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Arto Lipponen

Hippocampal Field Potentials in Animal Models of Alzheimer's Disease

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ABSTRACT

This project is providing new evidence how normal hippocampal dependent memory and pathological processes are related to electrical activity of local neuronal populations know as local field potentials (LFP) or brain rhythms. The focus of the first study was to study how hippocampal rhythms are expressing temporal context, an important aspect of hippocampal dependent episodic memory system. We recorded LFP during a prolonged lever pressing task enabling us to reduce other behaviorally related brain activity. As we noticed single brain rhythms as such cannot predict whether a rat pays attention to lever pressing for reward. More likely, the dynamics between theta and gamma rhythms are more related to the outcome in this task.

Similar results were obtained in a project in which we tested whether actually artificial electrical stimulation could improve memory or rescue it after lesioning nerve fiber bundles connecting hippocampus to medial septum. This lesion impairs memory in a hippocampal dependent memory task and abolishes naturally occurring theta rhythm. As we restored hippocampal theta by deep brain stimulation mimicking the amplitude and frequency of theta, we, surprisingly, observed no improvement but impairment in the memory. This impairment was as severe as the lesioning effect. As a conclusion, a single brain rhythm as such is not plausible crucial for normal memory functions. Rather, it is more likely that memory functions are related to more complex LFP dynamics involving several brain rhythms and their interactions.

Clinical observations have so far implied increase in the slower frequency range in EEG during progression of AD. However, recent reports also point to neuronal hyperactivity in AD. It seems that a small proportion of AD patients also has epileptic activity, which makes them more susceptible to faster progression of memory impairment. Indeed, when we recorded LFP of transgenic mice expressing amyloid pathology, we observed already before plaque formation hyperactivity in thalamo-cortical area and changes in the hippocampal theta-gamma-synchrony.

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Medical Subject Headings: Alzheimer Disease; Brain Waves; Hippocampus; Neurons; Memory; Behavior; Electric Stimulation; Cerebral Cortex; Thalamus; Epilepsy; Disease Models, Animal

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TIIVISTELMÄ

Aivojen sähköisessä rekisteröinnissä on kiinnitetty huomiota niin sanottuihin aivorytmeihin, jotka ovat pohjimmiltaan hermosolujoukkojen aiheuttamia jännitteen vaihteluja. Aivorytmien luokittelu perustuu niiden taajuuden mukaiseen luokitteluun, mutta on huomattavaa, että aivorytmejä esiintyy eri aivo-osissa eri aikoina ja ne ovat usein sidoksissa meneillään olevaan toimintaan ja käyttäytymiseen. Osatutkimuksessa selvitimmekin kuinka muistin toiminnan kannalta olennaisen hippokampuksen aivorytmit ilmenevät eläimen keskittyessä painamaan vipua pitkitetyn (2.5 s) ajan. Havaitsimme, että seuraamalla pelkkien yksittäisten aivorytmien voimakkuuksien muutoksia, emme voineet tarkasti ennakoida painaako eläin vipua riittävän kauan saadakseen palkkion. Pikemminkin kahden eri aivorytmin, thetan ja gamman, välinen vuorovaikutus ennakoi paremmin tehtävästä suoriutumista.

Löydöksemme sai tukea toisesta osakokeessa, jossa määritimme yksittäisen hippokampuksen rytmin osuutta muistin toiminnassa. Korvasimme poistetun aivorytmin keinotekoisella sähköstimulaatiolla, joka jäljitteli luonnollisen rytmin voimakkuutta ja taajuutta. Mikäli tämä aivorytmi itsessään olisi olennainen muistin kannalta, tulisi sähköstimulaation palauttaa muistin toiminta normaalille tasolle. Näin ei kuitenkaan käynyt, vaan havaitsimme, että sähköstimulaatio heikensi muistia jopa saman verran kuin aiheutamme vaurio. On siis selvää, ettei yksittäinen aivorytmi sinänsä ole tärkeä muistin kannalta tärkeä, vaan keinotekoisesti tuotettuna haitallisuudessa on verrattavissa jopa epilepsian aiheuttamiin muistihäiriöihin.

Vaikka aivorytmien väliset dynaamiset muutokset hippokampuksessa liittyvät oleellisesti muistin toimintaan Alzheimer-potilailta ei ole löydetty selkeitä muutoksia aivorytmeissä. Tulosten epätarkkuus voi johtua siitä, että suorat aivosähkömittaukset hippokampuksesta ovat hyvin ja toisaalta Alzheimerin taudin patologia ei välttämättä ole ollut kovin yhteneväinen tutkimusryhmissä. Kuitenkin tarkemmat tutkimukset ovat osoittaneet, että osalla Alzheimerin taudin potilaista taudinkuvaan kuuluu aivojen sähköinen yliaktiivisuus, ja jopa epilepsia, joka vaikuttaa ennustavan taudin nopeampaa etenemistä. Kolmas osatyömme osoittikin, että amyloidipatologiaan liittyy eri aivoaluieden yliaktiivisuus, ja mikä merkittävintä, hyperaktiivisuus oli osoitettavissa ennen varsinaisten amyloidiplakkien muodostumista.

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Yleinen suomalainen asiasanasto: Alzheimerin tauti; aivoaallot; hippokampus; hermosolut; muisti; käyttäytyminen; sähköstimulaatio; aivokuori; talamus; epilepsia; koe-eläimet

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List of the original publications

This dissertation is based on the following original publications:

I Lipponen A, Woldemichael BT, Gurevicius K, Tanila H. Artificial theta stimulation impairs encoding of contextual fear memory. *PLoS One. 7(11):e48506, 2012.*

II Lipponen A, Gurevicius K, Djupsund K, Tanila H. Theta and gamma oscillations in the rat hippocampus during attentive bar pressing.

III Gurevicius K, Lipponen A, Tanila H. Increased cortical and thalamic excitability in freely moving APPswe/PS1dE9 mice modeling epileptic activity associated with Alzheimer's disease. *Cereb Cortex. 23(5):1148-58.2. 2013*

Contents

Abbreviations

Aβ	Amyloid beta protein
AD	Alzheimer's disease
APP	Amyloid precursor protein
CA1,3	Cornu ammonis 1 or 3
CFC	Cross-frequency coupling
DG	Dentate gyrus
EEG	Electroencephalogram
EPSP	Excitatory postsynaptic potential
FFX	Fimbria fornix lesion
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
IPSP	EInhibitory postsynaptic potential
LFP	Local field potential
LIA	Large irregular activity
MS-DBB	Medial septum diagonal band of Broca
REM	Rapid eye movement (sleep)
SIA	Small irregular activity
TGC	Theta gamma modulation

1 Introduction

Alzheimer disease (AD) is the main cause of dementia (50-75% of all dementia cases). There is an urgent need for innovations in this field since it is estimated that worldwide by 2030 there will be 65.7 million people suffering from dementia and this figure is expected to double every 20 years, to 115.4 million by 2050 (1). Even though the ultimate cause of AD still remains elusive, a major correlate of AD is cerebral accumulation of amyloid-beta (Aβ) protein (2,3). The overexpression of Aβ leads to accumulation of extracellular amyloid plaques causing a progressive synaptic and neuritic injury and neuronal loss. Interestingly, the hippocampus, a key structure for learning and memory is the primary target of functional and pathological alternations in AD (3,4). Despite over 100 years of AD pathology research it is still unclear 1) what causes overexpression of Aβ, 2) how changes are related to the observed pathophysiology of specific brain areas and, 3) eventually, why these changes manifest themselves as memory impairment.

However, recently two strong lines of experiments have emerged to explain the plausible pathophysiological mechanism at single cell and neuronal network level. Recordings of single cell activity have found populations of hypoactive and hyperactive neurons in the close proximity in Aβ plaque-enriched regions in several brain regions (5,6). Moreover, intracranial recording of local field potentials (LFPs) monitoring the local activity of neuronal populations revealed epileptiform cortical and hippocampal synchronous discharges and generalized seizures in mouse models of AD (7,8). These animals have increased Aβ peptide production in these AD-vulnerable areas and cognitive deficits (9-11). Although still few in number and mainly suggestive, findings concerning hyperactivity have already at this stage important translational potential for clinical and therapeutic implications in humans. AD was found to be strongly linked to prevalence of unprovoked seizures and epilepsy even at the early stages of the disease (12,13). Notably, reduction of hippocampal hyperactivity by antiepileptic drug, levetiracetam, improved memory of mild AD patients (14). In addition, the same treatment was not only able to ameliorate memory but also reverse synaptic deficits in AD mice overexpressing Aβ peptide (15).

2 Review of the literature

2.1 ELECTRICAL ACTIVITY OF THE BRAIN – BRAIN RHYTHMS

One of the basic characteristics of neuronal tissue is its capability of creating current streams and voltage differences. Even though there are multiple phenomena related to electrical activity, brain rhythms or brain oscillations, presenting summated activity of neuronal assemblies, are among the most studied ones (16). These extracellular voltage deflections can be recorded from the scalp, termed as electroencephalography (EEG), or inside the brain tissue with microelectrodes, termed as local field potentials (LFP) (17).

2.2 HIPPOCAMPAL BRAIN RHYTHMS IN MEMORY FUNCTIONS

Recordings of local field potentials in the hippocampus have revealed multiple classes of oscillatory frequency bands or brain rhythms. These oscillations are often dependent on ongoing behaviour and, for instance, are distinguishably presented in different phases in wake-sleep cycle (18,19). In the hippocampus of a freely moving rat six prominent brain rhythms can be observed. The rhythmical patterns representing sinusoidal wave form are theta (6-12 Hz), beta 12-30 (Hz) gamma (30-100) and ripple (100-200 Hz), and the nonrhythmical rhythms include large irregular activity (LIA) and small irregular activity (SIA) (20). However, in the following section the main focus will only be on theta and gamma oscillations.

2.2.1 Theta rhythm

Theta rhythm is the most prominent LFP in the hippocampus, and it was first recorded from rabbits (3-7 Hz) (21,22) but was later found also in other species like rats (3-12 Hz) (23,24,24). Verification of hippocampal theta in human patients proved to be more difficult perhaps related to the pathological progress and medication of subjects (25). However, the current findings implicate the existence of hippocampal theta reset (26) and hippocampal theta-like oscillations (3 and 8 Hz) (27-29).

These findings led to active search of the mechanism that generates hippocampal theta rhythm (rhythmical slow activity, RSA), and focused mainly on the plausible roles of septohippocampal (21,30) and entorhinal-hippocampal connections reviewed in (31). The basis of the classical model of extracellular theta current is that LFPs are generated by postsynaptic potentials, and, therefore, extracellular fields are summed activity of inhibitory postsynaptic potentials (IPSPs) and excitatory

postsynaptic potentials (EPSPs) on the somata and dendrites of principal cells. These potentials are able to produce the sink, the site where positive charges enter the neuron, and the source, where positive charge flows out from the neuron. In hippocampal theta this model assumes that the medial septum and diagonal band of Broca (MS-DBB) are the pacemakers of hippocampal theta. Cholinergic and GABAerig neurons of MS-DBB provide the necessary slow depolarization and rhythmical hyperpolarization to interneurons linked to pyramidal cells. Therefore, activation of an inhibitory interneuron leads to rhythmic inhibitory postsynaptic potentials (IPSPs) in the pyramidal cell and induced perisomatic source. On the other hand, rhythmic excitatory postsynaptic potentials (EPSP) from entorhinal cortex through the perforant path produce the dendritic sink (32,33). Later it has come evident that there is also a wide diversity of cellular and circuit mechanisms underlying the generation of theta rhythm implying that theta rhythm reflects a dynamic interaction of various synaptic and cellular mechanisms (33-35). Notably, this could also explain why the exact theta frequency rangevaries in different species even though the physiological functions might be similar (19).

As theta became known, researchers were intrigued to find out the plausible functional significance of this rhythm. A large amount of data was collected of the relations of different behavioural stages and existence of the rhythm, sometimes even leading rather obscure findings, e.g. theta arousal in the cat hippocampus while presenting a mirror (36). In the long run, it came apparent that hippocampal theta is most distinguishable during two discrete behavioural stages: voluntary / exploratory movement and paradoxical (REM) sleep (24,37). At the same time, significance of the hippocampus in episodic memories and memory functions was revealed by lesion studies (4), and slowly more compelling evidence of the relation between theta and memory functions was established (38). Discrete lesion of MS is known to eliminate theta and simultaneously lesion produce severe learning and memory deficits (39). More compelling evidence on the role of theta in memory functions was shown in an experiment in which a high proportion of theta (8-22 Hz) during pretraining phase correlated with rapid learning rate of eye blink conditioning (40). This result was further supported by the observation that training during hippocampal theta was associated with faster learning than training during non-theta state (41). However, later studies have challenged these ideas. Contrary to previous findings, trials presented in the absence of hippocampal theta were found to lead to robust learned response while presenting conditioning trials during hippocampal theta had no effect on learning (42).

2.2.2 Gamma rhythm

Another prominent hippocampal rhythm is the so-called gamma rhythm (also known as the fast or beta rhythm). Despite the technical challenges comforted by the fact that this faster rhythm is of relatively low in voltage and often occurring with theta activity, one of the earliest studies was able to characterized gamma (fast) rhythm in the hippocampus of rabbit (43). It was noted that the usual frequency was around 40-50 Hz. In addition, although gamma was associated with theta, there were cases when it was not, e.g. after septal lesion (abolishing theta) physostigmine or reticular stimulation was still able to elicit gamma activity (43). More information of the behavioural correlates of gamma activity was revealed in a study showing more gamma activity (40-100 Hz) in rats during walking or REM sleep compared to immobility (44).

Naturally, these findings also led to speculations about the mechanisms of generating gamma. Based on the current understanding gamma models can be categorized as the inhibitory-inhibitory (I-I) or the excitatory-inhibitory (E-I). The I-I model is based on the assumption of synchronous activity of two interconnected inhibitory interneuron group. This model predicts that the first group of interneurons discharges together, and, therefore, generates synchronous IPSPs in the next group of interneurons. After hyperpolarization the second group is capable of spiking and inhibiting the first group. Since this process is dependent on multiple factors such as $GABA_A$ receptor kinetics, the timing of this process is flexible enough to cover 40-100 Hz frequency range (45). The E-I model is based on the reciprocal connections between pools of excitatory pyramidal and inhibitory neurons. If fast excitation and delayed feedback inhibition alternate with appropriate strength, oscillatory activity can emerge (34,46-48).

Hippocampal gamma has been shown to participate the dynamics between hippocampal areas and hippocampus-entorhinal connections. It has been proposed that there are two major gamma generators, one oscillator located in the dentate gyrus and the second in CA3 area, which are weakly coupled. In addition, the DG oscillator is dependent on the input from the entorhinal cortex while CA3 and CA1 gamma rhythms are coupled together (49). Supporting this idea, following a bilateral lesion of the entorhinal cortex the power and frequency of hilar gamma (40-100 Hz) activity significantly decreased or disappeared. Instead, a large amplitude but slower gamma pattern (25-50 Hz) emerged in the CA3-CA1 network (50).

The functional significance of gamma rhythm in memory functions is less clear. This relates to the fact that we still do not know exactly the conditions which enable hippocampal gamma to emerge (compared to hippocampal theta). A prominent idea of

gamma function comes from the field of vision research and is related to the so-called "binding problem", i.e. what is the neuronal mechanism of combining spatially separate features to form unified visual pattern (51). If two spatially separate sites in the visual cortex with the same orientation preference are stimulated with stimuli possessing global features such as coherent motion and continuity, the oscillations (40 – 60 Hz) become coherent. If the stimuli are not sharing the same global features, these two sites are still responding with same kind of oscillatory activity but, importantly, these rhythms are not coherent (52,53). However, this hypothesis has been under criticism since it is not able to clarify for instance why certain features seem bound together or what is the role of non-coherent gamma oscillations (54).

Nevertheless, this idea has been adapted also to concern hippocampal gamma. More specifically, it has been suggested that gamma rhythm (from ~25 to almost 150 Hz) actually consist of slow gamma (~25-50 Hz) and fast gamma (~65-140 Hz), which differentially couple CA1 area to inputs from medial entorhinal cortex, an area that provides information about the animal's current position, and from CA3, a hippocampal subfield essential for storage of such information (55). Still, future studies employing hippocampal gamma dependent tests will be essential for dissociating the contributions of gamma oscillations to various types of memory processing and to validate the hypothesis of the role of the gamma rhythm in the regulation of the spatial and temporal coordination of information flow.

2.2.3 Theta-gamma synchrony

In line with the hypothesis involving the binding problem, a modern view of cognitive science assumes that cognitive processes such as memory functions arise from functionally organized brain processes which act in coordinated fashion. How this coordinated action arises is one of the major challenges to be solved (56,57). As a hypothesis, brain rhythms and their coherent interactions are assumed to be well suited for the regulation of the spatial and temporal coordination of information flow and processing. Regulation of this multi-scale integration can be local or global interactions between different frequency bands, a phenomenon termed cross-frequency coupling (CFC) (58,59). The CFC interactions can principally be divided into 4 different categories: 1) power-to-power, 2) phase-to-phase, 3) phase-to-frequency and 4) phase-to-power (59).

Therefore, it also has been hypothesized that interactions between hippocampal gamma and theta rhythms are related to mnemonic functions of

hippocampus and connected brain structures. Based on the model proposed by Lisman and Idiart (60), memories are stored in a group of hippocampal pyramidal cells firing simultaneously in synchrony. It is thought that firing of these neuronal groups is tied to on-going gamma rhythm to keep their order. In addition, to prevent these memories and their order to fade away, they need to be refreshed periodically in which the role of theta is assumed to be crucial.

Actually, hippocampal theta and gamma rhythms occur together and interact in several way. Gamma activity is increased during theta-associated (phase to power cross-frequency) behaviors, such as exploration, sniffing, rearing and the paradoxical sleep in the hilus where gamma occurs with its' largest power (50). Interestingly, while an animal is exploring a familiar environment two separate bands of gamma in CA1 area, and their power is coupled to theta phase, slow gamma (25-50 Hz) to descending part of theta and fast gamma (65-140 Hz) to the through of theta (55).

The CFC has been attributed a role in hippocampal memory functions. It has been shown that hippocampal phase-to-power CFC is dynamically expressed only during certain periods of cognitive demanding task mainly while an animal is actively accessing sequential information during locomotion or navigation (61). However, it should be noted that in this study the phase of narrow theta (8-12 Hz) band modulated the amplitude of a wider range of frequencies even above the classic gamma frequency range (40-350 Hz). Interestingly, the amplitude of the low-gamma subband (30–60 Hz) becomes increasingly modulated by theta phase in CA3 area while an animal is learning which of the two stimuli are rewarded depending on the environmental context (conditional discrimination task). Furthermore, the strength of theta–gamma phase to power coupling was directly correlated with the increase in performance accuracy during learning sessions. These findings suggest a role for hippocampal theta–gamma phase to power CFC in memory recall. Interestingly, there was no difference between correct and error trials in any stage of learning (62).

Similar kind of results were observed in an experiment in which animals were tested in a one-trial, matching-to-place task in six-arm radial water maze. The single-trial spatial memory performance in rats was predicted by the power-to-power CFC of theta (4–10 Hz) and low gamma (30–50 Hz) rhythms in the hippocampal fissure. Theta–gamma power to power cross-frequency coupling / comodulation (TGC) was prominent during successful memory retrieval but was weak when memory failed or was unavailable during spatial exploration in sample trials (63). In addition, a phase-to-

phase CFC between theta (4-12 Hz) and gamma oscillations has been observed during running and REM sleep. More precisely, it seems that especially so-called slow gamma ($gamma_S$, 30-50 Hz) and midfreguency gamma ($gamma_M$, 50-90 Hz) and not fast gamma ($gamma_F$, epsilon, 90-150 Hz) are coupled with theta (64).

2.3 BRAIN RHYTHMS IN ALZHEIMER'S DISEASE

Dementia is characterized by multiple cognitive deficits that include impairment in memory. Alzheimer's disease (AD) is the most common dementing illness, accounting for approximately 50% to 80% of individuals presenting with dementia. Typical features include an insidious onset, a progressive course, and involvement in multiple areas of cognition in a patient who is otherwise alert, healthy, and free of motor or other neurological signs ((1,65). The hippocampus is the primary target of functional and pathological alternations in AD (2,3,66).

A number of studies have shown that moderate or severe AD influences EEG activity by slowing the EEG, i.e. reduces the proportional amount of higher alpha (8-15 Hz) and beta (16-31 Hz) rhythms and increases slower delta (< 4 Hz) and theta (4-7 Hz) rhythms (16). It's assumed that EEG slowing is related to simultaneous atrophy of cholinergic system but neurodegenerative processes also in other neurotransmitter systems may also play important roles in EEG changes in AD (67,68).

The use of EEG in AD is nowadays limited and EEG markers are not used as diagnostic criteria. However, recently there has been a growing interest to study the relationship between hyperactivity, epilepsy, and AD. Evidence from population studies and single case studies has shown that AD is associated with an increased risk for seizures and epilepsy. Unfortunately, these studies often underestimated or overestimated this relation due to methodological problems and the inherent difficulties in making the diagnosis of epilepsy in this population. However, it is currently estimated based on prospective and retrospective studies that 1.5–64% of all AD patients has at least one unprovoked seizure (69,70). In addition, a recent patient analysis revealed that if mild cognitive impairment (MCI) or AD is accompanied with epilepsy cognitive decline presents 5-7 years earlier than in control patients without epilepsy, and in AD patients even subclinical epileptiform activity associates with an earlier onset of cognitive decline (12).

These findings have been supported by studies in transgenic mice mimicking the amyloid pathology of AD emphasizing the possibility that aberrant excitatory neuronal activity represents a primary mechanism that may contribute to cognitive and

behavioral deficits. In vivo imaging of single cell activity has found populations of hyperactive neurons in the close proximity of amyloid plaques but hypoactive neurons distantly from plaques in several brain regions (Busche, Eichhoff et al. 2008, Busche, Chen et al. 2012). Moreover, LFP recordings monitoring the local activity of neuronal populations revealed epileptiform cortical and hippocampal synchronous discharges and generalized seizure activity in mouse models of AD. These animals have increased Aβ peptide production in these AD-vulnerable areas and cognitive deficits (Minkeviciene 2009, Palop 2007, Garcia-Alloza, Robbins et al. 2006).

These findings have important translational potential for clinical and therapeutic implications in humans. Notably, reduction of hippocampal hyperactivity by antiepileptic drug, levetiracetam, improved memory of mild AD patients (Bakker 2012). In addition, the same treatment was not only able to ameliorate memory but also reverse synaptic deficits in AD mice overexpressing Aβ peptide (Sanchez, Zhu et al. 2012). Therefore, targeting aberrant network activity with antiepileptic drugs seems to provide therapeutic benefit in the prevention or treatment of AD. However, the exact mechanisms by which Aβ peptide lead to neuronal hyperactivity and epilepsy, and how this hyperactivity may be related to memory impairment are still uncertain and controversial (Sanchez, Zhu et al. 2012, Qing, He et al. 2008, Ziyatdinova, Gurevicius et al. 2011, Cumbo, Ligori 2010)

3 Aims of the study

The aims of the study were linked to the above mentioned observations of the importance of hippocampal brain rhythms in cognitive function. The specific aims were the following:

1. to show that inducing theta oscillation by artificial electrical stimulation, which was mimicking the frequency of natural theta rhythm, could improve memory.
2. to validate the role of theta and gamma rhythm in the hippocampus during a prolonged lever pressing task requiring focused attention. This task was designed to reduce theta activity related to movement or exploratory behaviour allowing us to concentrate to brain rhythms induced by internal cues.
3. to test whether hippocampal brain rhythms show abnormalities in transgenic amyloid mice resembling the pathology of Alzheimer's disease.

4 Materials and methods

4.1 ANIMALS

In these studies both rats and mice were used to model hippocampal cognitive functions and their impairment. The rats used in the experiments were male Wistar rats from the Laboratory Animal Center at University of Eastern Finland. The transgenic mice used to model Alzheimer-related amyloid pathology were female APdE9 mice. The APdE9 colony founders were obtained from D. Borchelt and J. Jankowsky (Johns Hopkins University, Baltimore, MD, USA), while the mice were raised locally at the Laboratory Animal Center in Kuopio, Finland. Mice were created by coinjection of chimeric mouse/human APPswe and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The 2 transgenes cointegrated and cosegregate as a single locus (10). The line was originally maintained in a C3HeJxC57BL/6Jhybrid background. By the time of the present work, the mice were backcrossed to C57BL/6J for 14 generations. This mouse is later referred to as APdE9 line.

All the animals were caged in a controlled environment with temperature kept at +21°C and light on from 7:00 to 19:00, and water and food available *ad libitum.* All experiments were conducted in accordance with the guidelines of the Council of Europe and approved by the State Provincial Office of Eastern Finland.

4.2 SURGICAL PROCEDURES

Surgeries were performed under isoflurane anesthesia with the induction flow at 450 L/min (4.5%) and maintenance at 205–213 L/min (~2.0%). After all surgical procedures animals received carprofen (5 mg/kg, i.p., Rimadyl, Vericore, Dundee, UK) for postoperative analgesia, and antibiotic powder (bacitrasin 250 IU/g and neomycinsulfate 5 mg/g, Bacibact, Orion, Finland) was applied, if necessary, onto the wound.

In all studies the aim was to observe electrical activity of neuronal ensembles known as local field potentials (LFP). Therefore, animals were chronically implanted under isoflurane anesthesia with multiple intracranial bipolar or tripolar electrodes (Formwar insulated stainless steel wire, diameter 50 μm, California Fine Wire Company Co, GroverBeach, CA, USA), with designed vertical tip separation of 400 μm in the separate brain coordinates. In addition, screw electrodes were fixed on different locations of the skull to serve as ground, reference or recording electrodes. The screws served also

as anchors for dental acrylic cement and the connector (Mill-Max, NY, USA). In addition, silver wires ((PFA)-insulated, diameter 200 μm, A-M Systems, Sequim, WA, USA) were inserted into the neck muscles of the animal during surgery for electromyogram (EMG) recording.

4.3 VIDEO LFP ACQUISITION

After a recovery period of 5-7 days, the animals were accustomed to the recording setup. LFP was recorded while animals were performing behavioural task or when animals were freely moving in a box. The connector was attached to a custom-made preamplifier, and the signal was further amplified with an AC amplifier (e.g. gain 1000, Grass amplifier, 7P511K, Quincy, MA, USA). During recordings LFP sweeps were collected and digitized at 2 kHz per channel (DT2821 series A/D board; Data Translation, Marlboro, MA, USA). The signal was bandpass-filtered between 1 and 3000 Hz. The data were acquired by using Sciworks 5.0 program (DataWave Technologies, Loveland, CO, USA). The behavior of the animals was recorded using a camera (Live!Cam, Video IM Pro, Creative, Dublin, Ireland) that was positioned on top of the cage and synchronized with electrophysiological signals. AEPs were evoked using a pair of click tones (3 kHz, duration 10 ms, 70 dB, 500 ms between a pair of clicks, inter-stimulus interval 10 s). For AEP recordings, the mouse was continuously observed and all records for further analysis were obtained during immobility of the animal. A total of 30 responses were sampled and averaged.

4.4 LFP ANALYSIS

All signals were normalized to amplification, and offline analyses were conducted using MATLAB (Mathworks, Natick, MA, USA; R2008a). First, LFP was usually was preprocessed. To eliminate sweeps with artifacts, we first calculated the distribution for averaged power spectrum values between 80 and 90 Hz for each individual channel and for each 4 s sweep. Then, outlying sweeps were excluded using iterative implementation of the Grubbs test for outliers (MATLAB routine “deleteoutliers.m” by Brett Shoelson). This effectively removed occasional artifacts related to bad contact, jerky movements, or animal handling.

In most of the studies, power analysis was performed by using different methods. For instance in the study 3 Welch’s averaged modified periodogram method of spectral estimation (default MATLAB parameters) was used. However, in the first study LFP data seven representative 2-s epochs of movement and freezing were selected.

The selected sweeps were filtered (2 - 48 Hz) using a two-way least-squares FIR filter (the eegfilt.m routine from the EEGLAB toolbox. The frequency analysis was performed on each individual segment and the FFT (Fast Fourier Transformation) of each segment was used to create the total average. The effect of sham or fimbria-fornix lesioning was analyzed by comparing the average power of theta (4-12 Hz) of these groups during freezing and movement.

To quantify the gamma amplitude modulation by theta phase, we estimated a modulation index (MI) based on a normalized entropy measure, as described previously in Tort et al. (2008, 2010). This index is able to detect cross-frequency coupling between two frequency ranges of interest. First, we computed the bicoherence of the raw data, which indicates interacting frequency triplets (Kramer et al. 2008). Based on bicoherence, we chose to test theta (6–10 Hz) phase gamma (40–100 Hz) amplitude modulation. We estimated the mean power of gamma oscillation for each 1 Hz bin. Statistical significance of MI was estimated by creating shuffled versions of the time series (phase of one sweep and amplitude of another) and generated 200 surrogate MI values, where each MI value is calculated from 20 random pairs. Assuming a normal distribution of the surrogate MI values, a significance threshold was then calculated by using $P < 0.01$ as the threshold.

4.5 BEHAVIORAL TESTING

4.5.1 Contextual fear conditioning

The contextual fear conditioning paradigm was modified from (71) .First the animals were accustomed to the recording daily for 30 minutes during 5 consecutive days in a setup mimicking the actual testing and recording situation. Then the rat was placed in the testing cage (the preshock context, Context A) for 3 min. Right after this period three unsignaled foot shocks were delivered (0.7 – 0.8 mA, pulse duration 100ms, ISI 64 s). The pulses were delivered through a metal grid floor and generated by a Grass 88 stimulator connected with a stimulus isolator unit (Grass medical Instruments, Quincy, MA, USA). Twenty-four hours after the conditioning the animal was returned to the conditioning cage (Context A1) for behavioral follow-up for 5 minutes while no shock was delivered, and then returned to the home cage. Two hours later the animal was placed into a totally novel cage (Context B) for 5 minutes. Finally, two hours after the exposure to Context B

the animal was placed again in the original conditioning cage (Context A2). During these 5-min observation periods the behavior of the rat was video recorded. The recordings were used for offline analysis of freezing ("absence of any visible movement except for movement of the whiskers and respiration related movements, while the animal is in the crouching position" (72). Time spent in freezing was recorded and expressed as percent of total time in the context (5 min) (73) (**see Publication I Fig. 2**).

4.5.2 Lever pressing task

In the lever pressing task animals need to press a lever for 2.5 s to receive a water reward. The task took place in a plastic cage (width 31.5 cm, length 34 cm and height 40 cm) equipped with a lever and a water port on the same side of the cage. The lever and the water port were separated by a glass wall forcing animal to run to the water port after releasing the lever instead of just moving the upper part of the body. The task was guided by cue lights, a cue light above the lever and the second over the water port. During the actual task the cue light signaled the possibility to start a new trial. As soon as an animal started to press the lever the light was turned off. After constant 2.5 s lever pressing the light above the water port was switched on providing an external cue for the animal indicating successful trial (**see Publication II Fig. 1 and 3**).

4.5 HISTOLOGY

The animal received an overdose of either medetomidine (0.125 mg/kg) and ketamine (18.75 mg/kg) or Equithesin solution (0.1 mL/10 g) (chloral hydrate 425 mg +phenobarbital 60 mg/mL 1.75 mL + propylene glycol 3.3 mL + ethanol100% 1.2 mL, filled to 10 mL volume with distilled water. The electrode locations were marked by passing 1 mA of DC current for 8 - 10 s through the electrodes. Then the animal was perfused with ice-cold saline for 5 min at 13 ml/min followed by 4 % paraformaldehyde solution (PFA) for 13 min at 13 ml/min. The brain was moved from the skull and left for immersion postfixation for 4 h in 4 % PFA and after that in 30 % sucrose solution for two days. The brains were stored at -20 °C until slicing. Coronal sections (thickness 35 μm) were cut with a freezing slide microtome. The electrode locations were verified from the sections by cresyl violet with Prussian blue staining.

If the electrode marking with DC current was unsuccesful the electrode locations were confirmed by glial fibrillary acidic protein (GFAP) staining, and, in addition, verified by well-known hippocampal electrophysiological markers: phase of theta for maximum gamma oscillation (74), phase of theta for ripples (75), and depth profiles of auditory-evoked response (76).

4.6 STATISTICS

The LFP data were analyzed with Matlab R2008a, (MathWorks, Natick, MA, USA) software while SPSS 17 for Windows, (SPSS inc. USA) was used for statistical analysis. The graphs were created with GraphPad Prism 5 for Windows, 2009 Software (GraphPad Prism Software Inc. USA). The results are presented as means ± SEM. The threshold for acceptance of significance was set to 0.05.

5 Results

5.1 GLOBAL SYNCHRONIZATION OF HIPPOCAMPAL THETA IMPAIRS MEMORY

Lesioning the medial septum or fimbria-fornix, a fiber track connecting the hippocampus and the medial septum, abolished the theta rhythm and resulted in a severe impairment in hippocampal depedent memory (**see Publication I Fig. 3 and 4**). To test for a causal relationship between hippocampal theta and memory formation we investigated whether restoration of hippocampal theta by electrical stimulation during the encoding phase also restored fimbria-fornix lesion induced memory deficit in rats in the fear conditioning paradigm. Artificial theta stimulation of 8 Hz was delivered during 3-min free exploration of the test cage in half of the rats before aversive conditioning with three foot shocks during 2 min. Memory was assessed by total freezing time in the same environment 24 h and 28 h after fear conditioning, and in an intervening test session in a different context. As expected, fimbria-fornix lesion impaired fear memory and dramatically attenuated hippocampal theta power. Artificial theta stimulation produced continuous theta oscillations that were almost similar to endogenous theta rhythm in amplitude and frequency (**see Publication I Fig. 4**). However, contrary to our predictions, artificial theta stimulation impaired conditioned fear response in both sham and fimbria-fornix lesioned animals (**see Publication I Fig. 5**). These data suggest that restoration of theta oscillation *per se* is not sufficient to support memory encoding after fimbria-fornix lesion and that universal theta oscillation in the hippocampus with a fixed frequency may actually impair memory.

5.2 HIPPOCAMPAL LOCAL FIELD POTENTIALS AND CROSS-FREQUENCY COUPLING IN A LEVER PRESSING TASK

Even though hippocampus is known to be involved in the organization of episodic memories, it has proved challenging to understand how spatial and temporal information are organized in a cohesive manner. Characterization of the firing patterns of hippocampal neurons have revealed that some cells, so-called place cells, respond only when a rat is situated in a particular area of a testing platform, thus providing the framework for spatial context. However, some cells, so-called time cells, seem to be able to encode moments in temporally structured experience and, therefore, are able to

provide frameworks for temporal context. To verify to which extent single-cell activity related to temporal scale affects the activity of neuronal populations we measured local field potentials in the hippocampus while the animal was forced to sustain a lever press for at least 2.5 s to receive a water reward. We observed that in addition to the behavior related changes in theta (6-10 Hz), low-gamma (30-45 Hz) and high-gamma (55-90 Hz) there was a significant theta power difference between error and correct trials (**see Publication II Fig.4 and table 1**). Similarly theta (6–10 Hz) phase modulation of gamma amplitude in two ranges (30–45 Hz & 55-90 Hz) (TGPhase) as expressed as modulation index (MI) was specifically altered between different epochs but was also related to the performance accuracy (**see Publication II Fig. 5 and 6**).

5.3 HYPERACTIVITY OF CORTICO-THALAMIC NETWORK IN ALZHEIMER MODEL MICE

One of the hallmarks observed in neuronal pathology of Alzheimer's disease is accumulation of amyloid-β-protein. In this study we tested the hypothesis that numerous reported interactions of amyloid-β with cell surface molecules result in altered excitation–inhibition balance in brain-wide neural networks, eventually leading to epileptogenesis. For this purpose, we used amyloid precursor protein transgenic AD mice (APPswe/PS1dE9 or shortly APdE9 mice) which have been shown to display frequent occurrence of seizures peaking at an age (3-4 months) when amyloid plaques start to form in the cortex and hippocampus. We recorded local field potentials in freely moving 4-month-old APdE9 and wild-type (WT) control mice in the hippocampus, cerebral cortex, and thalamus during movement, quiet waking, non-rapid eye movement sleep, and rapid eye movement (REM) sleep. We observed that cortical LFP power was higher in APdE9 mice than in WT mice over a broad frequency range (5–100 Hz) and during all four behavioral states. Thalamic LFP power was also increased but in a narrower range (10–80 Hz; PublicationIII, Fig.3). While power and theta–gamma modulation were preserved in the APdE9 hippocampus, REM sleep related phase shift of theta–gamma modulation was altered (**Publication III Fig. 4**). Our data suggest that at the early stage of amyloid pathology, cortical principal cells become hyperexcitable and via extensive cortico-thalamic connection drive thalamic cells.

6 Discussion

One of the main aims of this study was to test the idea that hippocampal electrical rhythms, especially theta rhythm *per se* is necessary for successful memory functions. However, when we tested this hypothesis by restoring abolished hippocampal theta rhythm with artificial electrical stimulation mimicking naturally occurring theta, we observed no memory improvement but memory impairment (73). Most plausible explanation for this finding is that the artificial stimulation has a global effect on hippocampal networks. Theta rhythm is known to arise from interplay of a diversity of cellular and circuit reflecting a dynamic interaction between various synaptic and cellular mechanisms in the hippocampus and linked brain regions (35,77). Therefore, it is highly likely that the artificial global synchrony in this study actually prevented structured and selective way of communication between brain areas, thus resembling the pathological state during epileptic activity (78,79).

This finding is supported by the notion that multiple brain rhythms with distinct frequency band and behavioral correlates can be observed in the hippocampus (20). For instance, hippocampal theta and gamma rhythms occur together and interact, and are related to cognitive function as we also showed in the Publication II (50,61,63). It is questionable whether stimulation that mimicks a single rhythm could replace this complex interaction between different rhythms. On the other hand, it should be also noted that exogenous theta stimulation we used was fixed in its frequency and totally uncoupled to the rat's own behavior or processes related to memory. An influential hypothesis of memory trace formation suggests that information storage takes place in two stages characterized by a robust relation of hippocampal electrical activity and ongoing behavior. At first, during exploratory behavior with simultaneous theta activity the information is deposited in a labile form. These labile memories are later converted into long-lasting memories in the second stage characterized by rest or sleep with simultaneously occurring hippocampal sharp wave activity (78). Therefore, based on this hypothesis influencing memory formation by artificial stimulation is dependent not only on stimulation parameters but also the temporal / behavioral scale of naturally occurring behavior (80).

Therefore, attempts to influence learning and memory during naturally occurring specific LFPs might enhance cognition. As discussed in the introduction,

hippocampal theta seems to implicate a state of accelerated learning (40,41) even though this notion has lately been challenged (42). Accordingly, dominance of hippocampal theta oscillation might not be the only beneficial state for learning since training following a hippocampal ripple activity also led to faster conditioning (81).

Recent advances in controlling brain activity and influencing hippocampal LFP have provided new methods to influence memory functions (82). This was substantiated by a recent study utilizing optogenetics and online LFP recordings, although in a small set of animals(81). Fast inhibition as a function of ongoing theta phase was shown to be able to control both hippocampal LFPs and memory functions. More specifically, the study employed paced optogenetic close-loop stimulation based on light activation of channelrhidopsin-2 expressing parvalbumin-positive interneurons and online analysis of hippocampal LFP. Optogenetic feedback during the peak increased both peak frequency and amplitude of theta. Interestingly, stimulation during the trough and peak of theta also increased the power of lower gamma (25-35 Hz) but the change was more notable in the beta-band range (16-25 Hz) which could actually leak to faster frequencies. In addition, there were also reported changes in the behavior in hippocampal dependent spatial memory task during encoding and retrieval (83).

Consequently, it would be crucial to follow these preclinical guidelines in the clinical deep brain stimulation (DBS) trials aiming to improve memory or reduce the progression of dementia. However, it seems that the use of DBS has relied on serendipitous findings. DBS itself has been used therapeutically in several neurological and psychiatric disorders, such as Parkinson's disease (PD) and obsessive compulsive disorder, especially in drug resistant forms of disease. The exact mechanisms of action are still not exactly known, but most likely they are based on the DBS effect on changing the excitatory-inhibitory balance of neuronal networks, increasing neurogenesis or release of growth factors and/or activation of cholinergic transmitter system (84,85). However, a couple of rather obscure findings and following studies have led to a vivid hypothesis of its use in cognitive enhancement or even treatment for AD. One line of the experiments is based on the finding that stimulation of fornix and/or hypothalamus evokes *déjà vu* or sudden memory epochs dating back even 20 years (86). However, a follow-up study now aiming to AD patients was not that successful and led to more diverse and rather mild effects among patients (87). Another line of experiments has focused on stimulation of the nucleus basalis of Meynert. Stimulation of this area in one PD patient with related dementia improved memory but also attention and alertness, and

these findings have inspired a larger clinical trial (85,88,89). Notably, in these studies the stimulation is still based on standard DBS protocols utilizing, for instance, higher (50 or 100 Hz) or lower stimulation frequencies (< 20 Hz), which do fit the frequency range of natural hippocampal rhythms (84,85). However, it still lacks the relation of temporal occurrence of natural brain rhythms and it is not aimed at the hippocampal structures per se implying the mechanism likely not mimicking naturally occurring hippocampal oscillations.

Therefore, our finding of increased excitability in the early stage of amyloid pathology in cortico-thalamic networks perhaps provides more intriguing approach to understand pathological changes in electrical activity in AD. It is worth noticing that EEG findings related to AD are rather sparse and mainly emphasize the slowing of EEG, i.e. reduced proportion of higher alpha (8-15 Hz) and beta (16-31 Hz) rhythms and increased slower delta (< 4 Hz) and theta (4-7 Hz) rhythms (16). The recent studies linking hyperactivity of neuronal networks and especially hippocampus with memory impairment have provided an intriguing approach to understand and reduce cognitive decline not only in aged individuals and but also in AD patients. Excess neuronal activity in CA3 area of hippocampus occurs in aged rats (25-27 months) and it is linked with memory impairment (90). More importantly, this activity seems to be controllable by drug treatments such as commonly used antiepileptic agents sodium valproate and levetiracetam. Treating aged rats with established memory impairment with these drugs showed clear improvement in their performance in a hippocampal dependent memory task (91). Supporting evidence was further provided by a clinical study involving patients with amnestic mild cognitive impairment (aMCI) and, therefore, in a high risk to develop AD. It was shown that aMCI patients had increased activity in the DG / CA3 areas of hippocampus determined by fMRI. Importantly, the same antiepileptic agent as in the previous animal study (91), levetiracetam reduced hippocampal excitability to the level of the control group and improved cognitive performance (14).

Another line of experiments has focused on pathological aging and determining the role of neuronal hyperactivity in AD animal models and patients. Transgenic mice have enabled studies specifically on the role of amyloid pathology in AD related memory loss (3). Two-photon Ca2+ imaging in a mouse model of AD revealed a substantial decrease in neuronal activity overall but, surprisingly, near the amyloid plaques neurons displayed increased frequency of spontaneous Ca2+ transients (6). In addition, qualitatively identical changes in neuronal activity in the vicinity of plaques

were observed also in the hippocampal CA1 area but, notably there were hyperactive neurons already before the plaque formation (5).

Accordingly, our finding implies that hyperactivity observed at a single cell level can already in the early stage of plaque formation lead to changes in the activity of neuronal ensembles (92). In addition, at this stage animals start to express high probability of epileptic seizures, which in some case may be lethal (7,8). Therefore, the finding that levetiracetam can effectively suppress abnormal spiking activity and that chronic treatment also reverse cognitive impairments, offers plausible mechanism to control abnormal electrical activity of neuronal networks and work as a novel method to affect to the progress of AD (15). However, it should be noted that most of these findings only apply to amyloid pathology and, therefore, might not be able to recapitulate to whole spectrum of clinical AD.

7 Conclusion

It is evident that there is a need for better understanding of the electrical activity of the brain not only in AD animal models but also in clinical applications. As we and others have shown interfering naturally occurring electrical activity can indeed influence memory functions. However, an approach based on mimicking only the main characteristics of brain rhythms is not able to capture the complex biochemical processes manifested as oscillations, and, therefore, applying artificial brain stimulation to improve cognition is most likely to produce only modest outcome or even prevent memory functions. Fortunately, advanced recording technology and analysis methods allow us to pin-point in more detailed manner the rhythms of hippocampus and relate them on-going electrical activity not only at a single cell level but also at neuronal ensemble and network level. For instance, we showed that modulation between two hippocampal rhythm, theta and gamma, correlates with prolonged lever pressing performance and successive outcome. The clinical relevance of this finding needs to be verified in future studies, but our finding of hyperexcitability in AD transgenic mice is in line with reported neuronal hyperexcitability in AD transgenic animals and also in AD patients. Notably, as we showed these changes can be detected already in the early stage of the amyloid pathology. This possibility to detect plausible AD related pathology even before amyloid plaque formation, and accompanied progressive synaptic and neuritic injury and neuronal loss, as hyperexcitability and control it with antiepileptic agents provides a new intriguing possibility to slow down the progression of AD and cognitive decline.

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Arto Lipponen

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In the first study we noticed the dynamics between theta and gamma rhythms are related to attention. Similar results were obtained in the second experiment in which we attempted to restore memory function by replacing abolished theta rhythm. As a conclusion, a single brain rhythm as such is not plausible crucial for normal memory functions. This also applies to preclinical models of Alzheimer's disease. When we recorded LFP of transgenic AD mice, we observed hyperactivity and changes in the theta-gamma-synchrony.

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