Entrainment and its perturbation in the auditory cortex of the rat: phase and frequency modulations

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ABSTRACT

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Objective of the present study was to build oscillatory entrainment in the auditory cortex of the rat and perturb it selectively. There is hardly any previous research on entrainment so the study was of exploratory nature and a unique experimental design was used. Local field potentials (LFPs) were measured from a total of fifteen urethane-anesthetized rats via an extracellular microelectrode placed over the dura. In the four main experiments 2,5 Hz entrainment was created with tone trains of nine stimuli, the entraining frequency of the tone being 4000 Hz and the perturbation frequency of the tone being 4500 Hz. Perturbation was always in the eight tone of the tone train, in which the phase and/or frequency of the tone was changed. Also jitter in the entraining frequency and single tone experiments were studied. It was noted that the rat can be either in a so called *up & down* or *complex state* during the measuring, from which the complex state is the preferred state for analyzing response differences.

Phase perturbation results suggested that there is a specific influence when the phase is made longer rather than shorter. The longer phase response became more negative than the preceding entraining tone response approximately at 62-124 ms and rose to more positive levels at around 168-224 ms. Frequency perturbation, in turn, caused more positive levels than the preceding entraining tone response roughly at 86-138 ms suggesting a differing timeline of changes. When combining phase and frequency perturbation, both the longer and shorter phase perturbation presented more positive amplitudes than the preceding entraining tone response approximately between 41-160 ms. The longer phase response was again more negative than the shorter one, timeline of differences being 23-141 ms and from 252 ms onwards. It was found that the response to a frequency change remains even in the presence of a jitter in the entraining frequency, which suggests that it does not matter if entrainment is at a fixed interval or if it varies semirandomly. In addition, an important result was that at first responses to the two different tones do not differ from each other and do not change during the first single tone experiment. Differences were only observed after an entraining experiment, when the perturbation tone response became more positive than the entraining tone response. It is suggested from the results that entrainment can create memory traces in the nervous system, a probability being that the trace is actually stronger for the perturbation stimuli.

Keywords: entrainment, perturbation, local field potentials (LFPs), auditory cortex, rat

TIIVISTELMÄ

Tahdistaminen ja sen häirintä rotan kuuloaivokuorella: vaiheen ja taajuuden muuntelu

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Tutkimuksen tavoitteena oli luoda oskillatorista tahdistamista rotan kuuloaivokuorella sekä valikoivasti häiritä sitä. Tahdistamista ei ole juuri lainkaan tutkittu aiemmin, joten tutkimuksessa yritettiin löytää uutta tietoa käyttäen ainutlaatuista kokeellista asetelmaa. Viisitoista rottaa nukutettiin uretaanilla ja niiltä mitattiin paikallisia kenttäpotentiaaleja (*local field potentials, LFPs*) kovakalvon päälle sijoitetun solun ulkopuolisen mikroelektrodin avulla. Neljässä pääkokeessa luotiin 2,5 Hz tahdistamista yhdeksän ärsykkeen äänijonoilla, missä tahdistamisäänen taajuus oli 4000 Hz ja häiritsevän äänen taajuus oli 4500 Hz. Häirintä oli aina kahdeksannessa äänessä, jonka vaihetta ja/tai taajuutta muutettiin. Tutkimuksessa testattiin myös tahdistamistaajuuden viiveen vaihtelua sekä esitettiin yksittäisten äänien kokeita. Tulosten analysoinnissa huomioitiin se, että rotta voi olla mittaamisen aikana joko niin sanotussa *up & down* tai *kompleksissa tilassa*, joista kompleksia tilaa tulisi suosia vasteiden eroavuuksien tarkastelussa.

Vaihehäirinnän tulokset osoittivat, että vaiheen pidentämisellä on tietty ero verrattuna vaiheen lyhentämiseen. Pidemmän vaiheen vaste muuttui negatiivisemmaksi kuin edeltävän tahdistavan äänen vaste noin 62-124 ms kohdalla ja nousi positiivisemmaksi noin 168-224 ms kohdalla. Taajuushäirintä puolestaan aiheutti positiivisempaa tasoa kuin edeltävän tahdistavan äänen vasteella noin 86-138 ms kohdalla, mikä osoittaa muutosten aikajanojen eroavan toisistaan. Kun vaihe- ja taajuushäirintä yhdistettiin, sekä pidempi että lyhyempi vaihehäirintä olivat positiivisempia kuin edeltävän tahdistavan äänen vaste noin 41-160 ms välillä. Pidempi vaihe aiheutti edelleen positiivisemman vasteen kuin lyhyempi vaihe erojen aikajanan ollessa noin 23-141 ms ja 252 ms eteenpäin. Taajuushäirinnän vaste säilyi vaikka tahdistamistaajuudessa oli viiveen vaihtelua, mikä osoittaa että ei ole väliä onko tahdistamisessa aina tietty kiinteä väliaika vai vaihteleeko aika puolisatunnaisesti. Tärkeä tulos oli myös se, että alussa eri äänet eivät poikenneet toisistaan eivätkä muuttuneet ensimmäisen yksittäisten äänien kokeen aikana. Eroja tuli vasta tahdistamiskokeen jälkeen, jolloin häiritsevään ääneen tuli positiivisempi vaste kuin tahdistavaan ääneen. Näiden tuloksien pohjalta ehdotetaan että tahdistaminen voi luoda muistijälkiä hermojärjestelmään, jopa niin että jälki voi olla vahvempi häiritsevälle ärsykkeelle.

Avainsanoja: tahdistaminen, häirintä, paikalliset kenttäpotentiaalit, kuuloaivokuori, rotta

CONTENTS

1. INTRODUCTION

Neuroelectric oscillations have been a topic of growing interest in the field of neuropsychology. The focus has often been in amplitude modulations of different frequency bands and their connections to various cognitive processes (Başar, Başar-Eroglu, Karakaş, & Schürmann, 2001; for a review, see Sauseng & Klimesch, 2008). Less research has been devoted to entrainment of neuronal oscillations, when oscillations phase-lock in the presence of rhythmic stimuli. Understanding the mechanism of entrainment is important because it affects the very baseline excitability of neurons. This, in turn, can affect neuronal response amplitude and reaction time (Lakatos et al., 2008), amplification of sensory input and utilizing attention (Mathewson et al., 2009; Schroeder & Lakatos, 2008), as well as shifting brain operation modes between rhythmic and continuous mode (Schroeder & Lakatos, 2009). The purpose of this Master's Thesis is to study oscillatory entrainment and its perturbation in the auditory cortex of the rat. In the following sections, more specific information about oscillations and entrainment is provided, starting with neuronal excitability states.

1.1 Neuronal excitability

The electrical activity of a neuron is not only related to the excitatory and inhibitory synaptic inputs, but also to electrophysiological membrane properties known as voltage-gated channels (Hammond, 2008). Since nerve cells communicate through changes in their membrane potential (Vm), it is important to understand different variations in the Vm.

Membrane potential is the difference between the internal and external part of the membrane (Hammond, 2008). In resting state the potential is negative (-80/-50 mV), but when neurons are active Vm varies between -90 mV and +30 mV. Ionic currents change the Vm by depolarizing, repolarizing or hyperpolarizing it. Intracellular recordings have shown that even during cell silence membrane potential abruptly shifts between two

preferred levels: the hyperpolarized and depolarized state. When the membrane potential is less negative than the resting potential, the membrane is depolarized, which is also known as the *up state*. In contrast, when the Vm is more negative, it is said to be hyperpolarized or in the *down state*. A depolarization can be an action potential or a postsynaptic excitatory potential (EPSP), and a hyperpolarization for example a postsynaptic inhibitory potential (IPSP).

The voltage transition of the *up & down state* is an example of the spontaneous activity of cerebral cortex even in the absence of external stimuli (Holcman & Tsodyks, 2006). The up & down state is a dominant pattern during slow wave sleep and some forms of anesthesia; in the up state there is increase of neuronal discharge rate whereas in the down state there is suppression of impulse activity (Kropotov, 2009). In this Thesis some experiments had to be discarded from the analysis because the rat was in the up & down state and this deep sleep or anesthesia affected the local field potentials. The neural mechanism of the up & down state transitions remains unclear, but studies have suggested transitions may occur for example through activation of a hyperpolarization-activated cation current (H-current) or activation of the sodium current which depolarizes the membrane for spike generation (Kang, Kitano, & Fukai, 2004; Bazhenov, Timofeev, Steriade, & Sejnowski, 2002).

A depolarized up state and hyperpolarized down state are known to affect sensory-evoked responses, but whether and how they are modulated by sensory stimuli is not well understood (Gao et al., 2009). One aim of this Thesis is to explore how entrainment affects the evoked responses in a so called *complex state* of the rat, when the level of sleep or anesthesia is more shallow. This typical desynchronized electroencephalogram (EEG) pattern of arousal is evident both in the naturally sleeping animal and during anesthesia (Destexhe, Hughes, Rudolph, & Crunelli, 2007). Changes of the membrane potential are essential to neuronal communication so the state of the rat must be taken into account when analyzing responses.

1.2 Brain oscillations

Connections in the neuronal network work differently than a single neuron, because without inhibitory neurons excitation creates only further excitation. Depolarization of the principal cell, initiated by the excitatory input, is reduced by the hyperpolarizing effect of feedforward inhibition (Buzsáki, 2006). This narrows the temporal window of discharge probability. Cortical networks seem to gain their complexity primarily from the inhibitory interneuron system. Balance of excitation and inhibition often give rise to rhythmic behaviour known as *oscillations*.

The term oscillation refers to a cyclic process; some form of periodicity or rhythm. Any oscillation can be described by parameters such as the oscillation's frequency, amplitude and instantaneous phase (Sauseng & Klimesch, 2008). The frequency of oscillation depends only on the average duration of inhibition (Buzsáki, 2006). If some interneurons discharge together in a short time window, they will impose stronger inhibition on their targets. As a result more neurons will discharge together simultaneously upon recovery. Intervals between activation phases vary in proportion to the length of the oscillation period, so that lower frequencies allow larger areas of involvement whereas higher frequencies incorporate closely located regions with short synaptic delays (Penttonen & Buzsáki, 2003). This is how oscillations can constrain timing of action potentials of the individual cells.

Brain oscillations have been studied in relation to various cognitive processes, personality traits, mental disorders and even genetics. (Başar et al., 2001; Başar-Eroglu et al., 2007; Begleiter & Porjesz, 2006; Jaušovec & Jaušovec, 2007; Knyazev et al., 2003; Ward, 2003). It is somewhat unclear, however, what happens to the natural or spontaneous oscillations when stimuli appear. There has been some major debate stemming from event related potential (ERP) studies. Two models have been proposed: the evoked model, which states that evoked responses are independent of ongoing background EEG, and the phase reset model, which suggests a resetting of ongoing brain oscillations (for a review, see Sauseng et al., 2007). Also a mixture of both mechanisms has been suggested. There is extensive literature about the neural mechanisms of ERPs, but hardly any previous

study concerning what qualities of the external pace-maker affect the observed stimulus responses. This Thesis attempts to find answers to that question.

1.3 Perturbation

The brain maintains a particular composition of brain oscillations and their percent ratio during a particular functional state (Fingelkurts, Fingelkurts, Ermolaev, & Kaplan, 2006). Rosanova et al. (2009) used transcranial magnetic stimulation (TMS) to directly perturb different regions of the human corticothalamic system, and found out that each cortical area tended to oscillate at a rate close to its own natural frequency, even when not directly stimulated. The natural frequency was also preserved across a wide range of stimulation intensities. The research group points out that it is difficult to tell whether different cortical circuits are intrinsically tuned to generate oscillations at a particular frequency just by observing spontaneous EEG. That is why they suggest a straightforward way of probing the frequency tuning of a system: directly perturb it to detect the main rate of the ensuing oscillations, the "natural frequency". Perturbation can tell us about the structure, the properties, and the state of the system under study. In this Thesis the perturbation is in the phase of the presentation time of the entraining sound and/or in the frequency of the sound.

One solution to perturb the natural frequency has been to use steady-state-evoked responses (SSER), a physiological measure of the brain's sensitivity to a periodic stimulus that has been described for all sensory modalities (Simpson et al., 2005). In addition to SSER, a variety of linking terms have been used in different studies to describe the theoretical concepts, such as *entrainment* or *phase locking* of oscillations to stimuli. I find the term entrainment fitting to describe my own research since the purpose is to first time-lock oscillations using quick successive stimuli and then perturb the built rhythm. The word entrainment refers to determining or modifying the phase or period of something.

Auditory versions of steady-state responses (ASSR) are generated by repetitive stimulation of the ear to form a periodic response whose amplitude and phase characteristics stay constant over time (Bohórquez & Özdamar, 2008). The periodic stimulation is often referred to as *click/tone trains*. In studying auditory evoked steady-state responses Simpson et al. (2005) found out SSER reflects the component of brain activity that is linearly related to the driving stimulus, but its amplitude is influenced by other dynamic oscillatory processes that interact with it non-linearly. In waking human subjects, the ASSRs have shown to be particularly prominent at the gamma range of 40 Hz (Galambos, Makeig, & Talmachoff, 1981). The 40 Hz enhancement may be a more general property of the brain network, a neural resonator tuned to a frequency of 40 Hz (Azzena et al., 1995).

1.4 Entrainment

Gamma enhancement has been seen as critical for active brain operations, but Lakatos et al. (2005) have suggested EEG is actually hierarchically organized. They state that the amplitude of each oscillatory frequency is modulated by the phase of a local lower frequency oscillation. As I have pointed out earlier, oscillations control the excitability of the neurons thus influencing stimulus processing. The research group proposes that because the oscillatory hierarchy can entrain to repetitive stimulation, the auditory cortex can structure its temporal activity to optimize processing of rhythmic inputs.

According to Schroeder and Lakatos (2008) the brain seems to be especially biased towards two modes: *rhythmic mode* and *continuous mode*. Operation depends on the dynamics of task demands, so that when there is a task-relevant temporal structure to which the sensory systems can entrain to, lower-frequency oscillations become useful in sensory processing. In this rhythmic mode cortical activity phase-locks to the attended stream, high-excitability phases align with events in the stream and responses are enhanced to attended events.

On the other hand, when there is no relevant rhythm in the task to entrain to, low-frequency oscillations become detrimental for processing (Schroeder & Lakatos, 2008). It is less likely to detect subtle random stimuli when there are long periods of low excitability. In this continuous or vigilance mode, low-frequency oscillations are suppressed and the system is pushed into a continuous state of high excitability. A key component of the high-excitability state is enhancement of gamma amplitude/synchrony (Schroeder & Lakatos, 2009). Obviously the continuous mode is difficult to maintain for long periods as it consumes a lot of energy, so the rhythmic mode is preferred state of the system in which it spends the most time. In the light of this it seems useful to study entrainment, a rhytmic mode, more thoroughly.

Will and Berg (2007) have reported a significant increase in brainwave synchronization following periodic stimulation. They analyzed synchronization between auditory stimuli and EEG responses with stimulus-locked inter-trial coherence (ITC), which measures consistency across epochs of the EEG spectral phase at each frequency and latency window. Analysis of variance for the ITC values of periodic stimuli, noise and silence showed a significant effect for stimulus condition but not for EEG frequency bands (ranging from 1 to 44 Hz). The main effect was increased phase coherence under periodic stimulation. What is more, increased ITC seems not to be a consequence of the stimulus sequence, but is instead generated by specific brain responses to the stimuli. The researchers conclude that periodic auditory stimulation produces a mixture of evoked and induced, rate-specific and rate-independent increases in stimulus related brainwave synchronization that likely affect various cognitive functions.

The phase and in particular its modulations seem to be of extreme importance for the processing of sensory stimuli in the brain (Sauseng & Klimesch, 2008). For example two oscillations are said to be phase-locked if the phase difference between them is constant (Le Van Quyen & Bragin, 2007). The phase is usually expressed in degrees from 0° to 360°, or in radians from 0 to 2π. In this Thesis one form of perturbation is to change the phase of the entraining tone. Later I will present why using a constant cut-off value of the mean duration could be useful.

1.5 Intracranial studies

Physiological interpretation of the latencies of the scalp-recorded steady-state responses is fairly difficult, the main problem being that the recorded response may not derive from a single generator (John & Picton, 2000). For example Yuval-Greenberg et al. (2008) have pointed out that the induced gamma-band EEG response recorded on the scalp mirrors eye movements following the display of a new image, rather than neuronal oscillations. Intracranial and epidural animal models can provide more exact information about neuronal events, especially concerning localization. In this Master's Thesis *local field potentials* (LFPs) will be measured from the rats' auditory cortex via an extracellular microelectrode placed on the brain surface over the dura. LFPs are assumed to reflect mainly transmembrane synaptic input to the neuronal population in the vicinity of the electrode tip. I will now present two intracranial/epidural auditory entrainment studies that are similar to my study.

Using intracellular recordings from anesthetized guinea pigs Gao et al. (2009) found out that the transition of membrane potentials from up to down state could rapidly entrain to a rhythmic sound. Brief sound stimulus of 200 ms triggered an up-to-down transition in the auditory thalamus when the neuron was in the up state, but the stimulus had no effect in the down state. Repetitive sound stimulation at fixed inter-stimulus-intervals (ISI) caused entrainment so that there were periodic downward transitions in phase with the onset of sound. Varying the properties of ISI, the research group found that average durations of the up and down states increased with ISI. Entrainment was especially significant within 3-12 s range, and as the sound intensity increased, the amplitude of the downward transition also increased.

Gao et al. (2009) also studied spontaneous up to down transitions extracellularly in anesthetized animals. When the ISI was 8 s, the transitions gradually changed from variable intervals to a narrow range around the entrained ISI. What was interesting was that after entrainment was terminated, the spontaneous up/down transitions had intervals close to the entrained ISI. This effect lasted for a period of tens of seconds, suggesting a

short-term memory in the neural network. Anesthesia does not cause the effect because during slow wave sleep the research group found similar entrainment by rhythmic stimulation. These results suggest that oscillations can significantly affect sensory information processing, and conversely, sensory stimulation can affect the temporal pattern of oscillations.

Cotillon, Nafati and Edeline (2000) also found out that ISI (or inter-tone interval ITI) is a critical factor controlling occurrence of oscillations. They studied tone-evoked oscillations using simultaneous recordings from auditory cortex, auditory thalamus and auditory section of the reticular nucleus. I will only present findings from the auditory cortex since my Thesis focuses on that part of the auditory system.

Reliable oscillations which exhibited oscillatory pattern in more than 50% of the trials were observed in 28% of the recording from auditory cortex (Cotillon, Nafati, & Edeline, 2000). The frequency range was 6-14 Hz, with mean frequency of 10,8 Hz. Concerning the left and right auditory cortex, there were never two recordings exhibiting reliable oscillations simultaneously in both hemispheres. Oscillations were rarely present simultaneously in the three different structures, but in the same structure they were often simultaneous. As said before, the critical factor for the occurrence was the ISI. Tone-evoked oscillations were absent for ISIs smaller than 1 s, but could be observed for ISIs of 2 s or longer. In my Thesis the entraining ISI is 400 ms so it is interesting to see if any tone evoked shifts can be observed in the LFPs. The research group suggests the ISI cut-off value may be a function of the mean duration of the oscillations, which is reflected in my Thesis so that the phase perturbation is always $1/8\pi$ or $1/4\pi$ from the entraining stimulus presentation at π.

I will refer again to the fact that oscillations do not seem to be just a function of the stimulus characteristics. Also in the study of Cotillon et al. (2000) the occurrence, the frequency and the duration of the oscillations were not a function of the tone duration, binaurality/monaurality or the nature of the stimulus (pure tone/click). At the time of their study only four studies had mentioned tone-evoked rhythmic activity at the cortical level.

Until recently, there has been very little study of entrainment, especially at the epidural level. This introduction thus far has presented some theory behind entrainment and pointed out why these evoked oscillations are important to study. I will now present the research questions for this Thesis.

1.6 Objectives of the study

The main interest in this Thesis is how perturbation affects entrainment. Two modes of perturbation are tested: phase and frequency change. The four main experiments combine these features to test specific and combined features of perturbation. It is assumed that after creating entrainment with a tone train, there is a differing response when the phase and/or the frequency of the tone is changed. It is also hypothesized that the responses to phase and frequency change differ from each other. *Experiment 1* tests phase specific response whereas *experiment 2* tests frequency specific response to perturbation. Experiment 2 has a control procedure *experiment 4*, in which fixed interval entrainment is not created but there is still a frequency change in the tone. In *experiment 3* perturbation is both in the phase and frequency of the tone. The idea of *experiment 5* is to give the nervous system time to recover from the previous experiment and observe how the state of the animal changes during the measuring. It can also be used to study baseline responses to the two different tones and whether or not they change during the measuring session which lasts for hours.

The phase perturbation is especially interesting considering oscillations and entrainment, so it would be interesting to see some differences depending on the length of the phase perturbation (shorter or longer phase). Analogous tones were used by Astikainen et al. (*submitted*) to study the *mismatch response* and those results suggest that the rat brain encodes stimulus probabilities of the auditory past. However, as the experimental design of this Thesis is unique and there is no previous research of similar kind, the changes are hard to predict.

2. METHODS

The experimental design was planned together with my supervisor Markku Penttonen and he was also responsible for the anesthesia and surgery of the rats. I performed programming of the experiments during the fall of 2009 and carried out the measurements and analysis of data during the spring and summer of 2010.

2.1 Subjects and surgery

Fifteen Harlan Sprague Dawley rats (200-350 g) were used for the experiments. The rats were housed in metal cages, kept under a 12-h light-dark cycle and fed ad libitum. Anesthesia was administered with 1,1-1,4 g/kg urethane with intraperitoneal injections (Sigma-Aldrich, St. Louis, MO, USA). If necessary, supplemental doses were injected to retain the proper level of anesthesia, which was tested with withdrawal reflexes. Lidocane (20%, Orion Pharma, Espoo, Finland) was used as a local anesthetic for skin and muscles. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures, and procedures in them were approved by the Finnish National Animal Experiment Board. Consideration was used for the experimental design and application to minimize the number of animals used and their suffering.

The animals were placed in a stereotaxic instrument (Kopf series 962) using 45 degree ear bars and the scalp and muscles over the auditory cortex were removed. Two stainless steel watch screws, driven into the bone above the cerebellum (AP -11.0, ML 3.0) and frontal cortex (AP +4, ML 3.0) on the right side served as indifferent and ground electrodes in the recordings, respectively. An acrylic plate was fixed to the stereotaxic instrument with a screw and then to the ground screw of the rat with dental acrylic to fix the head securely. Craniotomy preserving the dura was performed to reveal a small area above the left auditory cortex (AP -5,5, DV 4,0 from the bregma). After this the right ear bar was removed and a teflon insulated stainless steel wire (0,2 mm diameter, A-M

Systems, Chantilly, VA) was carefully positioned with a stereotaxic electrode holder on the dura perpendicular to the brain surface. The electrode was bent to an angle to reduce the pressure. The position of the tip was defined by recording online electrical responses to the tones used in the actual experiments with an interval of 2 s between tones. The defining characteristics for the selection of the final recording site were a positive local field potential (LFP) response peaking at 50 ms with an amplitude of at least 300 μV, a clear additional positive peak at approximately 20 ms followed by a negative peak 10 ms later, and finally a wide negative peak between 100-200 ms in response to the tone. The positive & negative peak pair occurring at 20-30 ms is indicative of the cortical synaptic responses to thalamic activation.

2.2 Recording procedures

The EEG signal was 10-fold amplified using the AI 405 amplifier (Molecular Devices). Thereafter, the signal was low-pass filtered at 400 Hz (Cyberamp380, Molecular Devices) and finally sampled with 16-bit precision at 2 kHz (Digidata 1320A; Molecular Devices). The data was stored on a computer hard disk using Axoscope 9.0 data acquisition software (Molecular Devices). The data analyses were performed offline using Clampfit 9.0 (Molecular Devices), Analyzer 2.0 (BrainProducts, Gilching, Germany), Matlab 7.5 (MathWorks Inc., Natick, MA, USA), and SPSS 11 for Windows (SPSS Inc., Chicago, IL, USA).

Experiments were programmed and presented with E-Prime 2.0 (Psychology Software Tools Inc., Pittsburgh, PA, USA). Sinusoidal tones of 4000 Hz and 4500 Hz, 50 ms in duration with 5 ms rise and fall times, were used as stimuli. The tones were designed with Adobe Audition program, generated with the sound card of the experiment control computer and amplified with an active loudspeaker system (Studiopro 3, M-audio, Irwindale, CA, USA). The tones were directed to the right ear of the rat with a passive loudspeaker of the system placed at 20 cm from the right ear of the animal.

2.3 Experimental conditions

In all of the four main experiments, entrainment was created with a tone train of nine stimuli so that the perturbation was always in the eight stimulus. The entraining frequency was 2,5 Hz, the eight entraining frequency of the tone being 4000 Hz and the perturbation tone frequency being 4500 Hz. At first the inter-trial interval (ITI) was 10 s and the inter-stimulus interval (ISI) was 400 ms. To test the effect of a shorter ITI and obtain more tone train repeats the ITI was later changed to 3,6 s in all experiments. A total of fifteen rats were measured, seven rats with the 10 s ITI and eight rats with the 3,6 s ITI. Visual schemas of the experiments are provided in Figure 1.

Experiment 1: Phase perturbation

Every tone had the entraining 4000 Hz frequency and the perturbation was the changed ISI phase between the seventh and eight stimuli. The eight tone was randomized to appear either 1/4π before or 1/4π after the entraining stimuli at π. In this case the time was either 300 ms or 500 ms after the seventh stimuli. Number of tone train cycles was 200.

Experiment 2: Frequency perturbation

Entrainment occurred at 2,5 Hz, i.e. ISI was always 400 ms and perturbation was in the frequency of the eight tone, which was 4500 Hz. Number of tone train cycles was 100.

Experiment 3: Phase and frequency perturbation

Experiment 3 combined the perturbations of experiment 1 and 2. The ISI phase between the seventh and eight stimuli was randomized to be 300 ms or 500 ms and the frequency of the eight stimulus was 4500 Hz. Number of tone train cycles was 200.

Experiment 4: Frequency perturbation with a jitter in entrainment

ISI phase of all stimuli was randomized to appear either -1/4π, -1/8π, 0, 1/8π or 1/4π in relation to the 2,5 Hz entraining frequency (20 Hz jitter). In this case the intervals were

300 ms, 350 ms, 400 ms, 450 ms or 500 ms. Perturbation was the frequency of the eight stimulus, which was 4500 Hz. Number of tone train cycles was 100.

Experiment 5

The measurements started and ended every time with experiment 5, and experiment 5 was also between each experiment 1-4, the order of which were randomized. This tone train consisted of 50 ms pure tones with an ISI = ITI of 10 s. 4000 Hz and 4500 Hz tones were randomized and the number of stimuli was 100. As the ITI was switched to 3,6 s the amount of stimuli was changed to 200.

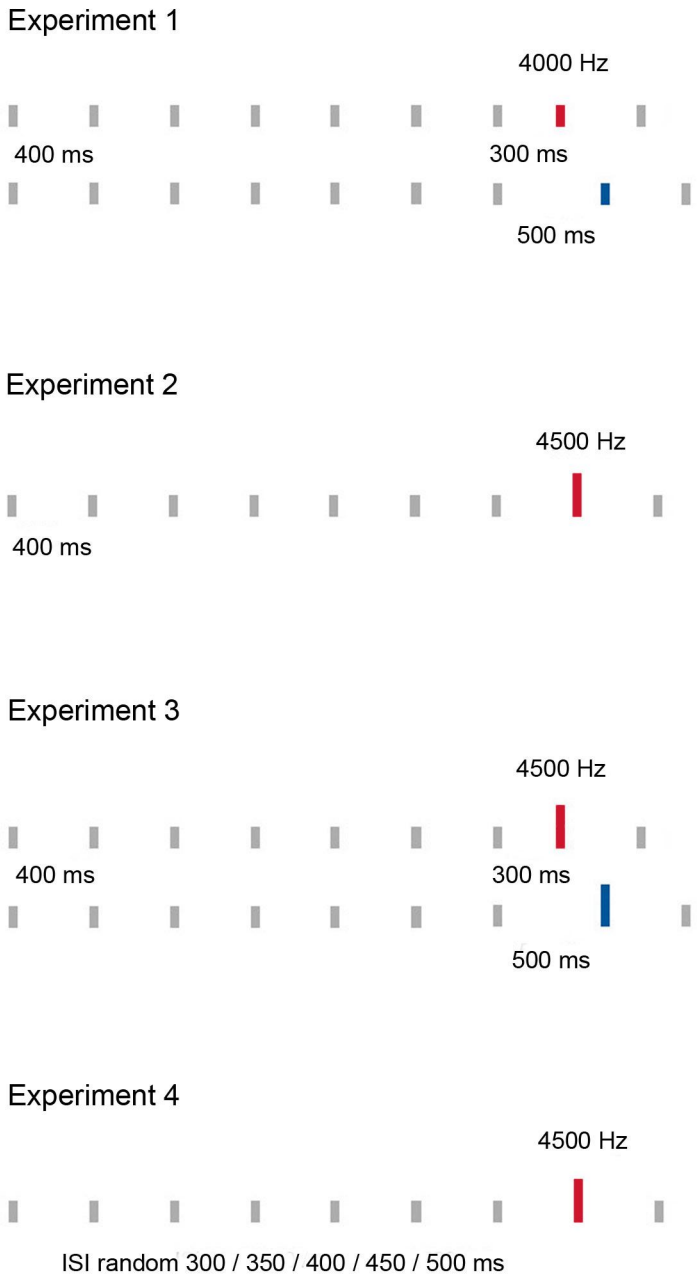

Figure 1. Visual schemas of the main experiments 1-4.

3. RESULTS

Data was preprocessed with Analyzer 2.0 by segmenting responses to the tones into 400 ms sweeps, each segment starting 50 ms before the tone and ending 350 ms after the tone. Segments with artefacts (usually random spikes from the environment) were removed from the analysis and baseline correction based on the preceding 53 ms was implemented for the averages before statistical testing. As the data points were gathered every 0,5 millisecond, each segment consisted of 800 data points. To avoid spurious statistically significant results the p-value was required to remain under the .05 level at least 10 ms for the difference to be considered significant. Figures were made with OriginPro 8.

As mentioned in the methods section, a total of fifteen rats were measured (seven rats with 10 s ITI and eight rats with 3,6 s ITI). All measurements were not, however, seen fit for statistical testing. Reasons for this lie in the location of the microelectrode and the spontaneous state of the rat. In the following analyses a small negative peak occuring approximately 30 ms after tone onset in responses is used as a reference for the electrode's optimal location on the auditory cortex. Measurements with these responses were preferred for the analyses over measurements without the negative peak. As discussed previously, the state of the rat also changes during the measuring day between the *complex* and *up & down state*. Experiments presented during the up & down state were discarded from the analysis. This was to make the group of measurements uniform and also to enable finding differences in responses since the up & down state represents a deeper level of sleep/anesthesia. In the end of the results section the up & down state measurements are also tested statistically to prove the state is not suitable for analyzing response differences.

3.1 Experiment 1: Phase perturbation

Seven measurements were chosen for statistical testing to represent good responses to the tones; six measurements with the 10 s ITI and one measurement with the 3,6 s ITI. This

criteria included the small negative peak occuring approximately 30 ms after tone onset. Grand averages over the seven measurements are provided in Figure 2. (top). As the seventh entraining tone was presented twice the amount of the eight perturbation tone, odd - even averaging was enabled to average only odd segments of the seventh tone when comparing the seventh and eight tone. The response to the seventh entraining tone is from here on referred to as *ent7* and the response to the eight perturbation tone as *pha300* (-1/4π phase) or *pha500* (+1/4π phase).

The average amplitude range of *ent7* was 387 μV, with a minimum value of -242 μV and a maximum value of 410 μV. The average amplitude ranges of *pha300* and *pha500* were 359 μV and 394 μV, respectively. *Pha300* had a minimum value of -137 μV and a maximum value of 442 μV, and same values were -304 μV and 412 μV for *pha500*. For each variable *ent7*, *pha300* and *pha500*, correlations between the seven measurements were statistically significant at the .01 level (2-tailed) between every measurement pair. *Ent7* correlations ranged from .24 to .93 correlation so that 86% of the pair combinations had above .50 correlation. *Pha300* and *pha500* correlations ranged from .43 to .96 (95% above .50 correlation) and from .11 to .90 (71% above .50 correlation), respectively. This means that the -1/4π phase perturbation responses tended to be fairly similar, whereas the seventh entraining tone and +1/4π phase perturbation responses had more variation.

Paired samples t-test was performed so that each measurement point of a variable was compared with the same point of another variable, the total count of data points being 800. For *ent7* and *pha300*, there was no statistically significant difference after the tone onset (<.05 significance level). For *ent7* and *pha500* differences were statistically significant (<.05 level) from 61,5 ms to 123,5 ms after tone onset and from 167,5 ms to 223,5 ms after tone onset, t(6) = 2,48- 3,73, p = .010-.048 and t(6) = -7,59- -2,46, p = .000-.049, respectively (Figure 2., under). *Pha500* was more negative during the 61,5-123,5 ms, the average of mean paired differences being 40 μV. During the 167,5-223,5 ms, *pha500* was more positive, the average of mean paired differences being 50 μV.

The main interest was the difference between the seventh and eight tone, but there was

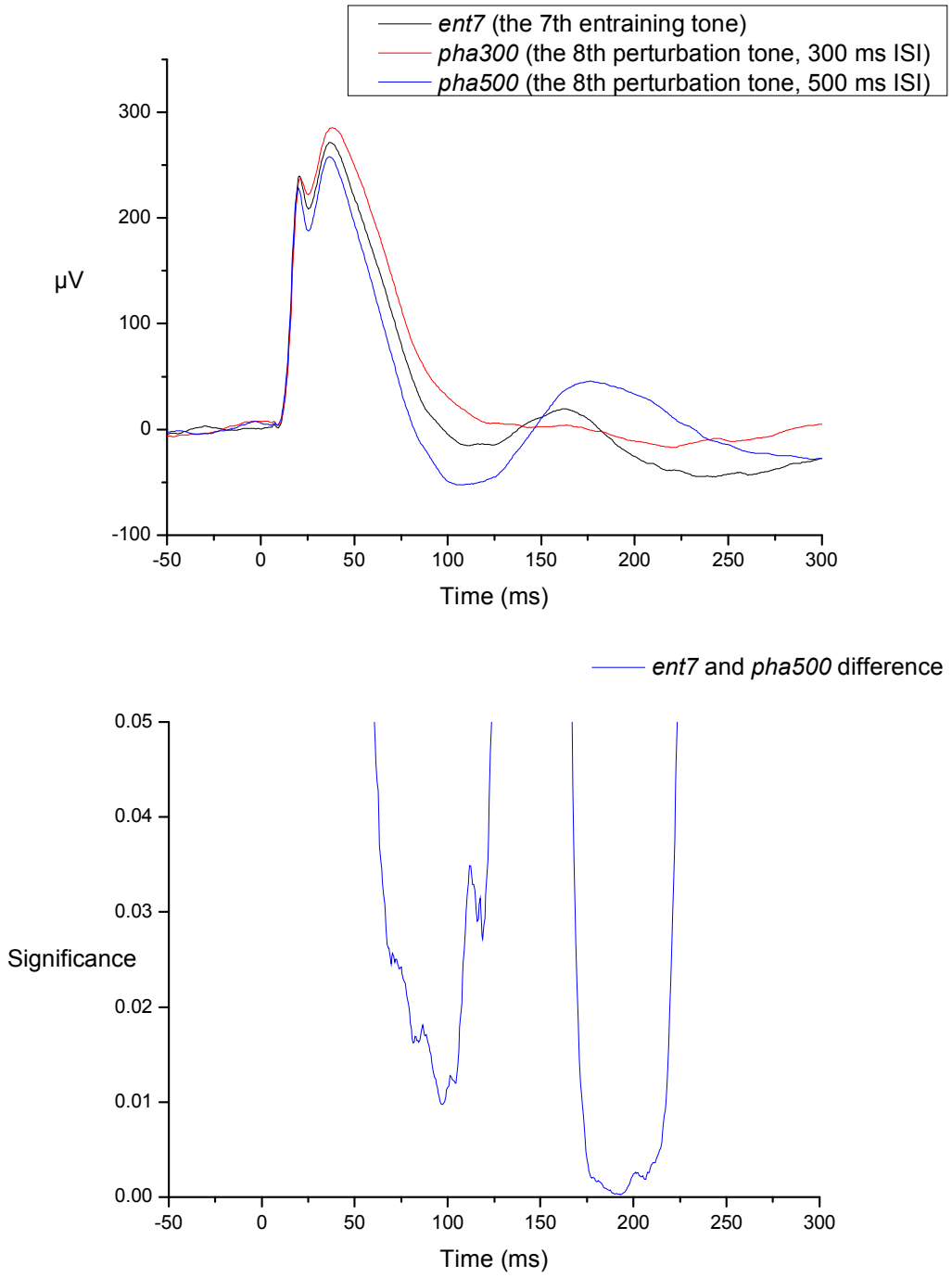

Figure 2. *Top*: Grand averages over the seven measurements of experiment 1. Tone was presented at 0 ms. *Under*: Timeline for statistically significant differences between the 7th entraining tone and the 8th perturbation tone with +1/4π phase.

statistically significant difference also between the different phases of the eight tone. For *pha300* and *pha500* the significance level was under .05 from 60,5 ms to 87,5 ms after tone onset and from 168,5 ms to 216 ms after tone onset, t(6) = 2,47- 2,72, p = .035-.049 and t(6) = -12,97- -2,47, p = .000-.048, respectively (not shown). Direction of changes was the same as in the *ent7* and *pha500* analysis, the average of mean paired paired differences being 75 μV and 44 μV for the respective phases.

These results suggest that the change of phase is not the only reason for the difference, but that there is some specific influence when phase is made longer rather than shorter. The +1/4π phase response tends to sink to more negative levels at approximately 100 ms and rise to more positive levels than the entraining tone around 200 ms.

3.2 Experiment 2: Frequency perturbation

Six measurements were chosen for analysis, three measurements with 10 s ITI and three measurements with 3,6 s ITI. It was harder to notice a clear trend in the responses of experiment 2; either the response to the eight perturbation tone of 4500Hz hardly differed from the seventh entraining tone or the change appeared as a positive rise about 60 ms after the tone in measurements with 10 s ITI. The six measurements chosen represent the positive rise group. Grand averages of the six measurements are shown in Figure 3. (top). From here on response to the seventh entraining tone will be referred to as *ent7* and the response to the eight perturbation tone of 4500Hz as *freq4500*.

The average amplitude range of *ent7* was 449 μV, with a minimum value of -198 μV and a maximum value of 452 μV. *Freq4500* had an average amplitude range of 427 μV, the minimum value being -138 μV and the maximum being 416 μV. Correlations between measurement pairs within both variables were all significant at .01 level (2-tailed). *Ent7* correlations ranged from .21 to .94 so that 80% of pairs had over .50 correlation. For *freq4500*, the correlations ranged from .29 to .95, 87% of pairs having over .50

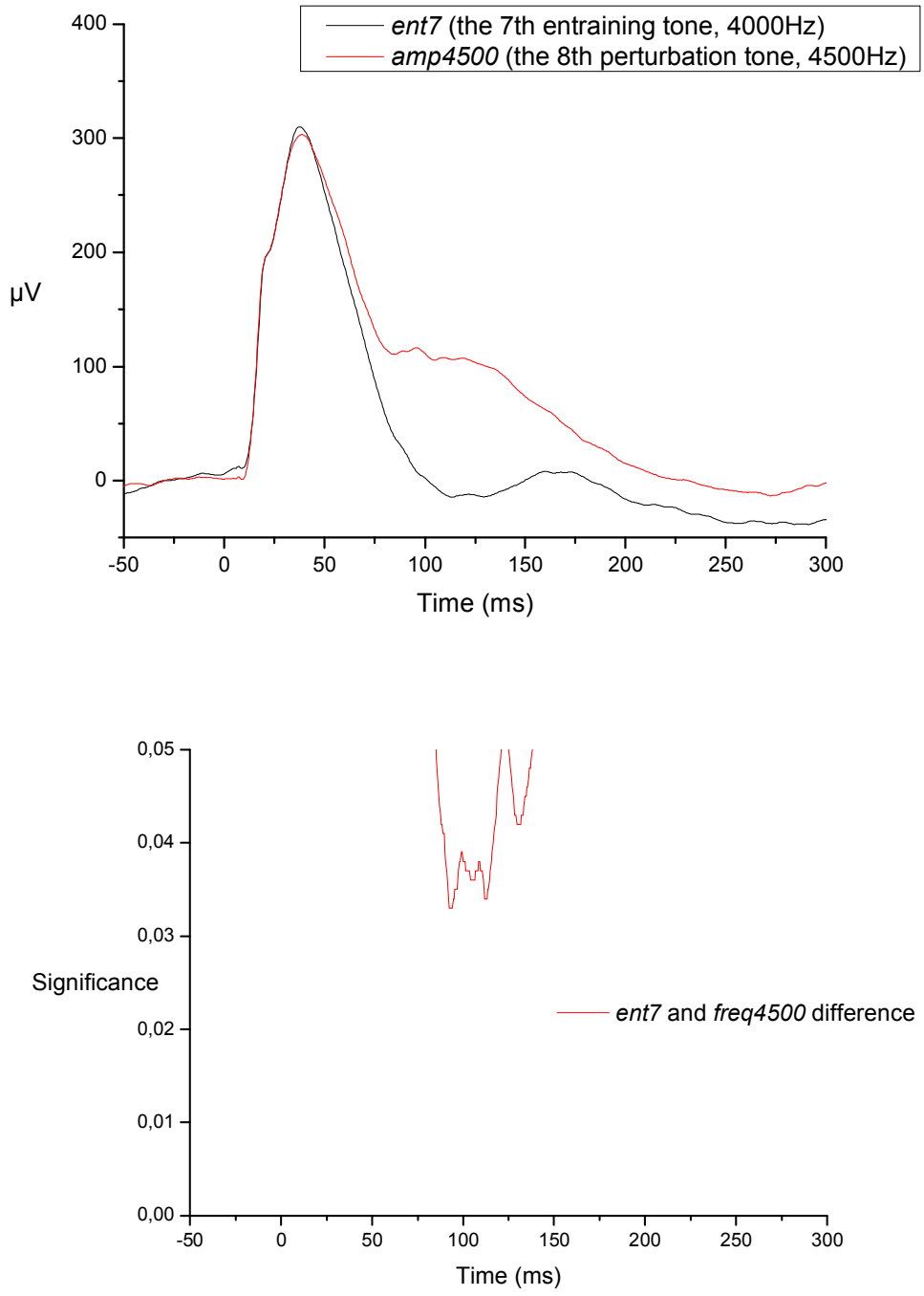

Figure 3. *Top*: Grand averages over the six measurements of experiment 2. Tone was presented at 0 ms. *Under*: Timeline of statistically significant differences between the 7th entraining tone of 4000Hz and the 8th perturbation tone of 4500Hz.

correlation. Thus, responses to the perturbation tone were somewhat more similar than responses to the entraining tone.

Again, the paired samples t-test was performed so that each data point was compared between the two variables *ent7* and *freq4500*. Differences between the two variables were statistically significant at <.05 level from 86 ms to 121 ms after tone onset and from 126 ms to 138 ms after tone onset, t(5) = -2,92- -2,60, p = .033-.049 and t(5) = -2,71- -2,60, p = .042-.049, respectively (Figure 3., under). The average of mean paired differences was 123 μV during 86-121 ms and 115 μV during 126-138 ms. Compared with the seventh entraining tone, as the frequency was increased there were more positive levels at approximately 90-140 ms. These results suggest that responses to frequency perturbation work differently than responses to phase perturbation.

3.3 Experiment 3: Phase and frequency perturbation

Nine measurements were chosen to represent good responses to the tones; five measurements with 10 s ITI and four measurements with 3,6 s ITI. The different ITI measurements seemed to represent somewhat differing responses, but as all the correlations were statistically significant at .01 level within each variable, the different ITIs were tested together. It can be noted that with the 10 s ITI there was a sudden negative peak approximately 30 ms after the tones but for the 3,6 s ITI measurements there seemed to be no such peaks. Grand averages over the nine measurements are presented in Figure 4.

Again, as the seventh entraining tone was presented twice the amount of the eight perturbation tone, only odd segments were chosen when comparing the seventh and eight tone. From here on response to the seventh entraining tone is referred to as *ent7*, response to the eight perturbation tone with -1/4π phase as *pha300+freq4500* and response to the eight perturbation tone with +1/4π phase as *pha500+freq4500*. Both perturbation tones were thus 4500Hz.

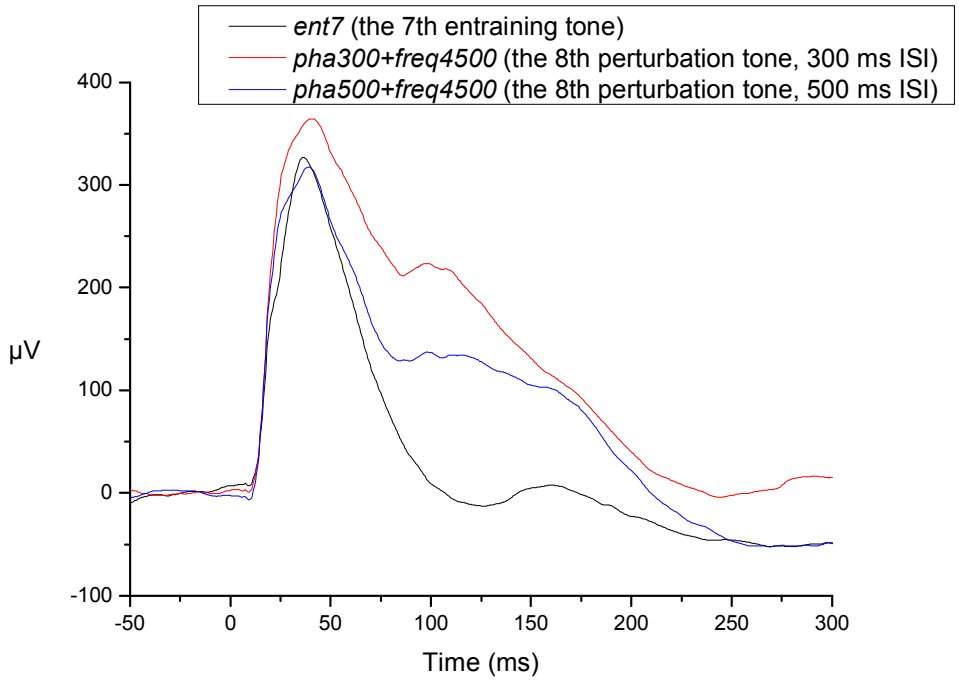

Figure 4. Grand averages over the nine measurements of experiment 3. Tone was presented at 0 ms and both perturbation tones were 4500Hz.

Ent7 had an average amplitude range of 439 μV with a minimum value of -192 μV and a maximum value of 645 μV. For *pha300+freq4500* the average amplitude range was 449 μV (min value -112 μV, max value 751 μV) and for *pha500+freq4500* the average range was 454 μV (min value -144 μV, max value 704 μV). As noted above, all correlations of measurement pairs within each variable were statistically significant at .01 level (2-tailed). *Ent7* correlations ranged from .30 to .98 correlation so that 83% of measurement pairs had over .50 correlation. *Pha300+freq4500* correlations ranged from .73 to .96 correlation so that all measurement pairs had over .50 correlation. For *pha500+freq4500* the correlations ranged from .30 to .96 correlation, 83% of measurement pairs having over .50 correlation. Thus, -1/4π phase perturbation responses represented good similarity, whereas there was more variation in the response to the seventh entraining tone and +1/4π phase perturbation.

As before, paired samples t-test was performed so that each data point of *ent7* was compared with the same point of *pha300+freq4500* and *pha500+freq4500*. For *ent7* and

pha300+freq4500 statistically significant (<.05 level) differences appeared from 41 ms to 154,5 ms after tone onset, t(8) = -4,64- -2,31, p = .002-.049 (Figure 5., top). During this time the eight perturbation tone with -1/4π phase had more positive levels than the seventh entraining tone, the mean paired difference being 154 μV on average. For *ent7* and *pha500+freq4500* there were statistically significant (<.05 level) differences from 0,5 ms to 12 ms and from 87 ms to 160 ms after tone onset, t(8) = 2,40- 3,65, p = .006-.044 and t(8) = -3,42- -2,32, p = .009-.049 (Figure 5., under). The respective mean paired differences during the two timelines were on average 12 μV and 121 μV. The +1/4π phase perturbation response had more negative levels 0,5-12 ms after tone onset and more positive levels 87-160 ms after tone onset.

The main interest was the difference between *ent7* and perturbation, but there were also statistically significant differences (<.05 level) between the two perturbation conditions *pha300+freq4500* and *pha500+freq4500*. These differences appeared from 23 ms to 141 ms and from 252 ms onwards from tone onset, t(8) = 2,36- 6,58, p = .000-.046 and t(8) = 2,32- 2,68, p = .028-.049 during 252-300 ms (not shown). The mean of pair differences was on average 68 μV during 23-141 ms and 59 μV during 252-300 ms after tone onset, *pha300+freq4500* having more positive levels than *pha500+freq4500* during both times.

Comparing these results to experiment 1 and 2, the frequency change probably makes the perturbation responses more positive after the tone has ended. In addition, there is the effect of phase perturbation in that the -1/4π phase has more positive levels than the +1/4π phase (also apparent in the results of experiment 1).

3.4 Experiment 4: Frequency perturbation with a jitter in entrainment

Eleven measurements were chosen for statistical testing, six with 10 s ITI and five with 3,6 s ITI. Again, the 10 s ITI measurements showed a small negative peak occuring 30 ms after tone onset, whereas in the 3,6 s ITI measurements it was absent during the first time experiment 4 was presented. From two rats the second presentation of experiment 4 was chosen instead of the first one. For statistical analysis the five different phases (-1/4π,

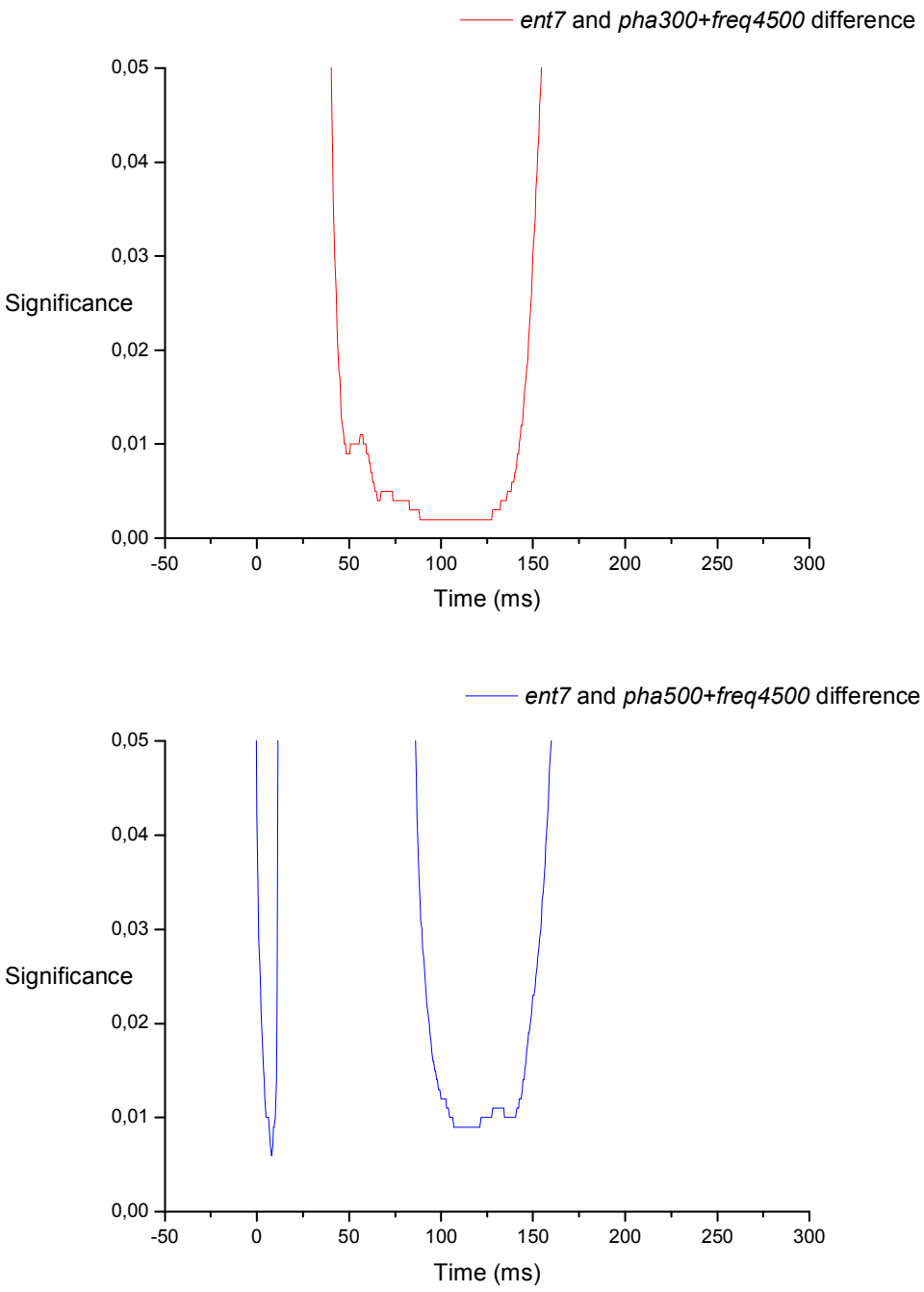

Figure 5. Timelines of statistically significant differences between the 7th entraining tone and the 8th perturbation tone in experiment 3. *Ent7* is the 7th entraining tone, *pha300+freq4500* the 8th perturbation tone with -1/4π phase and *pha500+freq4500* the 8th perturbation tone with +1/4π phase. Both perturbation tones were 4500Hz.

-1/8π, 0, +1/8π and +1/4π) of the eight perturbation tone of 4500 Hz were segmented as one response. From here on response to the seventh entraining tone will be referred to as *ent7* and the new combined response to the eight perturbation tone as *com4500*. Grand averages over the eleven measurements can be seen from Figure 6. (top).

The average amplitude range of *ent7* was 373 μV, with a minimum value of -125 μV and a maximum value of 489 μV. *Com4500* had an average amplitude range of 369 μV, the minimum value being -131 μV and the maximum value being 634 μV. With both variables, the within correlation of all measurement pairs was significant at .01 level (2-tailed). *Ent7* correlations ranged from .39 to .95 correlation, 93% of the pairs having over .50 correlation. For *com4500*, the correlations ranged from .25 to .96 correlation, 80% of the pairs having over .50 correlation.

Paired samples t-test was performed for *ent7* and *com4500*. Statistically significant differences (<.05 level) were found from 79 ms to 157,5 ms after tone onset, t(10) = -3,48- -2,23, p = .006-.049 (Figure 6., under). During this time *com4500* had more positive levels than *ent7*, the average of mean paired differences being 110 μV. Statistically significant differences occurred approximately at the same time in experiments 2 and 4, which suggests that it does not matter if entrainment is at fixed π or if it varies semirandomly at -1/4π, -1/8π, 0, +1/8π and +1/4π. Since the number of subjects differs (6 vs 10) it is difficult to evaluate whether differences are more pronunced when there is no entrainment (p = .033-.049 vs p = .006-.049).

3.5 Entrainment in the tone train

It is often thought that neurons are either stable or adaptive, meaning that a response to a repeated stimulus stays the same or changes. To test the possible changes during the entraining tone train, the entraining tones (4000 Hz) of *experiment 3* were cut in two parts. The response to the first tone was ignored at this point and analyzed later because it differs from rest of the tone train. The two halves of the tone train were compared so that the group of second, third and fourth tone response were compared to the group of fifth,

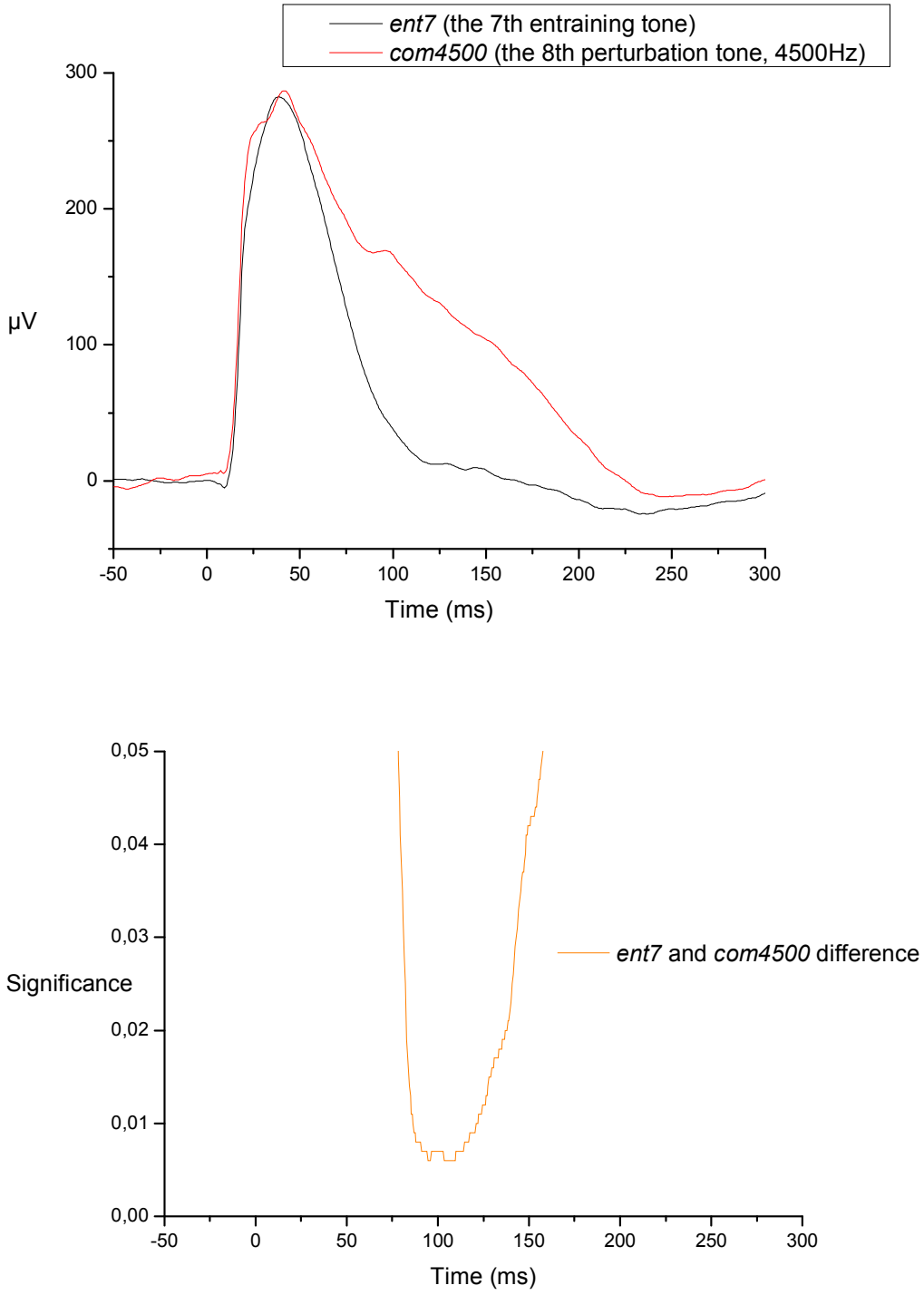

Figure 6. *Top*: Grand averages over the eleven measurements of experiment 4. All ISIs were random (-1/4π, -1/8π, 0, +1/8π or +1/4π) and the 8th perturbation tone was 4500Hz. Tone was presented at 0 ms. *Under*: Timeline of statistically significant differences between the 7th entraining tone and the combined 8th perturbation tone of 4500Hz.

sixth and seventh tone response. As noted before, the eight tone was the perturbation tone of 4500 Hz. The same nine measurements were used for the analysis as in the previous analysis of experiment 3. Grand average responses of the 2^{nd} - 4^{th} tone and 5^{th} - 7^{th} tone can be seen from Figure 7 (top).

Paired samples t-test between data points revealed statistically significant differences starting 26,5 ms after tone onset and lasting roughly until the next entraining tone (Figure 7., under). More specifically, the significance was under .05 level from 26,5 ms to 109 ms after tone onset and from 122 ms onwards, t(8) = -4,29- -2,32, p = .003-.049 and t(8) = -5,18- -2,33, p = .001-.048 during 122-300 ms. The last half of the entraining tone train had more positive values than the first half, the average of mean paired differences being 84 μV between 26,5-109 ms and 50 μV between 122-300 ms. These results suggest that responses to the entraining tone do change during entrainment, reflecting adaptivity in the nervous system.

3.7 First tone of the tone train

As the first tone was discarded from the entraining tone train analysis because the response is different after an inter-trial interval, the change in the first tone is analyzed here seperately. Responses to the first tone in *experiment 3* (same nine measurements as before) were cut in half and compared with the paired samples t-test between each data point. For the grand average responses of the two halves, see Figure 8. (top) where the first tone is referred to as *ent1*.

Statistically significant (<.05 level) differences were observed 178,5-199 ms and 275,5-285,5 ms after tone onset, t(8) = 2,32- 4,26, p = .003-.049 and t(8) = 2,35- 2,85, p = .021-.046 (Figure 8., under). During this time, the last half of the experiment was more positive, the average of mean paired differences being 29 μV and 36 μV, respectively. Results suggest that the change in the first tone follows a different timeline than the change in the following tones. It is also of interest that there are no differences at the time period of the usual perturbation effect.

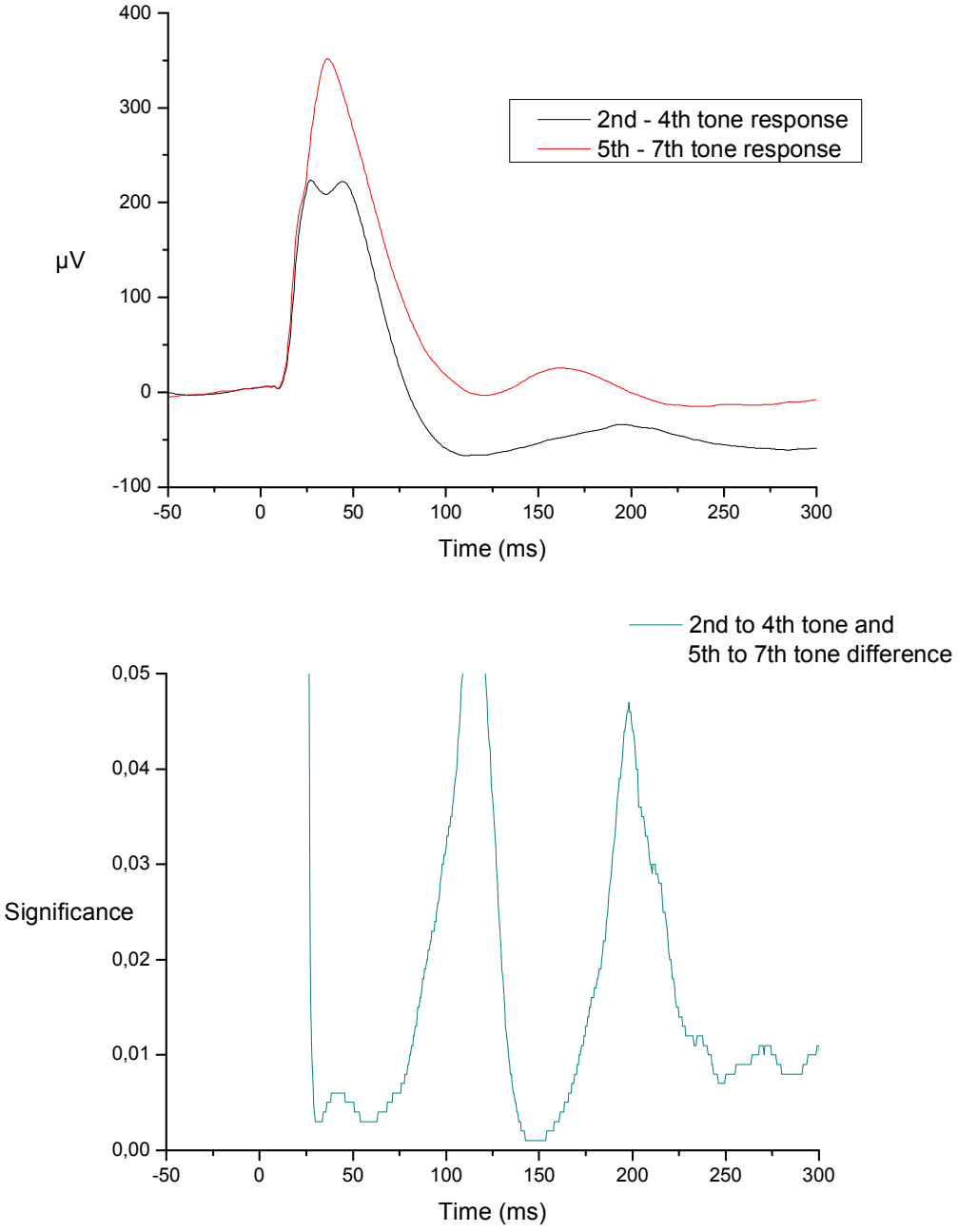

Figure 7. *Top*: Grand average responses of the two halves of the entraining tone train in experiment 3. *Under*: Timeline of statistically significant differences between the two halves of the entraining train.

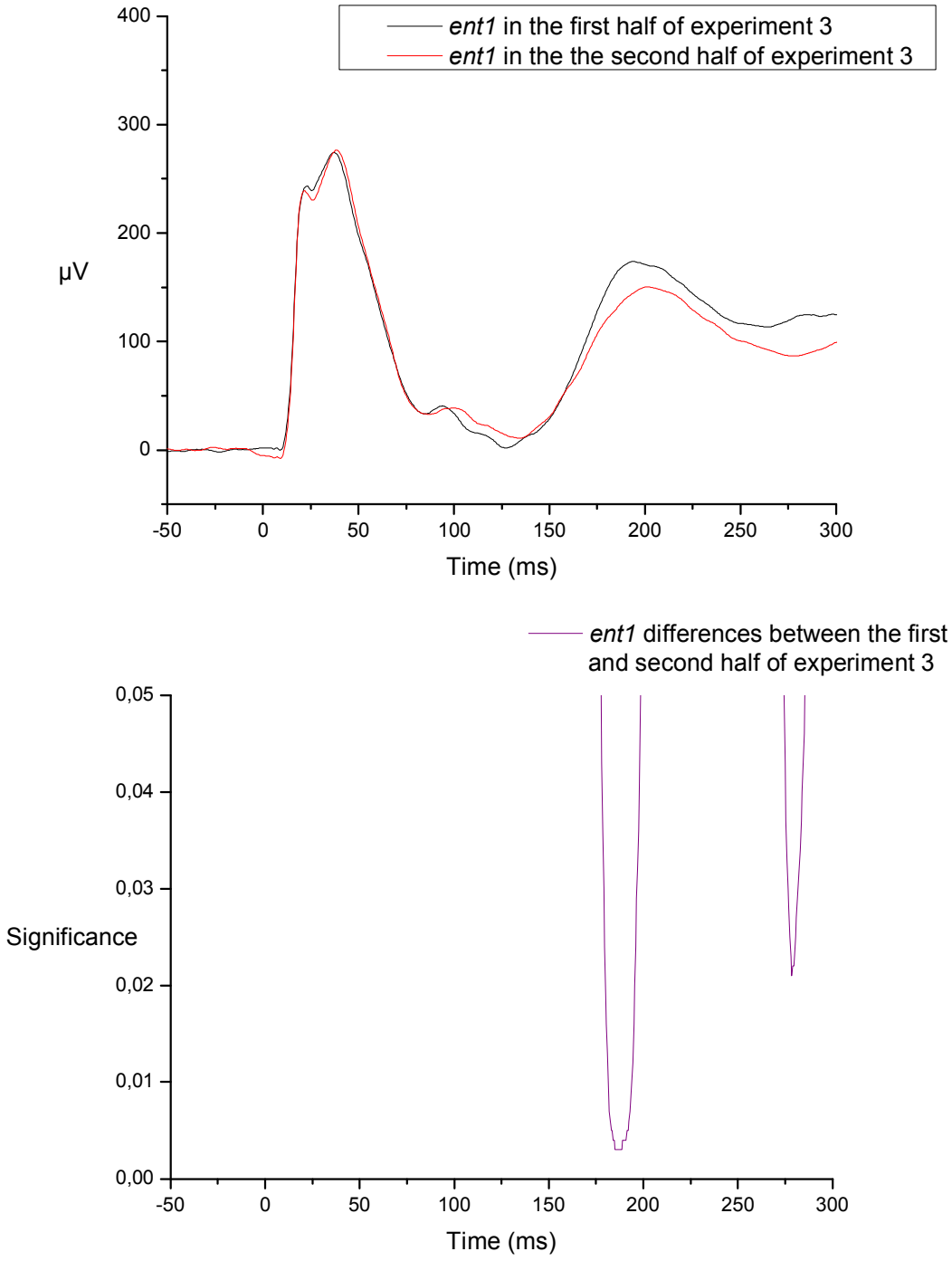

Figure 8. *Top*: Grand averages of the first and second half of the 1st tone (*ent1)* in experiment 3. *Under*: Timeline of statistically significant differences between the first and second half.

3.6 Entrainment and single tones

Analysis of the main experiments 1-4 has focused on the effects of perturbation, but it is also of interest to analyze the effect of entrainment on single tones in order to observe baseline changes. Seven measuring sessions of *experiment 5* were chosen for analysis based on good responses to the tones and within measurement correlations. These included three measuring sessions with 10 s ITI and four measuring sessions with 3,6 s ITI. Interestingly enough, in all except one measuring day the responses showed a clear negative peak of 30 ms even though it was absent in many experiments for the 3,6 s ITI.

The analysis began with the first presentations of experiment 5 to obtain the baseline responses of no-entrainment effect. First presentation grand average responses of the seven measuring days are presented in Figure 9. (top). From here on response to the entraining tone of 4000 Hz will be referred to as *ent4000* and the response to the perturbation tone of 4500 Hz as *per4500*.

Ent4000 had an average amplitude range of 454 µV, with a minimum value of -263 µV and a maximum value of 582 µV. For *per4500* the average amplitude range was 404 µV, the minimum and maximum values being -112 µV and 592 µV, respectively. Correlations within both variables were statistically significant at the .01 level (2-tailed). *Ent4000* correlations ranged from .39 to .90 and *per4500* correlations from .31 to .87 so that 90% of pairs had over .50 correlation within both variables.

Paired samples t-test between each data point of *ent4000* and *per4500* did not show any statistically significant differences in the first presentation of experiment 5. After this result the presentations of experiment 5 following an entraining experiment 3 were also tested statistically to study the effect of entrainment. Grand average responses over the seven measured subjects can be seen from Figure 9. (under). Paired samples t-test showed statistically significant differences (<.05 level) from 125 ms to 145,5 ms after tone onset, t(6) = -3,22- -2,46, p = .018-.049 (Figure 10.). *Per4500* was more positive during this time, the average of mean paired differences being 85 µV.

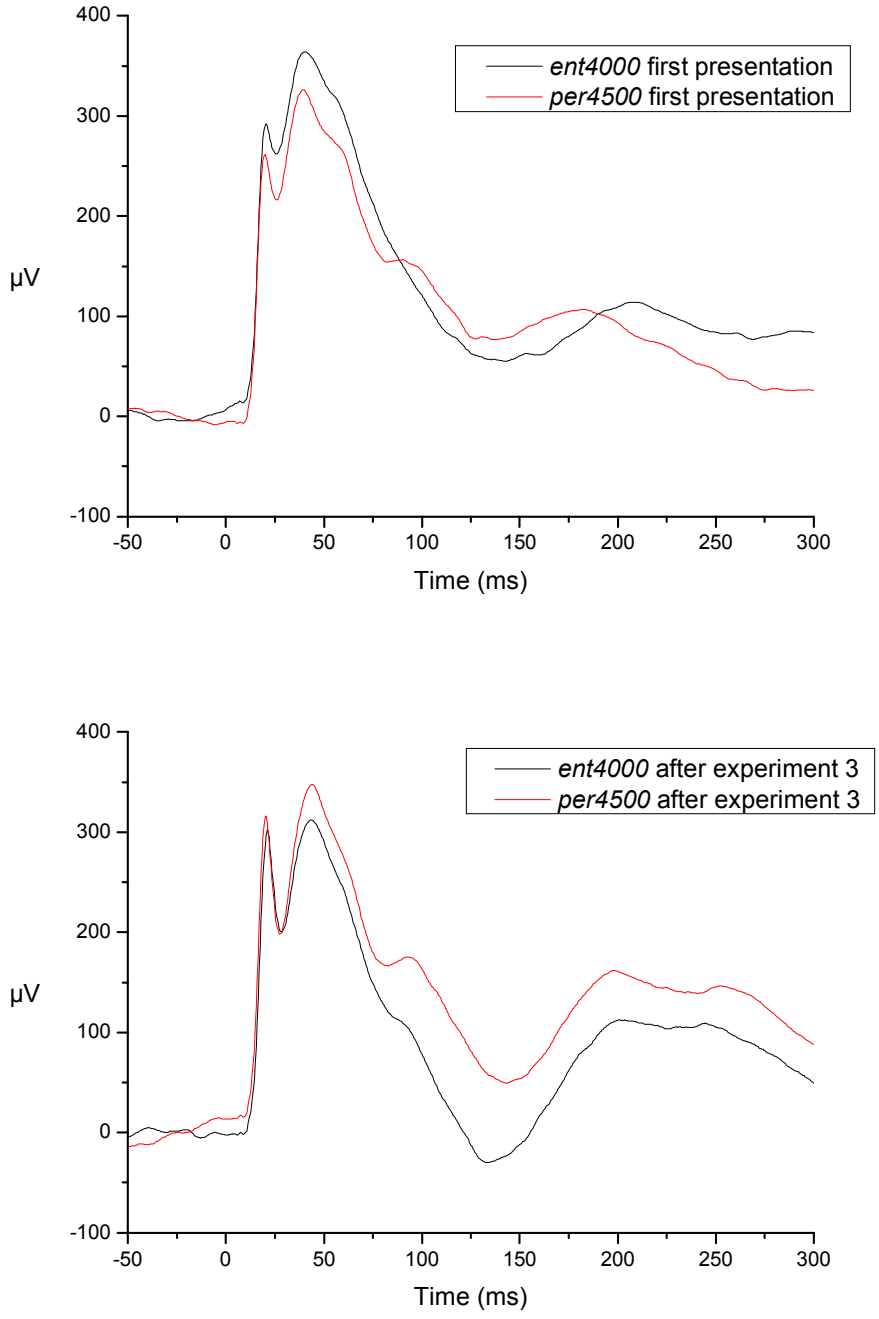

Figure 9. *Top*: Grand averages of the first presentations of experiment 5 over the seven measured subjects (no statistical difference). *Under*: Grand averages of experiment 5 following experiment 3 over the seven measured subjects (statistical differences).

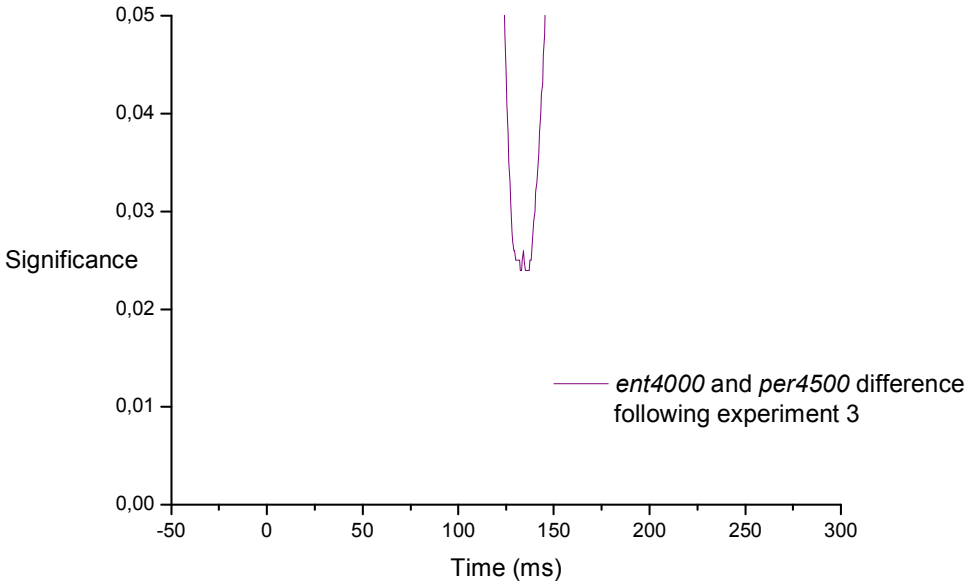

Figure 10. Timeline of statistically significant differences between the entraining tone and the perturbation tone of 4500 Hz in experiment 5 following entrainment.

As at first there is no difference between the two sounds, these results suggest the positive amplitude levels following the perturbation tone are not just because of the physical properties of the sound. Differences ensue only after the entrainment; the effect is retained afterwards either as more negative responses to *ent4000* or as more positive responses to *per4500*.

To test which response changes, paired samples t-test between each data point was performed within both variables *ent4000* and *per4500* for the first presentation of experiment 5 and experiment 5 following experiment 3. No statistically significant differences (<.05 level) were found for *ent4000*. For *per4500* differences were found from 248,5 ms to 304,5 ms after tone onset, t(6) = -3,60- -2,46, p = .011-.049. During this time the average of mean paired difference was 90 µV, presentation of *per4500* gaining more positive amplitude levels after an entraining experiment.

For statistical analysis of the response change during an experiment, responses in experiment 5 following an entraining experiment 3 were also cut in half and averaged. These halves were then compared with each other using the paired samples t-test for each data point. Five 3,6 s ITI measuring days were used for analysis since there was twice the amount of tones presented (200) compared with the 10 s ITI measurements (100). Grand averages of the first and second half of experiment 5 are presented in Figure 11. (top).

Statistically significant differences under the .05 level were observed only for the entraining tone of 4000Hz (*ent4000*). There were no statistically significant differences in the response to perturbation tone of 4500Hz (*per4500*). Differences of *ent4000* were under the .05 significance level 43,5-63,5 ms and 78-88,5 ms after tone onset, t(4) = -3,50- -2,81, p = .025-.048 and t(4) = -3,78- -2,79, p = .019-.049 (Figure 11., under). During this 30,5 ms time period the last half of the experiment had more positive values, the average of mean paired differences being 92 μV and 99 μV, respectively.

As the results of response change in the entraining tone train and single tones were similar and both suggested learning or adaptation in the system, also the first presentations of experiment 5 were analyzed from the five measuring days. While the same tones were used for searching the optimal location for the microelectrode, there were never entraining experiments before the first presentation of experiment 5. The following analysis was to control that the change was not simply because of repetition of the tone.

Paired samples t-test between data points did not show the same effect of more positive values during the last half of the experiment as in the experiment 5 following experiment 3. There were no statistically significant differences between the two halves of the experiment for either the entraining tone of 4000 Hz or the perturbation tone of 4500 Hz. These results suggest the change in the same response is a specific feature of entrainment and cannot be observed in presentation of single tones.

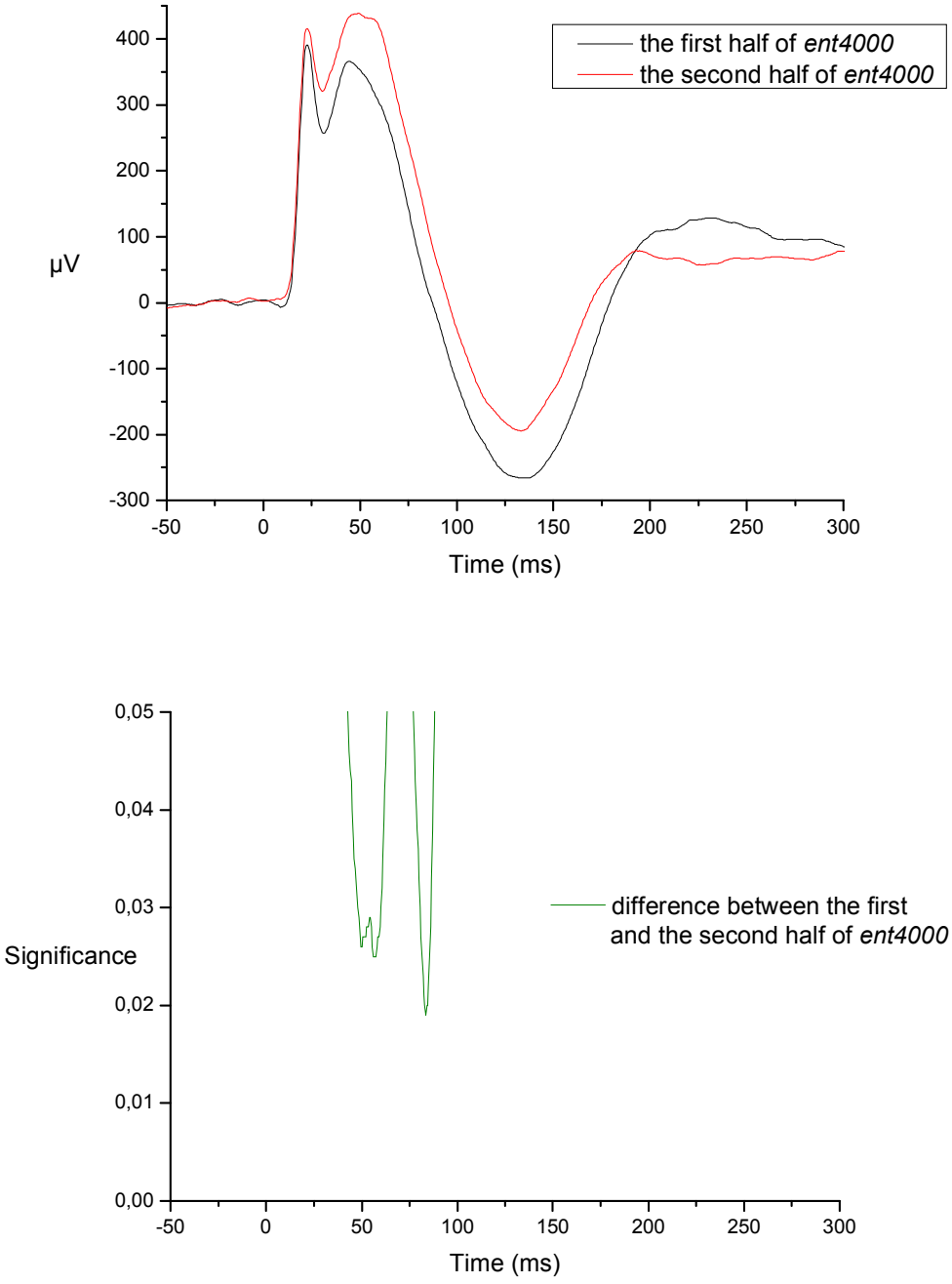

Figure 11. *Top*: Grand averages of the first and second half of experiment 5 for the entraining tone. *Under*: Timeline of statistically significant differences between the first and second half of experiment 5 for the entraining tone.

3.8 Effect of spontaneous states

Because the measurements taken into statistical testing are chosen based on certain characteristics, it is also important to consider the measurements left out of the analysis. As presented in the beginning of the results section, theoretical basis for understanding differences in responses lies in two domains: differing location on the auditory cortex and/or differing spontaneous state of the animal. Statistical analysis was implemented for *experiment 3* measurements to test the theory of different spontaneous states. As nine measurements were chosen to analyze the effect of perturbation, the six measurements left out of the analysis were also tested using the same method. *Ent7* will continue to be response to the seventh entraining tone, *pha300+freq4500* response to the eight perturbation tone with -1/4π phase and *pha500+freq4500* response to the eight perturbation tone with +1/4π phase. Both perturbation tones were 4500 Hz.

Grand averages of the six measurements left out are provided in Figure 12. Compared with the average amplitude ranges of 439 μV (*ent7*), 449 μV (*pha300+freq4500*) and 454 μV (*pha500+freq4500*) in the nine measurements chosen, the average ranges were respectively 551 μV, 500 μV and 470 μV in the measurements left out of the main analysis. Minimum and maximum values as well as within variable correlations can be seen from Table 1.

These descriptive statistics suggest that the spontaneous level of activity had more negative and positive values in the measurements left out of the analysis, the positive levels rising especially high (over 1000 μV). Within variable correlations of the dropped measurement pairs were all statistically significant at the .01 level (2-tailed), *ent7* having all correlations over .50 and 80% of measurement pair correlations being over .50 with *pha300+freq4500* and *pha500+freq4500*.

Paired samples t-test was performed as previously, so that every data point was compared to another data point between variables. There were no statistically significant differences between *ent7* and *pha300+freq4500* or *ent7* and *pha500+freq4500*. When comparing the

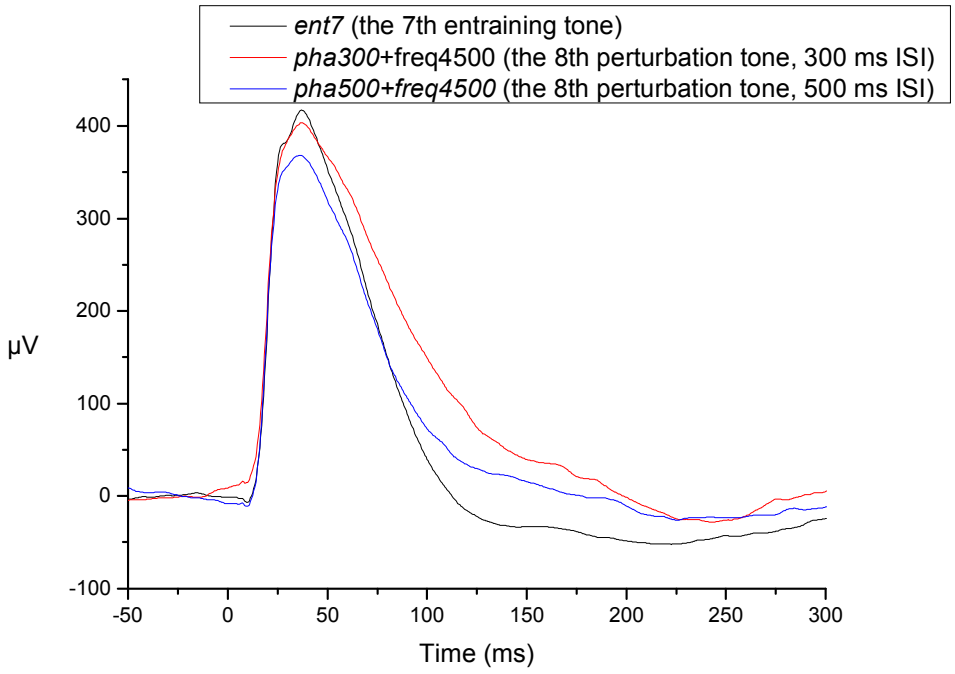

Figure 12. Grand averages over the six measurements of experiment 3 which were left out of the main analysis (up & down state). Tone was presented at 0 ms and both perturbation tones were 4500Hz.

	ent7		*pha300+freq4500*		*pha500+freq4500*	
	chosen	left out	chosen	left out	chosen	left out
min (μV)	-192	-222	-112	-162	-144	-121
max (μV)	645	1027	751	1079	704	862
min correlation	.30	.53	.73	.24	.30	.40
max correlation	.98	.96	.96	.93	.96	.91

Table 1. Minimum and maximum values and the within correlations of the seventh entraining tone (*ent7*), eight perturbation tone with -1/4π phase (*pha300+freq4500*) and the eight perturbation tone with +1/4π phase (*pha500+freq4500*) based on chosen measurements and measurements that were left out in experiment 3.

two perturbation tones, there was statistically significant difference -3 ms to 14 ms from tone onset, t(5) = 2,59- 3,10, p = .027-.049. The average of mean paired difference was 7 μV during that time, *pha500* being slightly more positive during the 17 ms.

As we compare these results with the nine experiments chosen for statistical testing, we see a clear difference in significance. Whereas in the nine measurements chosen for the main analysis *ent7* and *pha300+freq4500* differed statistically for 113,5 ms and *ent7* and *pha500+freq4500* differed for 84,5 ms, in the six measurements left out there was no statistical difference between these variables. Also when the perturbation tones *pha300+freq4500* and *pha500+freq4500* differed over 160 ms in the measurements chosen for analysis, the difference lasted only 17 ms in the six measurements left out.

Altogether these results suggest that the so called *up & down state* in the measurements is not appropriate for analysis, whereas the measurements with statistically significant differences reflect *complex state* of the rat. Examples of both states can be seen in Figure 13., where the complex state represents one measurement used in experiment 3 analysis (Figure 4.) and the up & down state represents one measurement used for the left out - measurement analysis (Figure 12.). From the results of statistical analysis and according figures it can be suggested that responses to the tones change according to the state of the animal. The up & down state could be considered as a deeper level of sleep/anesthesia, where responses lose their charasteristic shape and are more similar even in the presence of perturbation. Statistically significant differences are obtained only during the complex state of the rat.

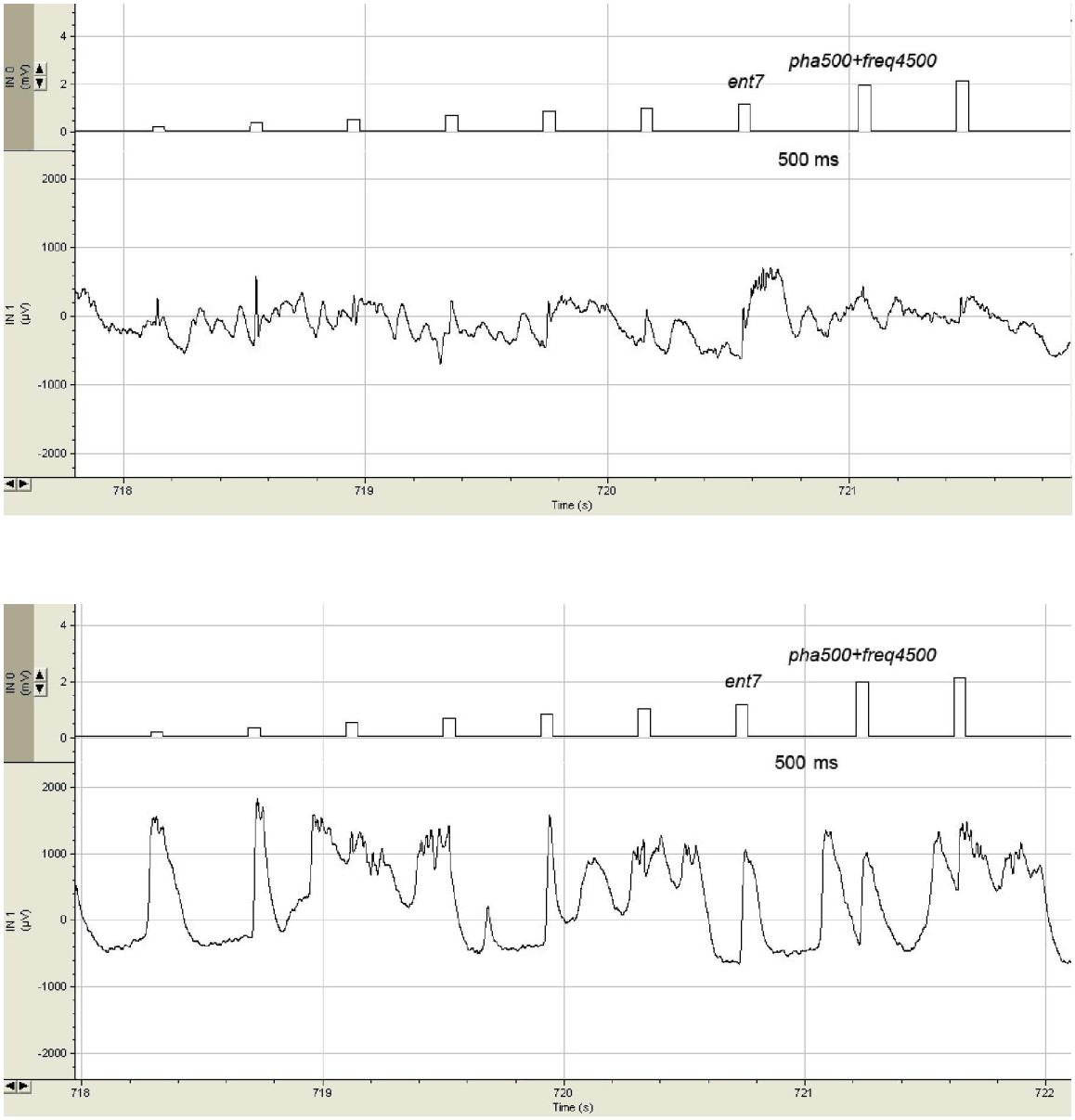

Figure 14. States of the rat viewed with Clampfit 9.2.

Top: *Complex state*. *Down*: *Up & down state*. Experiment presented was number 3 and the data shown is from approximately 12 min from the start of the experiment. *Pha500+freq4500* was the perturbation tone of 4500Hz with +1/4π phase. Note that the complex state tends to stay in [-500,500] µV zone whereas the up & down state rises above 1000 µV, having slow down periods of ~0,2 s.

4. DISCUSSION

The purpose of this Master's Thesis was to build entrainment in the auditory cortex of the rat and perturb it selectively. Two modes of perturbation were tested: phase and frequency modulation. It was hypothesized that disturbing the built entrainment would cause changes in the local field potential (LFP) responses, as measured by a microelectrode positioned over the dura. It was also believed that phase and frequency changes would differ from each other. As suspected, perturbation of auditory entrainment did cause a differing response, the direction and timeline of which depended on the mode of perturbation. Responses were also found to change both within an entraining tone train and after an entraining experiment. Results concerning perturbation experiments are discussed first, followed by results of entrainment and single tone analysis.

Effects of phase change were tested via *experiment 1* procedure. Interestingly enough, making the tone phase shorter did not have an effect, whereas making it longer caused differences when compared to the preceding entraining tone. Response to the increased phase turned more negative at approximately 60 ms and rose to more positive amplitudes at around 170 ms. This result is fascinating because it suggests differences do not ensue only because of change of phase; there seems to be some specific influence when the phase is made longer rather than shorter. Astikainen et al. (*submitted*) studied the *mismatch response* using a pseudorandom sequence with at least two standard tones between consecutive deviant tones and found out the response was observable when the inter-stimulus interval (ISI) was 375 ms but not when the ISI was 600 ms. The optimal ISI range for the rat remains to be tested, but from these combined results it would seem the ISI should be somewhere over 300 ms and under 600 ms. For future studies it is of interest that the negative change observed in this Thesis seems to be phase specific because it was not observed in the presence of frequency change alone.

The effects of frequency perturbation were tested via *experiments 2* and *4*. *Experiment 2* showed that following entrainment, frequency perturbation caused a more positive

response than the response to the previous entraining tone roughly at 90-140 ms. Similar results were also achieved in *experiment 4* when there was a 20 Hz jitter in the 2,5 Hz entrainment, suggesting that it does not matter if entrainment is at a fixed interval or if it varies semirandomly. With humans, responses to auditory stimuli of high frequency generally occur at earlier latencies than responses to low frequency stimuli (John & Picton, 2000). Perhaps because of the small frequency change or because the LFPs are extremely fast responses such latencies are unobservable in the current results. It could, however, be noted from *experiment 2* figures that the 4000 Hz tone response has a gently sloping positive peak starting at around 130 ms and the 4500 Hz tone response has a similar peak starting at 90 ms. Nevertheless it is clear that the amplitude difference is the most visible change in frequency perturbation. One could suggest the positivity to a higher frequency tone could merely be a physical property of the tone, but as *experiment 5* results are discussed this is proved to be otherwise.

Experiment 3 provided some interesting results since it combined the effects of both phase and frequency perturbation. Because of the timeline of differences and both perturbation tone responses being followed by more positive levels compared with the preceding entraining tone, it can be suggested results were more similar to frequency-only perturbation. The longer phase did have a very short period of negativity compared with the preceding entraining tone just as the tone began, again raising the question why a longer phase change implies some negative changes. There was also similarity to phase-only experiment when comparing the two perturbation tones as the longer phase change continued to be more negative than the shorter phase change. To sum up the main effect, differences tended to start at a very early stage for the shorter phase response, even before the tone presentation had ended. The positive change also lasted for a longer time with the shorter phase. This result is surprising considering the shorter phase did not differ from the entraining tone in the phase-only experiment. It would be intriguing to study more why a shorter phase causes more positive levels than a longer one.

Analysis of entrainment during a tone train suggested responses to an entraining tone change even in a short tone train of seven tones. Responses in the last half of the tone

train had more positive amplitudes than responses in the first half, starting as early as 27 ms after tone onset and lasting roughly until the next tone. It is especially surprising that the response turns more positive, as it is often thought a response stays steady over time or there is habituation or refractoriness. For example an important feature of the N1 peak of the auditory event-related potential is its systematic reduction in amplitude when the stimulus is repeated, suggested to result from a refractory process (Budd, Barry, Gordon, Rennie, & Michie, 1998). It was also interesting that a separate analysis of the first tone in the tone train showed a differing timeline of changes, the response turning more positive at around 180-200 ms and 280-290 ms. This timeline clearly differs from the perturbation effects.

Results of *experiment 2* and *4* brought up a question whether the observed positivity reflects only physical properties of the perturbation tone. Theoretically, we can think of *experiment 5* as a design where there is first the baseline no-treatment measuring, then treatment (entrainment) and lastly the second measuring. If differences between the two tones of 4000 Hz and 4500 Hz can already be seen in the baseline measuring, the effect could be a physical property of the tone. However, results revealed that at first there are no differences between the two tones. Only after an entraining experiment the perturbation tone was followed by more positive amplitudes than the entraining tone, starting at 125 ms. This suggests plasticity or "memory" in the nervous system because differences between the responses to the single tones in *experiment 5* are the same but less pronunced as in the frequency perturbation experiments. The neuronal system could keep a memory trace after its activation, in a similar way like beta rhythms work as postactivation traces (Kropotov, 2009). In opposion to the assumption that the trace is stronger for relevant stimuli, it is observed the trace is stronger when stimulus is irrelevant (cf. perturbation tone) and memory is not needed anymore. This was reflected in the results when the 4000 Hz tone response and the 4500 Hz tone response were further analyzed to see which one caused the difference after entrainment; it was indeed the perturbation tone becoming more positive at around 250 ms, not the entraining tone response becoming more negative.

In opposition, it is interesting that *during experiment 5* after an entraining experiment only the response to the entraining tone changed. The last half of the experiment became more positive for the entraining tone response roughly at 45-90 ms after tone onset. These combined *experiment 5* results could reflect a different mechanism of neuronal adaptivity between a short entrainment tone train and exposure to entrainment experiments lasting for hours. It has been suggested that a mismatch response cannot be observed in a repeated tone design, so it is a fascinating question to the field of neuropsychology whether the perturbation tone response change observed here reflects something entrainment-specific (Astikainen et al., *submitted*).

One limitation of this current study stems from the different states of the rat. If the main interest would have been different states of sleep or anesthesia, experiments of this study could have been classified strictly into *complex* or *up & down state* measurements and compared with each other. However, as the objective of this Thesis was to study perturbation and entrainment, it was seen appropriate to discard the up & down state measurements to unify the baseline local field potential responses. Discarded measurements of *experiment 3* were analyzed to give information about the up & down state and point out why the state is not optimal for analysis. The amplitude range of the up & down state was shown to be much wider than the amplitude range of complex state, and the only difference between responses was observed between the two perturbation tones. As pointed out in the results, this difference lasted only 17 ms, compared with the 160 ms of the complex state. Thus the main limitation comes from the fact that the number of measurements discarded was not the same in each experiment, states being spontaneous and unpredictable. This prevented from doing certain analyses and comparative statistics.

Since the inter-stimulus interval is suggested to be retained after conditioning and found to be a critical factor controlling the occurrence of oscillations, it can be assumed the change of inter-trial interval (ITI) from 10 s to 3,6 s has some effects (Gao et al., 2009; Cotillon, Nafati, & Edeline, 2000). As seen in the analysis of the first tone in *experiment 3*, after a silent interval even the timeline of differences between the first and second half

of presentation diverge from the difference timeline of following tones. Studying the ITI change was not, however, an objective of this Thesis. Practical difficulties arise since the high pass amplifier used in this study is 0,1 Hz, meaning that changes lasting over 1-2 s disappear from the measuring. A direct current amplifier was not seen appropriate because of large current and baseline level changes which make data analysis more difficult. Also the steel wire used was not optimal for studying slow changes. As the results of this current research are known, it would be very interesting to use the direct current and study longer changes in responses with a silver chloride electrode. Like Cotillon, Nafati and Edeline (2000) found out in their study, stimulus-locked oscillations were only present for ITIs of 2 s or longer. With thalamic neurons significant entrainment appears to be within 3-12 s (Gao et al., 2009).

It can also be considered how the age of the rat affects auditory responses. Out of the fifteen rats measured, the last six rats measured were younger than the first ones. For example transformation in the peripheral auditory system coupled with alterations in brainstem auditory pathways have been seen as electrophysiological correlates of aging (Cooper, Coleman, & Newton, 1990). It has also been suggested the majority of cells recorded from young rats respond most vigorously to fast and medium speeds, whereas slow speed is optimal for old rats (Mendelson & Ricketts, 2001). However, as both groups of rats were used for the analyses any differences observed cannot be only age-related effects.

One interesting theoretical question is whether the response changes observed in this Thesis reflect a learning process. Usually it is thought an awake state is a prerequisite for learning. However, learning modifies the responses of primary sensory cortex to sensory stimuli and this receptive field plasticity constitutes a physiological memory: modality specific experiences can be stored in primary sensory cortices (Weinberger, 1997). The primary sensory cortex can code the behavioral importance of a stimulus by increasing its magnitude of response to that stimulus; the only question is does learning *specifically* modify processing of a stimulus or *generally* increase responses to similar stimuli. In the auditory cortex it has been observed the tuning shifts to the frequency of a behaviorally

important tone during classical conditioning – is same possible for urethane-anesthetized rats when there is no behaviorally relevant reinforcer? It may be safer to use the term neural memory concerning the response changes in this Thesis since learning can be seen as a whole organism property and neural plasticity as a substrate of learning. In any case biology and learning are unavoidably intertwined, a recent example being that merely pairing a tone with stimulation of the *nucleus basalis* (provider of acetylcholine to the cerebral cortex) induces auditory memory (Miasnikov, Chen, & Weinberger, 2008).

The study of entrainment has only started to emerge recently. Although somewhat similar methods have been applied previously, the combination of stimuli and experimental design used in this Master's Thesis have never been used before. From this kind of complex animal model it may remain vague why the study of entrainment is useful, but it has far reaching consequences that the brain is biased towards a rhythmic mode and that perturbation can be used to study the structure and properties of the system (Lakatos et al. 2005; 2008, Rosanova et al., 2009, Schroeder & Lakatos, 2008; 2009). For example children are best equipped to learn about the environment when it is appropriately paced for them; attentional perspective changes with age so that the once inaccessibly "slow" will come to seem "just the right speed", and eventually even rather "fast" (Drake, Jones, & Baruch, 2000). A tempo that is "just right" for picking up speech relationships should cause rapid learning during a critical period in infant's development. Examples like these show that expanding oscillatory research to include entrainment could shed new light on brain operations and have practical applications. A challenge for future research is to clarify how and under what circumstances the nervous system creates memory traces of entrainment.

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