral ventricle) has been reported to have neuroprotective effects (Kakuda et al 2000). The mechanism of action seems to involve glutamate receptors (Kakuda 2002). This is in line with the results reported here, of a decrease in synaptic action due to impairment of AMPA- and kainite-receptor-mediated transmission (Kakuda et al 2002) or possibly interference with glycine-mediated modulation of the NMDA receptor (Yamada et al 2005).

In summary, the studied enriched green tea extract has several active components. The original belief that L-theanine would dominate the effect of the total extract could not be proven. On the contrary, the total extract revealed a strong concentration-dependent stimulatory action on the hippocampal pyramidal cells, leading to an increase of long-term potentiation, presumably due to the action of theogallin or its possible metabolite quinic acid. This kind of effect is representative for an enhancement of spatial and time-dependent memory. The results, therefore, supplement and strengthen the interpretation of a recent study performed with the total extract in man, where positive effects on cognition and relaxing properties have been found (Dimpfel et al in press).

Conclusion

The results provide evidence for the involvement of several active principles in the action of decaffeinated green tea extract on electrical brain activity. The overall enhancement of hippocampal pyramidal cell responses, as observed for the total extract, seems to be due to the combined action of glutamic acid and theogallin (or its presumable metabolite quinic acid), whereas L-theanine seems to have an opposite effect. However, this action was not strong enough to antagonize the effects of glutamic acid and theogallin. Since no other ingredients are left in possibly effective concentrations, theogallin and L-theanine (and possibly quinoic acid) are probably responsible for the effects of the total extract. The results are in line with the observation that the tested decaffeinated green tea extract has stimulating properties and improves time and spatial memory parameters as derived from increases in long-term potentiation.

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