Aus dem Universitätsklinikum Münster

Institut für Physiologie I
Direktor: Univ.-Prof. Dr H. C. Pape

# Acute effects of garlic extract on spreading depression and synaptic activity in rat brain slices

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**Marschollek, Claudia**
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Dekan: Univ.-Prof. Dr. Wilhelm Schmitz

1. Berichterstatter: Prof. Dr. med Ali Gorji
2. Berichterstatter: Prof. Dr. Hansdetlef Wassman

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Referent: Prof. Dr. med Ali Gorji
Koreferent: Prof. Dr. Wassmann

**ZUSAMMENFASSUNG**

**Wirkung von Knoblauch auf kortikaler Spreading Depression und synaptische Plastizität**

Claudia Marschollek

Durch die Geschichte hindurch haben verschiedene Kulturen das Potential von Knoblauchöl erkannt um damit verschiedene Krankheiten vorzubeugen und diese zu behandeln. Die neuesten Untersuchungen unterstützen, dass Knoblauchöl und dessen Auszüge einen großen Anwendungsbereich hat. Die unterschiedlichen Bestandteile in Knoblauch reduzieren das Risiko einer kardiovaskulären Erkrankung und Krebs, haben einen Antitumoreffekt, haben eine Wirkung auf die Blutzuckerkonzentration, beugen neurologischen Störungen vor und werden in der Behandlung dieser eingesetzt. In mittelalterlicher Literatur wurde Knoblauch häufig empfohlen um Kopfschmerzen zu behandeln. Um die neurophysiologische Wirkung von Knoblauchöl zu beurteilen, wurde dieses auf Effekte bei kortikaler Spreading Depression, indiziert durch KCL Mikroinjektion, elektrisch hervorgerufene exzitatorische postsynaptische Feldpotentiale (fEPSP) und Langzeitpotentialen (LTP)am somatosensorischen neokortikalen Gewebe von Ratten getestet. Bei der CSD zeigte sich eine Abnahme der Amplitudeabhängig von der Dosis (1-500ml/l) von Knoblauchöl, allerdings keinen Einfluss auf die Dauer und Geschwindigkeitsausbreitung. Die Konzentration von 500ml/l Knoblauchöl reduzierte die Amplitude von fEPSP reversibel. Ebenfalls blockierte sie die Auslösung von LTP und fEPSP in der dritten Schicht der neokortikalen Scheiben signifikant. Somit kann Knoblauchöl CSD unterdrücken, wahrscheinlich ähnlich der Hemmung der synaptischen Plastizität.

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**Abstract**

Throughout history many different cultures have recognised the potential use of garlic for prevention and treatment of different kind of diseases. Recent studies support the effects of garlic and its extracts in a wide range of application. Different compounds in garlic are thought to reduce the risk for cardiovascular diseases and cancer, to have anti-tumour effects, to show effect on blood glucose concentration, and to prevent and treat neurological disorders. Garlic was suggested by many medieval literatures to be beneficial in treatment of headache. To investigate the neurophysiologic properties of the action of garlic oil, its effects on CSD induced by KCl microinjection, electrically evoked field excitatory post-synaptic potentials (fEPSP), and long-term potentiation (LTP) were tested in rat somatosensory neocortical tissues. Garlic oil dose-dependently (1-500µl/l) decreased the amplitude of CSD whereas has no effect on CSD duration and propagation of velocity. Garlic oil (500µl/l) also reversibly reduced the amplitude of fEPSP. Garlic oil at concentration of 500µl/l significantly blocked induction of LTP of fEPSP in the third layer of neocortical slices. Thus, garlic oil can suppress CSD, likely via inhibition of synaptic plasticity. The data indicate the potential for garlic oil to use in the treatment of CSD-related neurological disorders.

## Introduction

Dietary factors play a crucial role in the development of various human diseases. Across different cultures, there are several dietary patterns, which are believed to promote human health. Despite cultural and geographical differences, there are some shared characteristics of healthy dietary patterns. Perceiving plant foods as beneficial diet is advised by the folklore of many cultures over centuries. Garlic acquired a reputation in different traditions as a formidable prophylactic and therapeutic medicinal herb.

*The history of garlic*

Garlic has been used throughout history for both culinary and medicinal purposes. Although sometimes maligned, garlic has had an amazing array of nutritional and medicinal applications throughout human history, and it is still improving the health of many today. Some of the earliest references to medicinal effect of garlic were found in *Avesta*, a collection of Zoroastrian holy writing that was probably compiled during the sixth century BC (Darmestetar, 1898). Archaeologists have discovered clay sculptures of garlic bulbs and paintings of garlic dating about 3200 B.C. in Egyptian tombs in *El Mahasna*. A recently discovered Egyptian papyrus dating from 1,500 B.C. recommends garlic as a cure all for over 22 common ailments, including lack of stamina, heart disease and tumors, and it's been said the Egyptians fed garlic to the slaves building the pyramids to increase their strength (Lasinski, 2005). In ancient Greece and Rome, garlic enjoyed a variety of uses, from repelling scorpions to treating dog bites and bladder infections to curing leprosy and asthma. Ancient Chinese and Indian medicine recommended garlic to aid respiration as well as digestion and to treat leprosy and parasitic infestation (Rivlin, 1998). Garlic is mentioned in the Bible and the Talmud. In the medieval period, garlic also played an important role in the treatment of different diseases. Avicenna, in his well-known book Canon, recommended garlic as a useful

compound in treatment of arthritis, toothache, chronic cough, constipation, parasitic infestation, snake-biting, gynaecologic disorders, and infectious diseases (Ibn Sina, 1989). With the onset of Renaissance, special attention was paid in whole Europe to the medicinal benefits of garlic. In the middle Ages, garlic was thought to combat the plague and was hung in braided strands across the entrances of houses to prevent evil spirits from entering (Lasinski, 2005).

In seventeenth century England, garlic was considered unfit for ladies and anyone who wished to court them, and it was avoided in America even early into the 20th century. However, garlic has attracted particular attention of modern medicine because of its widespread belief that it helps in maintaining good health. In some Western countries, the sale of garlic preparations ranks with those of leading prescription drugs.

*Allium sativum*, commonly known as garlic (German: Knoblauch), is a species in the onion family Alliaceae (German: Lauchgewächse). Garlic is a bulb of a lily-like plant, belonging to the same family as onions, chives, leeks and scallions. Garlic is the strongest flavoured member of the onion family. The majority of *Allium* species including garlic are native to the Northern hemisphere, mainly in Asia. A few species are native to Africa and Central and South America. China is by far the largest producer of garlic, with approximately 10.5 million tonnes annually, accounting for over 77% of world output (Economic Research Service, 2006).

The herbaceous bulb is characterized by a pungent odour. The bulb is compound, consisting of anything up to twenty segments, called 'cloves'. Usually there are about ten cloves to a bulb, packed side by side around a thin central core. These cloves separated by scaly membranes and enclosed by a brittle parchment-like skin. The flesh of the clove is ivory coloured, and should be hard and firm though easily cut with a finger nail. The skin is usually white, but may have a pale pink or purplish tinge. Garlic is widely variable in size, based on

its variety. Many varieties of garlic exist in different parts of world. In South East Asia a small variety with only four to six cloves grows and is similar to Spanish garlic, llium sativum ophioscorodon. A giant variety is grown in California. There are different types or subspecies of garlic, most notably hard-neck garlic and soft-neck garlic. The latitude where the garlic is grown affects the choice of type as garlic can be day-length sensitive. Hard-neck garlic is generally grown in cooler climates; soft-neck garlic is generally grown closer to the equator. The leaves, and flowers on the head are also edible, and being milder in flavour than the bulbs. The root cluster attached to the basal plate of the bulb is the only part not typically considered palatable in any form. Garlic propagates easily and is one of the simplest plants to grow. The flowers are hermaphrodite and are pollinated by Bees, insects.

The plant prefers light (sandy) and medium (loamy) soils and requires well-drained soil. The plant prefers acid, neutral and basic (alkaline) soils and can grow in very alkaline soil. It can not grow in the shade. Garlic plants can be grown close together, leaving enough room for the bulbs to mature, and are easily grown in containers of sufficient depth. It requires dry or moist soil. August or early fall is considered the best time for sowing cloves, as this allows the roots time to develop before the first frost sets in. New leaves will appear before winter and in the spring. Plants will rapidly reach a height of 18 inches. Garlic will do best in full sun but can be grown with satisfactory results in partial shade.

*Garlic properties*

When crushed, *garlic* yields allicin. Allicin has been found to be the compound most responsible for the hot sensation of raw garlic. Allicin (allyl 2-propenethiosulfineate or diallyl thiosulfinate) is the principal bioactive compound present in aqueous garlic extract or raw garlic homogenate. When garlic is chopped or crushed, allinase enzyme, present in garlic, is activated and acts on alliin (present in intact garlic) to produce allicin. Other main compounds present in garlic homogenate are 1-propenyl allyl thiosulfonate, allyl methyl thiosulfonate,

(E,Z)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide (ajoene), and γ-L-glutamyl-S-alkyl-L-cysteine. The adenosine concentration increases several-fold as the homogenate is incubated at room temperature for several hours.

Another widely studied garlic preparation is aged garlic extract. Sliced raw garlic stored in 15-20% ethanol for more than 1.5 years is refereed to aged garlic extract. This process is supposed to cause considerable loss of allicin and increased activity of certain new compounds. These compounds include S-allylcysteine, S-allylmercaptocysteine, allixin (3-hydroxy-5-methoxy-6-methyl-2-penthyl-4*H*-pyran-4-one), and selenium which are stable and significantly antioxidant. Medicinally used garlic oil is usually prepared by steam-distillation process. Steam-distilled garlic oil consists of diallyl, allylmethyl, and dimethyl mono to hexa sulfides (Lawson, 1998).

Allyl methyl sulfide cannot be digested and is passed into the blood. It is carried to the lungs and the skin, where it is excreted. Since digestion takes several hours and release of AMS several hours more, the effect of eating garlic may be present for a long time.

*Garlic adverse effects*

Multiple cases of bleeding have been associated with garlic use, and caution is warranted in patients at risk of bleeding or prior to some surgical/dental procedures. Garlic can cause a skin rash in sensitive people. Over consumption can cause heartburn. Garlic may interact with anti-inflammatory medications, such as ibuprofen or naproxen, aspirin, ASA, warfarin, heparin, and other drugs that affect bleeding or platelets (Gattu et al., 2009).

A  B 

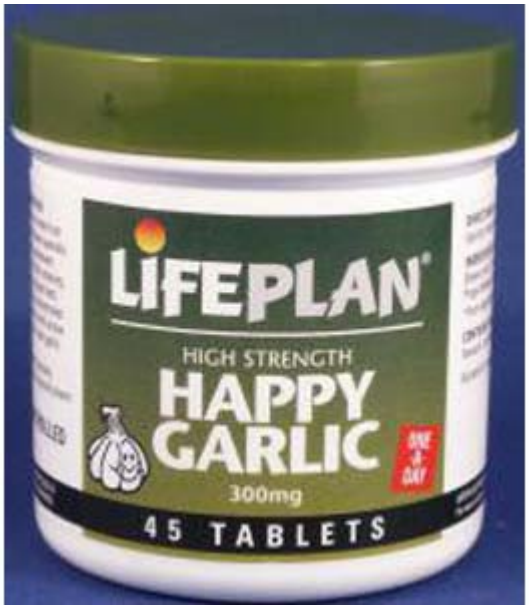
C 


**Figure 1.** *Allium sativum*, commonly known as garlic, is a species in the onion family Alliaceae. A: different forms of whole plant. B: the bulb of garlic. C: garlic preparations.

*Medicinal use and health benefits*

There are appreciable epidemiologic evidences that demonstrate therapeutic and preventive roles of garlic. Several experimental and clinical investigations suggest many favourable effects of garlic and its preparations. These effects have been largely attributed to *i)* reduction of risk factors for cardiovascular diseases, *ii)* reduction of cancer risk, *iii)* antioxidant effect, *iv)* antimicrobial effect, *v)* enhancement of detoxification of foreign compound and hepatoprotection, and *vi)* neurological disorders.

Garlic and its preparations have been widely recognised for prevention and treatment of cardiovascular diseases. The wealth of scientific literatures supports the proposal that garlic consumption have significant effect on lowering of blood pressure, reduction of atherosclerosis, decreasing serum cholesterol as well as triglyceride, inhibition of platelet aggregation, and increasing fibrolytic activity. In *in vivo* animal experiments, intravenous administration of garlic extracts in hypertensive animals brought the blood pressure back to the normal levels (Sial and Ahmed, 1982). Several clinical studies showed that garlic reduced blood pressure in more than 80% of patients suffering from hypertension (Pektov, 1979; Konig and Scincider 1986; Auer et al., 1989). Most of human studies on lipid lowering effects of garlic and its compounds described significant decrease in serum cholesterol and triglyceride (Gardner et al., 2001; Ziaei et al., 2001). Allicin and S-allyl cysteine present in aged garlic extract as well as diallyl-di-sulfide in garlic oil are the active compounds responsible for anti-atherosclerotic effect (Gebhardt and Beck, 1996; Yeh and Liu., 2001). In addition, it was reported that garlic decreases the risk of peripheral arterial occlusive diseases, plasma viscosity, as well as unstable angina and increases elastic property of blood vessels and capillary perfusion (Banerjee and Maulik, 2002). Garlic significantly inhibited intracellular ca2+ mobilization, thromboxane-A2 synthesis, and protected against

thrombocytopenia induced by collagen or arachidonate application in rabbits (Banerjee and Maulik, 2002).

Many experimental and clinical studies pointed to possible cancer-preventive effects of garlic. In rodents, garlic and its constitutes have been reported to inhibit the development of chemically induced tomurs in the liver, colon, bladder, mammary gland, esophagus, lung, skin, and stomach (Lau et al., 1986; Sparnins et al., 1986; Wattenberg et al., 1989; Amagase and Milner, 1993; Wargovich et al., 1988; Ohaeri, 2001; Lawson, 2001; Knowles and Milner, 2003; Kweon et al., 2003). Garlic compounds have been found to block covalent of carcinogens to DNA, enhance degradation of carcinogens, have anti-oxidative and free radical scavenging properties, and regulate cell responses. Reduction of some malignancies risk by consumption of selenium-enriched plants like garlic has been reported (Finley, 2003).

On oral administration of and garlic sulfoxide amino acids, S-methylcysteine sulfoxide and S-allylcysteine sulfoxide to alloxan-diabetic rats for a month, their diabetic condition, being characterized by glucose intolerance, weight loss, depletion of liver glycogen, etc., was ameliorated as comparable to rats treated with glibenclamide and insulin (Sheela et al., 1995). However, anti-diabetic effect of garlic in human is still controversial. It was suggested that allicin may effectively combine with compounds like cysteine and enhance serum insulin. In some clinical investigations chronic feeding of garlic extracts showed significant reduction of blood glucose level whereas some other studies reported no change on glucose concentrations (Banerjee and Maulik, 2002). Garlic also can protect the liver cells from some toxic gents like in overdose of acetaminophen (Patten et al., 1993).

Garlic has been used for centuries in various societies to combat infectious diseases. Garlic has been proven to be effective against a plethora of gram-negative, gram-positive, and acid-fast bacteria. These include Salmonella, Escherichia coli, Pseudomonas, Proteus,

Staphylococcus aureus, Klebsiella, Closteridium, Mycobacterium, Micrococcus, and Helicobacter (Cavallito et al., 1944; Jezowa et al., 1966; Johnson and Vaughan, 1969; Sharma et al., 1977; O'Gara et al., 2000; Adler and Beuchat, 2002). An inhibitory synergism effect was observed when garlic applied together with vancomycin (Jonkers et al., 1999).

In addition to antimicrobial effect, garlic has been reported to act against several protozoa such as Candida, tinea pedis, Leishmania, and Lepromonas (Lemar et al., 2002) as well as several fungi like Cryptococcus, Trichosporon, aflatoxin, and Aspergillus (Tansey and Appleton, 1975; Tadi et al., 1991). Furthermore, anti-viral action of garlic was reported to affect influenza A and B, Cytomegalovirus, HIV, herpes simplex virus 1 and 2 as well as rhinovirus (Weber et al., 1992; Tsai et al., 1985 a,b). Allicin, diallyl trisulfide, and ajoene have been shown to have antiviral effect (Weber et al., 1992).

Garlic and its extract have been shown to be effective in prevention of different neurological disorders. *S*-methyl-*L*-cysteine , a substrate in the catalytic antioxidant system mediated by methionine sulfoxide reductases, is an abundantly substance in garlic and some other vegetables such as cabbage, and turnips. It has been shown that *S*-methyl-*L*-cysteine prevents the $\alpha$-synuclein-induced abnormalities in rats. Application of this substance is a new prevention and therapeutic approach for Parkinson disease and potentially for other neurodegenerative diseases involving oxidative stress (Wassef et al, 2007). Some members of the transient receptor potential (TRPA) family of cation channels mediate sensory responses to irritant substances. TRPA1 is an important component of the transduction machinery through which environmental irritants and endogenous proalgesic agents depolarize nociceptors to elicit inflammatory pain. TRPA1 has been proposed to function in diverse sensory processes, including thermal (cold) nociception, hearing, and inflammatory pain. It is well known that TRPA1 channels are activated by pungent compounds found in garlic. This

substance may act on nocieceptors and modulate noxious stimuli (Salazar et al., 2008). Alzheimer's disease involves amyloid beta accumulation, oxidative damage and inflammation. S-allyl-l-cysteine, a water-soluble organosulfur component present in garlic is known to prevent cognitive decline by protecting neurons from amyloid beta induced neuronal apoptosis (Gupta and Rao, 2007). Feeding of aged garlic extract prevented deterioration of hippocampal based memory tasks in mice, suggesting that aged garlic extract has a potential for preventing Alzheimer (Chauhan and Sandoval, 2007). *N*-acetylcysteine, a compound available in garlic, protects neuronal death in cerebellar granule neurons. This neuroprotective effect is due, at least in part, to preservation of mitochondrial membrane potential and intracellular intracellular glutathione levels (Arakawa et al., 2006).

*Spreading depression*

Cortical spreading depression (CSD), is a pathophysiological phenomenon which occurs as a propagating wave of short neuronal hyperexcitability followed by a transient wave of depression, first identified in the neocortex of rabbits (Leao, 1944). CSD is an "all-or-none" process and spreads in the manner of a wave through gray matter. CSD appears first at the stimulated site and propagate in all directions at the velocity of 2–3 mm/min, so that increasingly distant areas undergo successively a similar temporary depression. An important characteristic feature of CSD is a propagating negative potential with amplitude of 10–30 mV and duration of more than 30 sec, which may be preceded or succeeded by a positive fluctuation of variable amplitude and duration (figure 2).

CSD can be initiated by different stimuli and so can be directly studied in experimental investigations. It was first induced by applying a brief tetanus of faradic stimulation to the rabbit neocortex by Leao group (Leao, 1944; Fig. 1). However, such stimuli could lead to convulsive activity spreading from the stimulated area and so subsequent authors preferred to

employ direct current (DC) stimuli (Leao & Morrison, 1945; Ochs, 1962). Mechanical stimulation, for example, by stroking of the cortical surface with a blunt instrument, a falling weight or even lightly tapping the cortex also initiates SD (Leao, 1944). More recent studies have achieved more reliable and reproducible induction of CSD by rapidly inserting and retracting hypodermic steel needles (Kaube and Goadsby, 1994; Lambert et al., 1999; Ebersberger et al., 2001). However, one of the most common models of SD initiation is KCl application to the neuronal tissues (Wernsmann et al., 2006; Dehbandi et al., 2008). This model has been proven to be the most reliable stimulus leading to reproducible events on earlier investigations (Martins-Ferreira et al., 2000; Bradley et al., 2001). Changes in extracellular $K^+$ concentration might be involved in such pathophysiological processes in human brain tissue (Mayevsky et al., 1996; Nicholson & Sykova, 1998). CSD can also be induced by applications of the excitatory amino acids glutamate and aspartate; metabolic inhibitors such as NaCN that poison oxidative metabolism and NaF and iodoacetate that primarily interfere with glycolysis; the $Na^+$-$K^+$ ATP-ase inhibitor ouabain has also been used in cortical brain slices; local cooling by depressing energy metabolism below a critical level but has proven an irreproducible experimental method (Smith et al., 2006).

CSD involves a temporary localized redistribution of different ions between extracellular and intracellular compartments. These ions redistribution are energy dependent. During eliciting of CSD the concentration of extracellular $K^+$ rapidly rises (between 40-60mM), causing brief neuronal excitation then depolarization and a period of electrical silence during which DC potential at the brain surface falls. In tandem, $[Na^+]_o$ and $[Cl^-]_o$ levels decrease as these ions enter cells. Consequently, water enters cells, the extracellular compartment is reduced, and cells swell. $Ca^{2+}$ ions also move inwards, but slightly later than the outward movement of $K^+$, suggesting that $Ca^{2+}$ movements follow $K^+$ fluxes. Additional negative ion species move outwards to maintain electrical balance, the excitatory neurotransmitter glutamate probably

being the most important (Somjen et al., 2001). Widely accepted hypotheses hold that the primary event responsible for both the initiation and the propagation of CSD is the release of potassium and glutamate from neuronal elements to the extracellular space, which initially excites and then depresses adjacent neurons (Somjen, 2001).

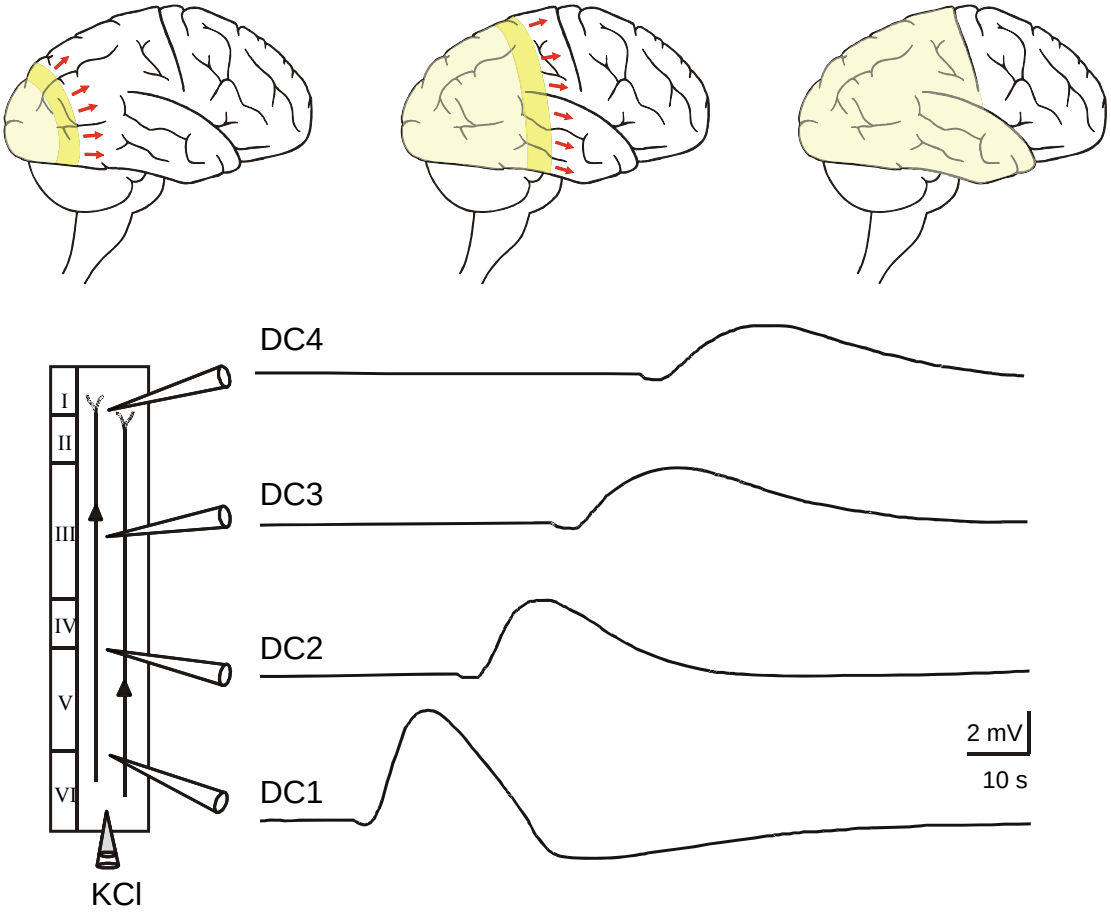



**Figure 2.** Vertical propagation of a negative DC-potential wave after injection of KCl in a neocortical slice. Injection of KCl solution (3 M) via a microelectrode elicited spreading depression-like fluctuation during superfusion with artificial cerebrospinal fluid. Injecting and recording electrodes arranged as shown. Voltage variations were recorded simultaneously by four electrodes (DC1–DC4) which set apart by 1 mm (Adopted from Gorji et al., 2001).

There are evidences to suggest that CSD plays an important role in different neurological disorders (Gorji, 2001; Somjen, 2001). Subdural recordings in patients demonstrated that CSD is involved in various disorders associated with acute neuronal injury including traumatic and spontaneous intra-cerebral hemorrhage (Strong et al., 2002; Fabricius et al., 2008) as well as subarachnoid hemorrhage and stroke and contribute to tissue damage. Furthermore, propagation of a CSD wave in human neocortical tissues has been shown to generate aura symptoms in patients suffering from migraine attacks (Hadjikhani et al. 2001).

The designation migraine with aura denotes the syndrome of headache associated with characteristic sensory, motor, or visual symptoms, usually gradually developed over 5–20 min and lasting less than 60 min. Direct alterations of electrical activity of cortical neurons by the locally spreading wave can lead to clinical symptoms (e.g. the aura phase of migraine). The most common symptoms in aura phase are visual arising from dysfunction of occipital lobe neurons. The excitatory neurological symptoms, e.g., flashing lights are usually followed by suppressive ones, e.g., scotoma or hemianopia in this phase. Magnetoencephalographic studies in human noted that the magnetic signals were seen in migraineous patients but not in patients suffering from other forms of headache or normal controls. Three distinctive signal patterns; suppression of spontaneous cortical activity, slow field changes and large-amplitude waves, were observed strictly in migraine patients. In some migraine patients, magnetic signals were also recorded between attacks. The same magnetic fields appeared during the propagation of CSD in the cortex of anesthetized animals. High-field functional MRI was used to detect blood oxygenation level-dependent (BOLD) changes during visual aura in three migraineurs. A focal increase in BOLD signals developed first in extrastriate cortex and spread at the velocity of 3.5 ± 1.1 mm/min over occipital cortex. These initial BOLD features were consistent with scintillations and paralleled by decreases in the stimulus-driven MR

oscillations. Increasing in BOLD signals was followed by a decrease in the mean signal. This phase appeared to correspond to the localized scotoma and MR stimulus-induced response remained suppressed. Within 15 ± 3 min, both BOLD signals and MR stimulus-induced response recovered. During periods with no visual stimulation, but while the subject was experiencing scintillations, BOLD signal followed the retinotopic progression of the visual percept. Spreading BOLD signal changes as neocortical SD did not cross prominent sulci (Hadjikhani et al., 2001). Recent investigations provide early insights into mechanisms that lead to trigeminovascular activation.

CSD is also a well-known phenomenon in experimental epilepsy. CSD can be elicited in susceptible area by a single discharge of an epileptic focus. Epilepsy and migraine are both disorders characterized by transient paroxysmal neurological dysfunction, usually with a normal neurological examination between attacks. A number of syndromes in which migraine and epilepsy are related have been described. Headaches are observed quite frequently following epileptic attacks and seizures provoke a syndrome similar to the headache phase of migraine in 50% of epileptics. A number of anticonvulsive drugs have the capacity to stabilize migraine and some anti-migraine drugs increase the epilepsy threshold. It was reported that combination therapy with anticonvulsant and anti-migraine drugs in some intractable epileptics improves seizure control (Gorji, 2001).

Consistent with an upstream role for CSD, prolonged application of migraine prophylactic drugs suppresses CSD in experimental models as a proposed mechanism of action. In line with clinical recognition that prolonged administration of prophylactic medicaments is important to achieve maximum therapeutic efficacy, treatment extension beyond 3–4 weeks also maximizes the inhibitory effects of drugs such as topiramate, valproate, methysergide, amitriptyline, and propranolol (Gorji, 2001). Application of new substances with potential of

efficacy in CSD-related neurological disorders on CSD threshold in *in vitro* as well as *in vivo* studies is a well-known model to investigate of possible efficacy of these substances.

Naturally occurring substances derived from plants currently have, and will continue to have, a relevant place in drug discovery. The use of medicinal plants for the treatment of headache in Persia can be traced back to the 6th century BC. Despite progress in the development of therapy in recent years, effective and potent drugs are still required for the treatment of headache. The search for new pharmacologically active analgesics obtained from plants has led to the discovery of some clinically useful drugs that, during the past two centuries, have played a major role in the treatment of human diseases. However, most medicinal plants prescribed by Persian physicians remain largely unexamined (Gorji, 2003). One of substances which were suggested to act effectively on treatment of headache attacks in medieval literature is garlic. The aim of the present study to investigate the effect of garlic extract at different concentrations on CSD as well as its effect on synaptic strength in rat neocortical tissues.

## Material and methods

The experiments were performed on adult rat (200-350g) somatosensory neocortical slices. The brain was removed under deep methohexital anaesthesia and placed in cold (1–4°C) artificial cerebrospinal fluid (ACSF) pre-equilibrated with 5% $CO_2$ in $O_2$ to give a pH of 7.4. The ACSF contained (in mM): NaCl 124, KCl 4, $CaCl_2$ 1.0, $NaH_2PO_4$ 1.24, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10. The somatosensory neocortices were dissected and cut into slices of 500 μm thickness. The slices were incubated in ACSF solution for >1 h at 28°C. After 30-min incubation, $CaCl_2$ was elevated to 2.0 mM. Slices were transferred to an interphase-type experimental chamber and superfused with ACSF at 32°C (1.5–2 ml/min).

*Electrophysiological recordings*

Extracellular field potentials were recorded with glass microelectrodes (150 mmol/l NaCl; 2–10 MΩ) connected to the amplifier by an Ag/AgCl–KCl bridge in the third and the fifth layers of neocortical tissues. Field potentials were traced by an ink-writer and recorded by a digital oscilloscope.

*Induction of neocortical SD*

SD was elicited by KCl microinjection. A glass electrode filled with 2 M KCl was fixed in a special holder connected with plastic tube to a pressure injector and the tip inserted into the sixth layer of the neocortical slices. A high-pressure pulse was applied to inject an amount of $K^+$ in the tissue sufficient to induce cortical SD (tip diameter: 2 μm; injection pressure 0.5–1.0 bar applied for 200–300 ms, two injections, 1–3 nl per pulse). Cortical SD-like events were evaluated with respect to their amplitude, duration and velocity rates. SD duration was defined

as the interval between the time of half-maximal voltage shift during onset and recovery of the negative DC potential deflection.

*Long-term potentiation*

Single pulses of electrical stimulation were applied through a bipolar platinum electrode attached to the white matter perpendicular to the recording electrodes. Evoked field excitatory postsynaptic potentials (fEPSP) were recorded in the third layer of neocortical slices. The fEPSP was elicited by adjusting the intensity of stimulation to ~50% of that at which population spikes after fEPSP began to appear. The amplitude of fEPSP 1 ms after the onset was measured for data analysis. In long-term potentiation (LTP) experiments, the cortex was sequentially stimulated once every minute. Ten trains of four pulses (pulse duration 0.1 msec; interpulse interval 50 msec; intensity 5 V) were repeated at intervals of 10 msec. LTP was operationally defined as the mean change in fEPSP amplitude in response to five stimuli given 30 min after tetanic stimulation compared with the mean response to five test pulses applied immediately before the stimulation. Thus % potentiation = [(posttetanus amplitude of fEPSP/baseline amplitude of fEPSP) 1] 100. Tetanic stimulation was applied 60 min after application of drug.

*Experimental protocols*

The experimental protocol consisted of four periods as follows: (a) control period, neocortical slices were superfused with ACSF (30 min), tested for spontaneous CSD; (b) KCl injection, induction of SD (CSD1); (c) application of garlic oil (1-500 μl/l) before the second injection of KCl (CSD2); (d) washout of garlic oil with ASCF (45 min, second control period), third injection of KCl (CSD3). Only a single concentration of garlic oil was used in a given slice.

*Drug*

Garlic oil purchased from Caelo (Hilden, Germany).

*Statistical analysis*

All data are given as mean ± SEM. The data were statistically analysed using the Mann–Whitney Rank Sum test. Multiple comparisons were performed by analysis of variance test (ANOVA) for repeated measures followed by a Dunn's test. Significance was established when the probability values were less than 0.05. The investigations were approved by the local ethics committee (Tierversuchsgenehmigung, Bezirksregierung Münster, Deutschland, AZ: 50.0835.1.0, G79/2002).

## Results

*The effect of garlic oil on CSD*

Focal application of KCl in the sixth layer of neocortical tissues induced negative DC deflections followed by positive waves (amplitude of 14.7 ± 2.2 mV; duration of 121 ± 6 sec). Negative DC-fluctuations were sometimes preceded by small positive waves. These cortical CSD waves propagated opposite to the direction of the ACSF flow at propagation velocity of 3.3 ± 0.1 mm / min. The effect of five different concentrations of garlic oil (1, 10, 100, 500 µl/l; n = 6 for each concentration) was tested on potassium-evoked CSD in neocortical slices. The ratio between the second and the first DC potential waves (SD2/SD1) was calculated in slices treated with garlic oil. Sixty minutes of garlic oil application at concentration of 1, 10, 100, 500 µl/l reduced the amplitude of CSD by 34.6 ± 5.7%, 38.6 ± 9%, 49.4 ± 7.4%, and 69.6 ± 9%, respectively. Garlic oil dose-dependently decreased the amplitude of CSD (Fig. 3; $P = 0.04$ ; ANOVA test, Dunn's method). Garlic oil at these concentrations did not affect the duration or propagation velocity of CSD.

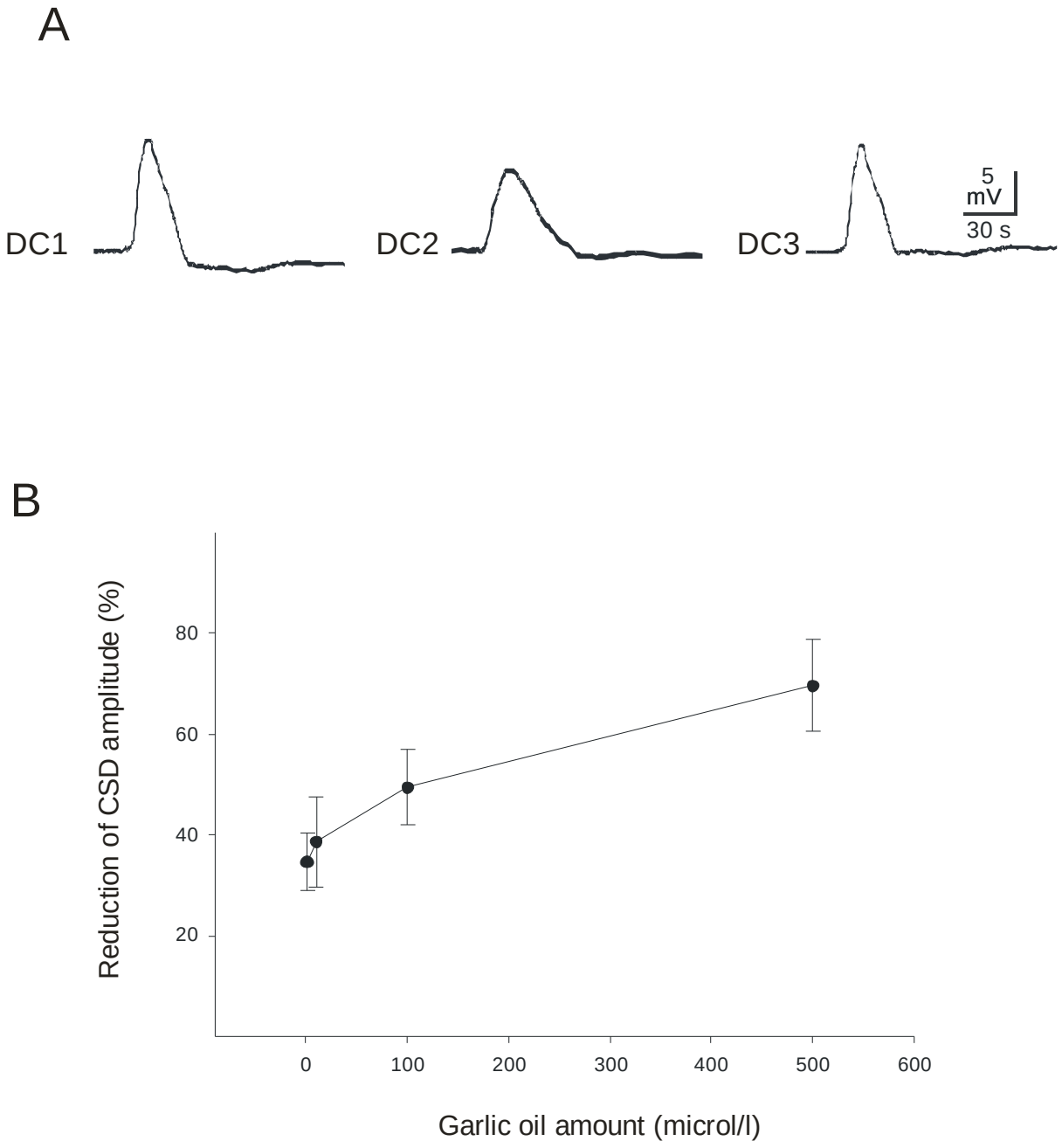



**Figure 3.** Effects of garlic oil on cortical spreading depression (CSD) in somatosensory neocortical tissues. A: Recording of DC potential shifts in the third layer of a neocortical slice before (DC1), during (DC2), and after (DC3) application of garlic oil (100 µl/l). Field potentials were recorded by an ink-writer. CSD was elicited by KCl microinjection. B: The curve indicates the plot of percentage reduction of CSD amplitude vs. garlic oil concentrations (n = 6 for each concentration). Garlic oil dose-dependently decreased the

amplitude of CSD. The percentage of CSD amplitude enlargement was measured by division of the amplitude of CSD induced after application of garlic oil to the amplitude of CSD elicited before superfusion of the substance. Values represent mean ± SEM. Significance was determined by ANOVA test followed by Dunn's post-test (B; $P = 0.04$).

*The effect of garlic oil on EPSP and LTP*

Garlic oil at concentration of 500µl/l decreased the fEPSP amplitude by 28.9 ± 8.8% after 60 min of continuous application. After 30 min washout of garlic oil, the amplitude of fEPSP returned to the pre-application level. A conditioning tetanic stimulation was delivered to the white substance of neocortical slices followed by pulses with stimulation parameters identical to control values. The evoked fEPSP was stable for at least 30 min before application of tetanic stimulation (less than 10% variation; Fig. 4). Administration of tetanic stimulation produced a rapid and stable enhancement of the amplitude of the fEPSP in all tested preparations (n = 7, 154 ± 11 % control; Fig. 4). LTP lasted as long as the fEPSP were recorded (at least for 90 min). The potentiation rose within 1–2 min and stabilized within 5 minutes after the train of stimulations. Application of garlic oil (500µl/l; n = 12) sixty min before tetanic stimulation significantly suppressed LTP induction in all tested slices (134 ± 6 % baseline, Mann–Whitney Rank Sum test; $P \leq 0.001$, Fig. 4).

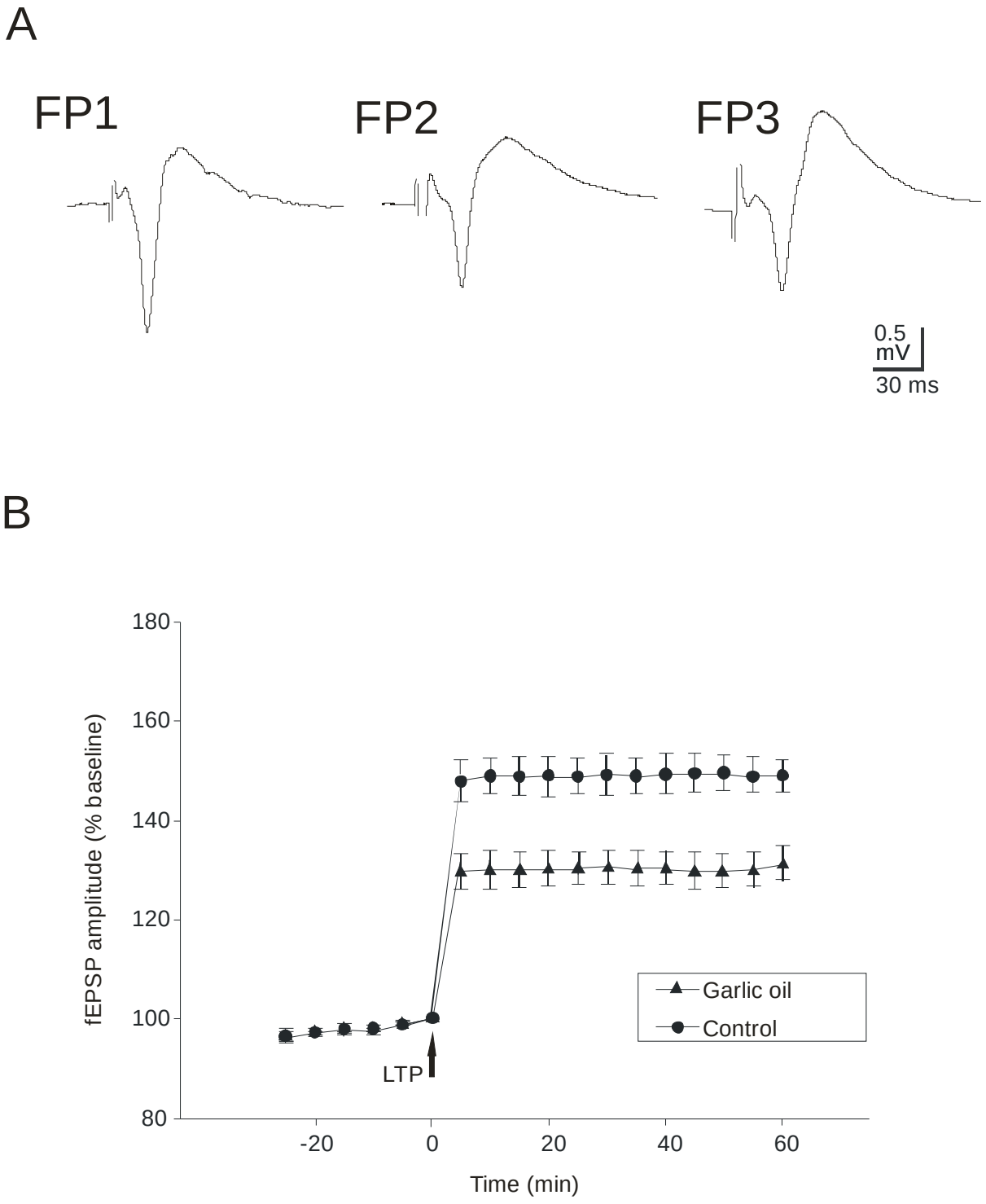



**Figure 4.** The effect of garlic oil on the evoked field excitatory postsynaptic potentials (fEPSP) and long-term potentiation (LTP) of fEPSP in neocortical preparations. (A) Garlic oil at concentration of 500µl/l decreased the fEPSP amplitude by 28.9 ± 8.8% after 60 min of continuous application (FP2). After 30 min washout of garlic oil, the amplitude of fEPSP returned to the pre-application level (FP3). B: Tetanic stimulation (Ten trains of four pulses;

pulse duration 0.1 msec; interpulse interval 50 msec; intensity 5 V) produces a rapid and stable potentiation in the amplitude of the evoked field potentials, calculated as a percentage of baseline mean response amplitude. Closed triangel and closed circles show the evoked fEPSP after application of garlic oil (500µl/l) and control, respectively. Arrow shows the time of tetanic stimulation, 60 min after application of substances. Application of garlic oil significantly inhibited LTP of the evoked field potentials (Mann–Whitney Rank Sum test, $P$ = 0.001), calculated as a percentage of baseline mean response amplitude.

## Discussion

The beneficial effects of garlic in the treatment of cephalic pain were recommended emphatically by many medieval medical literatures. The present data indicate the strong effects of garlic oil to suppress CSD in *in vitro* animal brain models. Furthermore, garlic oil significantly inhibited the evoked fEPSP and decreased LTP induction in neocortical slices.

CSD is a complex neurovascular event that triggers a cascade of mechanisms leading to the up-regulation of genes, plasma protein extravasation, sensitization of perivascular nerve fibers, and central transmission of pain signals via the brainstem (Gorji, 2001). Observation of trigeminovascular activation by CSD within cerebral cortex indicates that CSD increases the trigeminal neuronal discharges, which could explain the underlying mechanism of the subsequent headache (Gorji et al., 2004). The effects of several anti-headache substances including both classic (such as paracetamol and acetylsalicylic acid) and new compounds (such as sumatriptan and tonabersat) on different *in vivo* and *in vitro* models of CSD were examined. CSD also affected by application of some natural substances such as eugenol (Muller et al., 2006) or some hormones such as estrogen and progesterone (Sachs et al., 2007). These chemical compounds influenced different characteristic features of CSD such as the amplitude of the fluctuation of DC potential, the propagation velocity, the recovery of the optical and electrical signals (Wiedemann et al., 1996), the latency of initiation and the frequency of occurrence (Takagi et al., 1998), elevation in cGMP (Read et al., 2001), nitric oxide release (Read and Parsons, 2000), COX-2 expression (Miettinen et al., 1997), biphasic blood oxygenation level-dependent magnetic resonance imaging waves (Netsiri et al., 2003), and CSD-associated hyperemia (Gold et al., 1998). CSD, therefore, serves as a model to examine the efficacy of substances that may have clinical usefulness in the treatment of cephalic pain. The suppressive effects of garlic oil on CSD indicate the probable clinical benefits of this substance for the treatment of migraine headache with aura.

LTP is an experimental phenomenon, which can be used to demonstrate the repertoire of long-lasting changes of which individual synapses are capable (Collingridge & Singer 1990, Malenka and Bear 2004). LTP points to long-lasting enhancement in signal transmission between two neurons, which results from stimulating them synchronously. LTP can be induced experimentally by applying a few trains of high-frequency stimuli to the connection between two neuronal cells. LTP improves synaptic transmission in neuronal network.. LTP enhances the ability of communication between pre-synaptic and postsynaptic neurons with each other at the synaptic site. The magnitude of pre-synaptic depolarization determines whether LTP will be produced in the postsynaptic neuron. LTP enhances the postsynaptic sensitivity to neurotransmitter by increasing the activity of existing receptors especially NMDA receptors and by increasing the number of receptors especially AMPA receptors on the postsynaptic surface. Induction of LTP occurs when the concentration of $Ca^{2+}$ inside the post-synaptic cell flow via NMDA-receptors exceeds a critical threshold. While NMDA-receptors are present at most postsynaptic membranes, at resting membrane potentials they are blocked by a magnesium ion that prevents the entry of calcium into the postsynaptic neuron. Strong depolarization through the summation of excitatory postsynaptic potentials relieves the magnesium blockade of the NMDA-receptor and allowing calcium influx to the cell (Malenka and Bear 2004). LTP activates CaMKII and PKC use phosphorylation to carry out the two major mechanisms underlying the induction of LTP. These mediators phosphorylate existing AMPA receptors to increase their activity and mediate or modulate the insertion of additional AMPA receptors into the postsynaptic membrane.

LTP is expressed in spinal cord as well as several brain areas, including neocortex and hippocampus and have been proposed to represent the cellular mechanisms underlying pain as well as learning and memory (Ji et al., 2003). Central sensitization plays a major role in the

generation of migraine, tension-type headache and neuropathic pain (Burstein and Woolf 1999). The present data showed that LTP in the neocortical tissues was significantly inhibited after garlic oil application. This demonstrates that garlic oil produces a sustained inhibition in the synaptic plasticity of neocortical tissues. CSD induces an LTP-like effect in rat neocortical slices (Footitt and Newberry, 1998) and enhances LTP induction in human neocortical tissues (Berger et al., 2008). Both inhibition of LTP induction and CSD elicitation were observed by eugenol or esterogen and progesterone applications in rat neocortical tissues (Muller et al., 2006; Sachs et al., 2007). Both aberrant presynaptic and postsynaptic connectivity contribute to epileptogenesis and migraine (Cohen, 2005). The neurophysiological events that were inhibited by garlic oil in the present study all involve modulation of synaptic transmission. CSD, a phenomenon suggested to be involved in migraine with aura, was suppressed drastically by garlic oil. Triggering of SD also requires activation of excitatory synaptic transmission in animal and human neuronal tissues (Gorji et al., 2001).

LTP is a phenomenon in which a constant pre-synaptic high stimulation of excitatory amino acids neuronal pathways results in a prolonged enhanced postsynaptic response. In the present study, garlic oil reversibly suppressed fEPSP as well as CSD and inhibited LTP in neocortical slices. It is well established that NMDA receptors are a molecular detector of the coincidence of both the presynaptic release of glutamate and a postsynaptic depolarization at the origin of LTP induction (Sourdet and Debanne, 1999). Pre-synaptic NMDA receptors have recently been suggested to potentate the afferent field volley evoked by Schaffer axon stimulation (Suarez et al., 2005). However, other pre-synaptic mechanisms such as involvement of kainate receptors (Schmitz et al., 2001) or transient outward potassium current could also modify presynaptic excitability (Gu et al., 2004).

Furthermore, garlic suppressed platelet aggregation via inhibition of the release of mediators such as thromboxane A2, prostaglandin E2, and calcium (Qi and Wang, 2003).

Lipoxygenases and cyclooxygenases are the most common therapeutic drug target in human pain (Warner and Mitchell, 2004). Arachidonic acid by modulation of a postsynaptic transient $K^+$ current strongly regulates synaptic integration by means of suppression of postsynaptic A-current and subsequently facilitates induction of LTP in hippocampal pyramidal cells (Ramakers and Storm, 2002). Lipoxygenase and cyclooxygenase inhibitors of arachidonic acid attenuated potentiation of the population excitatory postsynaptic potential and reduced pre-established LTP in CA1 area of hippocampal slices (Williams and Bliss 1989; Shaw et al 2003). Arachidonic acid increased markedly during the negative DC shift and returned to normal levels in few minutes after SD (Lauritzen et al., 1990). COX-2, the inducible form of the enzyme converting arachidonic acid to prostaglandins, is induced within hours after SD in cortical neurons by a mechanism dependent on phospholipase $A_2$.

However it should be noted that some studies point to activation of pain sensing by garlic. Allicin is the active ingredient of garlic that causes a burning sensation through activation of TRPA1 and TRPV1. Fresh-cut garlic and allicin, one of its constituents, activate TRPA1 and TRPV1, two noxious thermo TRPs found in pain-sensing neurons that innervate the mouth and tongue. Activation by garlic and allicin is specific to neurons expressing these channels; no other populations of trigeminal ganglia neurons are activated by these stimuli. Among the chemical constituents of garlic extracts, allicin is by far the most potent activator of TRPA1 and TRPV1. Furthermore, the activity of allicin, given its concentration in garlic, is sufficient to explain all of garlic extract's activity on these thermo TRPs. Extracts of baked garlic (which differ from fresh extracts primarily in their lack of allicin) are unable to activate thermo TRPs. Allicin and other garlic constituents are expected to stimulate olfactory and gustatory neurons as well; however, the burning sensation that fresh garlic can produce must act via the trigeminal system (Macpherson et al., 2005).

## Conclusion

In conclusion, the present data revealed that garlic oil suppressed the amplitude of CSD in rat neocortical slices as well as fEPSP and LTP. The inhibitory effect of garlic oil on CSD is likely to be mediated by inhibition of synaptic transmission. This indicates the potential for garlic oil to use in the treatment of CSD-related neurological disorders such as migraine headache with aura.

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## Genehmigung der *in vivo*-Versuche

Bezirksregierung Münster

Bezirksregierung Münster • 48128 Münster

Gegen Empfangsbekenntnis
Herrn
Prof. Dr. med. Erwin-Josef Speckmann
Institut für Physiologie
-Institut für Experimentelle Epilepsie-
forschung-
des Universitätsklinikums Münster
Robert-Koch-Str. 27 a
48149 Münster

Dienstgebäude:
Domplatz 1-3
Telefon: (0251) 411-0
Durchwahl: 2110
Telefax: 2115
Raum: 431
Auskunft erteilt:
Frau Möllers
E-Mail:
irmgard.moellers@bezreg-muenste
Aktenzeichen:
50.0835.1.0 (G 79/2002)

19. Dez. 2002

**Tierschutz;**
**Durchführung von Versuchen an Wirbeltieren**

Ihr Antrag vom 10.10.2002, hier eingegangen am 06.11.2002

**Genehmigungsbescheid:**

Sehr geehrter Herr Professor Speckmann,

gemäß § 8 Tierschutzgesetz (TierSchG) vom 25. Mai 1998 (GBGl. I S. 1105) in der zur Ze geltenden Fassung wird Ihnen die Genehmigung zur Durchführung nachstehenden Versuchsvorhabens erteilt:

**„Experimentelle Epilepsieforschung". (10 Teilprojekte gem. Antrag)**

Leiter des Versuchsvorhabens und seine Stellvertreter sind:

Herr
Prof. Dr. med. E.-J. Speckmann
Institut für Physiologie
-Institut für Experimentelle
Epilepsieforschung-
des Universitätsklinikums Münster
Robert-Koch-Str. 27 a
48149 Münster

Frau
Prof. Dr. rer. nat. Heidrun Straub
Institut für Physiologie
des Universitätsklinikums Münster
Robert-Koch-Str. 27 a
48149 Münster