THE EFFECT OF HYPOTHALAMIC STIMULATION DURING TRACE CONDITIONING IN RABBITS

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Aikaisemmat tutkimukset ovat osoittaneet että pikkuaiivot ovat olennainen ja välttämätön rakenne kanien vilkkuloumiehdollistamisessa, kuin myös muissa diskreeteissä, somaattisissa lihasvasteissä. On myös osoitettu, että muut aivorakenteet ovat osallisina tietyissä puolissa tään kaltaisessa oppimisessa, vaikkakin on esitetty että ensisijainen alue muistijäljen muotoutumisessa on pikkuaivojen interpositustumakkeessa. Useat aikaisemmat tutkimukset ovat osoittaneet että hermosolujen toiminnassa tapahtuu muutoksia myös hippokampuksen soluissa, muutoksia jotka korreloivat ehdollistuneisiin käyttäytymisvasteisiin.

Erityisesti hippokampuksen on todettu olevan välttämätön trace-ehdollistumisessa. Trace-ehdollistumisessa hippokampuksen solut aktivoituvat melko heti ehdollistetun ärsykkeen alkamisen jälkeen ja aktiivisuus vähenee sen loppumisen jälkeen. Aktiivisuus ilmaantuu uudelleen ehdottoman ärsykkeen aikana ja mallintaa suuruudeltaan ja ajallisesti käyttäytymisvastetta ennen sen ilmaantumista. Kun ehdollistunut käyttäytymisvaste ilmaantuu ja lisääntyy, aktiivisuus siirtyy ehdottoman ärsykkeen ajalta aikaisemmaksi trace ajalle. Aikaisemmat tutkimukset ovat osoittaneet, että kun hypotalamuksen tietyä tumaketta (MFB) ärstetään ennen tai jälkeen ehdollistuniskäsittelyä, se vaikuttaa oppimiseen joko sitä ehkäisevästi tai lisäävästi.

Tässä tutkimuksessa monisoluuaktiivisuutta (MUA) mitattiin hippocampuksen CA1 alueelta kanien vilkkuluomiehdollistamisessa käytäen trace-asettelmaa, kun kanit samaan aikaan saivat palkitsevaa hypotalamus ärstystä joko käsittelyä ennen tai sen jälkeen. Tulokset osoittavat, että sekä käyttäytymisvasteet että hippocampuksen aktiivisuus lisääntyivät niiden käsittelyjen aikana, jolloin ehdollistuneen ja ehdottoman ärsykkeen parittaisten esittäminen jälkeen annettiin hypotalamukseksi ärstystä. Jos hypotalamuksen ärstystä annettiin ennen parittaisten ärsykeiden esittämistä, käyttäytymisvasteissa ja hippocampuksen aktiivisuudessa ei tapahtunut lisäystä. Monisoluuaktiivisuuden mitaus osoitti, että hippocampuksen CA1 solut aktivoituvat ennen käyttäytymisvasteen ilmaantumista.
ABSTRACT

Previous studies have shown that cerebellum is essential and critical in delay conditioning of rabbit nictitating membrane response as well as other discrete, somatic muscle responses. It has also been found that other brain structures are involved in some aspects of this type of learning, although it is suggested that the primary place for memory trace formation is in the cerebellar interpositus nucleus. Many earlier studies have shown that neuronal changes takes place also in hippocampus cells, changes that correlate with behavioral conditioned responses.

Especially, hippocampus has been found to be essential in trace conditioning. During trace conditioning the firing of hippocampal pyramidal cells show activity that starts shortly after conditioned stimulus (CS) onset and decays after its offset. The activity appears again in the unconditioned (UCS) period, and it models the amplitude-time course of the behavioral response before its overt appearance. When the delayed conditioned responses (CR) appear and increase, the activity shifts from the UCS period to the earlier portion of the trace period. Earlier studies show that adding of brain stimulation to hypothalamic medial forebrain bundle (MFB) before or after the conditioning treatment has either suppressing or facilitating effect on learning.

In the present study, multiunit activity (MUA) was recorded in CA1 region of the hippocampus during classical trace conditioning of the nictitating membrane response when rabbits at the same time received rewarding post- or pre-trial hypothalamic stimulation (HS). The results showed that both behavioral responses and hippocampal activity increased during sessions when paired presentation of the conditioned and unconditioned stimuli (CS and UCS respectively) was followed by hypothalamic stimulation. If the hypothalamic stimulation was given prior the paired presentation of the stimuli, there was no increase in behavioral responses and hippocampal activity. Multiunit activity recordings showed that the hippocampal CA1 cells were activated before any behavioral responses could be observed.
INTRODUCTION

Classical conditioning of the nictitating membrane (NM) or eyeblink conditioning has become a widely used and useful method for studying the neural pathways underlying learning and memory. It has appeared to be the paradigm the most appropriate to get information of brain structures and associative learning (Steinmetz, 2000).

The methods and the techniques for behavioral NM studies were first presented by Gormezano (1966). Since then the procedure has been adopted by several laboratories (Arikoski, Korhonen, Penttonen, Ruusuvirta & Wikgren, 1997; Thompson, 1990; Weiss, Kronforst-Collins, Disterhoft, 1996).

The paradigm provides stimuli that are well defined and can be precisely controlled. In addition to external eyelids, rabbits also have a third eyelid or nictitating membrane. In normal conditions the animal rarely blinks, only to protect its eye from external stimuli. This makes the responses highly controllable (Gormezano, 1966).

In this paradigm a discrete conditioned stimulus is paired with a discrete unconditioned stimulus with particular temporal relationship between CS and UCS. The UCS is an aversive stimulus (corneal air-puff) that always evokes a reflexive unconditioned response (UCR). The CS is a neutral stimulus that does not usually evoke any behavioral responses. With several pairings of the CS and the UCS, the CS alone starts eliciting a behavioral response, the conditioned response (CR). Tone is usually used as a CS and airpuff or a periorbital shock as an UCS in the conditioning of the rabbit nictitating membrane.

Due to the large amount of research the neural pathway for classical conditioning of the rabbit nictitating membrane is well known. It includes the UCS pathway, the CS pathway, the CR pathway and the UCR pathway. The UCS pathway consists of somatosensory projections to the dorsal accessory part of inferior olive and its climbing fibre projections to the cerebellum (see figure1). The CS pathway consists of auditory projections to the pontine nuclei and their mossy fiber projections to the cerebellum. The CRs are formed in cerebellum and projected through the red nucleus to motor neurons in cranial nerve nuclei, which are responsible for generating the conditioned nictitating membrane response. The accessory abducens and facial nuclei also receive input from the trigeminal nucleus. This pathway forms a trisynaptic brain stem pathway that mediates execution of the UCR. The red nucleus may also inhibit transmission of
Figure 1. A simplified scheme of the areas involved in learning the conditioned somatic defensive eyeblink response.

UCS information to the inferior olive, so that UCS activation of the climbing fibres is attenuated when a CR occurs. The essential convergence and retention site is assumed to be the interpositus nucleus in the cerebellum.

Several studies have confirmed that in addition to the cerebellum another higher brain system, the hippocampus is activated as a result of associative learning in the paradigm. The pyramidal neurons in the hippocampus change their activity as a result of paired learning. Learning results in long-lasting increases in excitability in these cells. A probable cellular mechanism for this kind of plasticity is long-term potentiation in postsynaptic neurons in CA1.

However the hippocampus does not seem to be essential in the basic classical conditioning (Solomon, Vander Schaaf, Thompson & Weisz., 1986). When animals with hippocampectomy are conditioned with long-interval trace conditioning the learning is disrupted, but their learning is at the same level as in naïve animals when delay paradigm is used (Solomon et al., 1986). On the other hand, cerebellum and its associated brain-stem circuitry does appear to be essential for the conditioning (Thompson, 1990).

Two types of paradigms can be recognized on the basis of different temporal arrangements of the CS and UCS, delay and trace. In delay paradigm the CS is
presented slightly before the UCS and they overlap. In trace paradigm CS and UCS are separated by an empty interval, trace in which neither stimulus is present.

The hippocampus has been found to play an important role in trace conditioning, which requires more complex aspects of learning. In trace conditioning, the animal must maintain an image of the CS long enough to be associated with the UCS. This interval is called the trace interval. Especially hippocampus has been found to be essential in long-interval trace conditioning, when the trace interval is as long as 500 ms (Disterhoft, Thompson & Moyer., 1994; Solomon et al., 1986; Weiss et al., 1996). The hippocampus has a time-limited information storage capability. Kim, Clark and Thompson (1995) found that the hippocampus is necessary for the retention of recently acquired, but not remotely acquired trace conditioned responses. It has also been found to be important in modulating temporal characteristics of learned behavior (Port, Mikhail & Patterson, 1986).

Several studies have confirmed that the firing of the pyramidal neurons of the hippocampus correlate highly with the amplitude–time course or shape of the behavioral CR and UCR (Berger, Rinaldi, Weisz & Thompson, 1983; Solomon et al., 1986). Multiunit activity shows bursting that spans the trace interval. The hippocampal neurons activate approximately 50 ms prior any behavioral responses. McEchron & Disterhoft (1997) used the trace conditioning paradigm and noticed that there are several stages of learning related activity in the pyramidal cells of the hippocampus in CA1 region. They found two pyramidal cells firing profiles and suggested that these might be involved in assessing temporal properties of the CS-UCS conditioning trial.

When a third stimulus is presented, in addition to the CS and UCS, it can, depending on the nature of the stimulus produce either facilitation or retardation in the development of the association between the CS and the UCS (Arikoski et al., 1997). This stimulation can be applied either before or after the CS-UCS pairing. The most frequently studied brain stimulation reward sites are those of the diencephalic medial forebrain bundle (MFB) (Wise & Rompre, 1989). Stimulation to this site produces strong reward, without any motoric or aversive side effects (Wise & Rompre, 1989). Other stimulation is traditionally compared to the stimulation to lateral medial forebrain bundle (Wise & Rompre, 1989). The MFB is the main pathway for the ascending dopamine fibers, and there is important dopaminergic innervation of the habenula, a way-station in the dorsal diencephalic bundle. Early studies have shown that electrical
stimulation of several regions of the forebrain is rewarding. These are the regions innervated by dopaminergic fibers (Wise & Rompre, 1989).

The first purpose of this experiment is to find out whether learning-related changes could be found in the hippocampus CA1 region using the multunit activity recordings and what is the relation of the hippocampus CA1 cells activity to the behavioral responses observed. The hypotheses is that MUA changes during conditioning sessions and that the hippocampal cells show activity that correlates with the amplitude-time course of the nictitating membrane conditioned responses. The second purpose is to find out whether a rewarding hypothalamus brain stimulation has a facilitating or retarding effect on the learning procedure, here on the trace conditioning. The hypotheses is that the rewarding brain stimulation has an effect on the learning procedure.

METHOD

Subjects
The subjects were eight New Zealand albino rabbits (Oryctolagus cuniculus) weighing 2.8–3.4 kg at the time of the surgery. The animals were individually housed in a temperature- and humidity controlled metal cages, on a 12:12 hr light-dark cycle with free access to food and water. All the experiments were carried out during the daylight portion. The animals were taken care of by the experimenters, staff and veterinarians of the University of Jyväskylä. Experiments were carried out in accordance with the EU Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures.

Surgery
The animals were anesthetized with i.m. injections of a ketamine-xylazine cocktail (Ketalar®, 50 mg/ml; Rompun®, 20 mg/ml, physiological NaCl ad 10 ml). The initial dosage was 3–4 ml and the anesthesia was maintained by additional injections of 2 ml every 20–40 min. The eyes were treated with Oftan® to prevent infections and dryness during the operation. After a deep general anesthesia had been achieved, the animals were placed in a stereotaxic instrument (Kopf Instruments) with bregma 1.5 mm above lambda. A longitudinal incision was made to reveal the skull onto which the headstage (designed to hold the minitorque potentiometer, an airpuff delivery nozzle and tone
tubing) was cemented using four stainless steel anchoring screws. After drilling a hole over the cerebral cortex, three Teflon insulated, stainless steel stimulation electrodes (100 μm exposed tips) were lowered stereotaxically into the lateral hypotalamus (β1.0, R2.0, H-6.0; λ0.0, R2.0, H-5.0 and λ1.0, R2.0, H-4.0). Two recording electrodes were implanted into CA1 region of the hippocampus (β5.0, R5.0, H+6.0; β5.0, R6.0, H+5.5) and two recording electrodes into cerebral cortex regions N VI and VI (λ1.0, R6.0; β8.0, R6.0). The electrodes were implanted using stereotaxic atlases of the rabbit brain and their final depths were determined by observing the characteristic activity on the oscilloscope.

The electrodes were connected to two 15-pin D-type connectors that were cemented onto the skull with dental acrylic together with four anchoring screws. The implantation of the electrodes was done by the pressure fitting method developed by Korhonen (1991). Also a headstage designed to hold a minitorque potentiometer, an air puff delivery nozzle and tone tubing were attached to the complex.

Finally, a small loop of 4-0 nylon thread was sutured at the nictitating membrane of the right eye. Analgetic (Temgesic® 0.02 mg/ml) was given s.c. at the end of surgery and every eight hours for the following 48 hours. The subject were given at least one week to recover after surgery before the actual experimental procedures.

**Experimental apparatus**

The apparatus and methods used are basically those described by Gormezano (1966). The animals were restrained in a ventilated, sound-attenuating and electrically shielded Plexiglas box. Adjustable ear plate and ear clamp were securing the head and a second plate placed over the animals back to restrict general movement. The NM movement was measured by attaching a small nylon loop saturated to the NM of the animal to a stainless steel wire and connected to the headholders minitorque potentiometer.

Head movements were recorded with a solid-state piezoresistive accelerometer (ICSensor, 3021-002-P) and videotaped for later analysis. The sessions were observed by means of a video monitor. The data were collected by an IBM compatible computer and the experiment was controlled by another computer.

The tone was directed to the rabbits left ear by a tone generator placed outside the training box. The airpuff was delivered through a tube attached to the animal’s headgear.
Behavioral training procedures

Each animal was observed in the chamber for the purpose of determining the behavioral threshold current levels of the HS electrodes and reassuring that the electrode and current level selected for conditioning would not induce struggling while under restraint. The HS consisted of electrical stimulation of the medial forebrain bundle at a pulse width of 0.5-ms, a train duration of 250-ms, a stimulus intensity typically of 100-250 μA, and a pulse frequency of 100Hz. Stimulation typically resulted in heightened exploratory activity. In the present study, a current level inducing orienting and approach movements, without any aversive effects, was selected.

The CS was a 50-ms tone (1 kHz, 78 db SPL) delivered through a plastic tube placed in front of the left ear at a distance of 1 cm. The UCS was a 100-ms air puff (2.1 N/cm²). Before training the animals were adapted to the experimental situation by placing them in Plexiglas restraining box in the sound-proof conditioning chamber for two 45-min adaptation sessions.

The first three daily sessions consisted of 60 unpaired stimulus presentations. CS, UCS and HS were presented pseudorandomly. The five daily trace conditioning sessions followed the three unpaired sessions. First five sessions consisted of four different types of trials; 60 CS-UCS paired trials followed by a 250 ms train of HS, 10 CS-only trials, 10 UCS-only trials, and 10 HS-only trials. The order of HS presentation was reversed after these first five conditioning sessions so that the animals received the HS before the CS-UCS pairing.

In the trace paradigm CS offset preceded UCS onset creating 200 ms stimulus-free period, trace interval. The interstimulus interval was 50 ms. The intertrial interval was randomly ranging from 30-50 s.
Figure 2. Schematic illustration of the training procedures. a) Poststimulation trial. b) Prestimulation trial.

Conditioned responses were defined as any 0.5 mm or greater NM movement starting after CS but preceding UCS. The UCR was defined as any NM movement greater than 0.5 mm starting after UCS.

Trials, during which the NM-movement exceeded 0.5 mm prior to the CS onset were excluded from the analysis.

Histology
After the experiments, the animals were given a lethal dose of pentobarbital (10-15 cc i.m. and i.v.) and then perfused intra-aortically with saline followed by 10 % formalin. The brains were removed and fixed in formalin-sucrose solution for at least one week. Frozen coronal sections of 100 μm were taken from the sites of the electrodes. Slices were mounted on gelatinized slides and stained with cresyl violet. The locations of the electrodes were determined according to the stereotaxic atlas. The placement of electrodes can be seen in figures 3 and 4 (see appendix 1 and 2).

Data analysis
The signal analysis was based on a 1500 ms sampling period. The data was collected by using BRACE® computer program. SPSS for Windows 8.0 was used for all numerical processing, ANOVA for repeated measures and Paired Samples t-tests. Two channels
from each animal for recording multiple unit activity in the hippocampal regions CA1 were chosen for analysis. The MUA recordings from the subject 39 were excluded from the analysis, because the recording electrodes were not in the CA1 region in the hippocampus.

Two parts of the interstimulus interval (ISI) period were chosen for evaluation of the effect of trace conditioning. The CS1 part was the time period 100 ms after the onset of the CS and CS2 was the time period 100 ms before the onset of the UCS.

The MUA was band-pass filtered (500-6000 Hz) and digitized at the rate of 15000 samples/s with a microcomputer. Frequencies of spikes were calculated using a custom-programmed DTVee for Windows program. After setting a threshold for amplitude of spikes, the spike frequency exceeding this threshold was counted per 10 ms bin. Subsequently, standard scores of each bin were computed (activity of a bin minus 100 ms pre-stimulus activity (average of 5 bins) divided by the standard deviation of pre-stimulus activity over the averaged trials).

RESULTS

Behavioral results

![Nictitating membrane response](image)

Figure 5. Behavioral data
The behavioral data are shown in figure 5. During unpaired sessions there was no increase from the first unpaired session to the third unpaired session. It was found that during the pre-trial HS procedure there was no increase in CRs, while during the post-trial HS procedure there was an increase in the nictitating membrane responses.

The results showed a significant increase in the rabbit nictitating membrane response during post HS trace conditioning sessions \( F(1.2,1.3) = 6.166, p < 0.05 \). While the changes were not statistically significant during pre HS conditioning sessions. The results of t-test showed that there is a significant difference between the first and last post HS sessions [t-test \( p < 0.05 \)]. The difference was not significant comparing the first and last pre-trial sessions.

*Electrophysiological results*

*Figure 6. Electrophysiological data.*
The electrophysiological data from CA1 are shown in figure 6. During the unpaired sessions there was no increase in the multi-unit activity from the first unpaired session to the third unpaired session during either CS1 or CS2 parts. Comparing the first and last unpaired sessions, the difference was not significant either in the CS1 or CS2 parts.

The results showed that there was an increase in the activation of the hippocampus CA1 cells during acquisition of the tone CS2 at the post HS sessions, while the firing of the hippocampus cells did not change during the pre HS sessions. The interaction of hypothalamus stimulation (post vs. pre) and conditioning sessions was significant [F(1.5,19.6) = 5.825, p < 0.05] (see figure 6). The results showed a significant conditioning session effect [F(1.9,24.6) = 3.926, p < 0.05]. The MUA activity showed significant change over sessions. The results showed a significant difference between the first and last post HS sessions during CS2 part [t-test, p < 0.05]. A significant difference was also found between the first and last pre HS sessions during CS2 period [t-test, p < 0.05]. Interaction of stimulation and conditioning effects had a significant linear trend [F(1,13) = 7.697 p < 0.05] (see figure 7).

![Graph showing MUA activity over sessions](image.png)

**Figure 7.** Post and pre trial HS sessions during CS2 part.
The interaction of hypothalamus stimulation (post vs. pre) and conditioning sessions was significant [F(1.8,23.3) = 5.214 p < 0.05] during CS1 part (see figure 6). The results of multiple unit activity recordings showed that during acquisition of the tone CS1 part the conditioning session effect was not significant. The t-test result showed a significant difference between the first and last pre HS sessions during CS1 part [t-test, p < 0.05]. Interaction of stimulation and conditioning had a significant linear trend [F(1, 13) = 5.273 p < 0.05] and a significant quadratic trend [F(1,13) = 6.842 p < 0.05] (see figure 8).

![Graph showing MUA vs Sessions](image)

Figure 8. Post and pre trial HS sessions during CS1 part.
The t-test result show that the difference was not significant between the first and last post HS sessions during CS1 part.
DISCUSSION

This study shows that learning related changes can be found in the MUA of the hippocampus CA1 cells. When the unpaired sessions were compared to the trained sessions an ascending curve was found at the CS2 part during post-trial HS sessions. In contrast, when the HS preceded the CS-UCS pairing, the curve was slightly descending. When comparing the MUA results to the behavioral results, it can be seen that the hippocampus cells activity precedes the behavioral responses.

Earlier studies have shown that hippocampal responses to the CS always precede behavioral responses in latency from stimulus onset (Thompson, 1990). During trace conditioning, when the CRs appear the activity shifts to earlier in trace interval and forms a model of amplitude time course of the behavioral CR (Solomon et al., 1986; Weiss et al., 1996). The same tendency can be seen in the present study, the hippocampal neurons in the CA1 regions showed activity before any overt behavioral responses occurred. Later in training when the behavioral CRs emerge the neural activity shifts to earlier in the trace interval modelling the time-amplitude course of the behavioral CR (see appendix 3, figures 9 & 10).

Laroche, Falcou and Bloch (1983) compared multi-unit activity changes in rats dentate gyrus and entorhinal cortex using a mild post-trial stimulation of the mesencephalic reticular formation. Multiunit activity increased in response to an auditory signal after pairing the signal with a footshock in dentate region but not in entorhinal cortex (Laroche et al., 1983). Thus post-trial stimulation facilitated the development of associative changes in dentate multiunit activity as in the present study.

Arikoski et al. (1997) compared the results of classically conditioned rabbits in delay paradigm receiving rewarding electrical stimulation of hypothalamus to the rabbits conditioned without this stimulation. In their study they found that the rewarding electrical stimulation of hypothalamus given after the paired trials enhanced CR performance, the animals receiving the stimulation showed faster learning than the control group (Arikoski et al., 1997). It also facilitated learning related changes in hippocampal cells while using delay paradigm (Arikoski et al., 1997). When the rewarding stimulation was given prior the paired trials both the behavioral responses and the hippocampus activity showed no learning related responses (Arikoski et al., 1997). The increase in the CR was also much larger in the control group that did not
receive any hypothalamus stimulation than in the prestimulation group (Arikoski et al., 1997). Their second phase of the study was to reverse the post-trial and pre-trial treatments (Arikoski et al., 1997).

In the present study trace paradigm was used and the animals received first the post-trial treatment and then the procedure was reversed to pre-trial treatment. The results of this study showed the same tendency as seen in the delay conditioning study of Arikoski et al. (1997). As in the previous study it can be seen that on the first day of conditioning the mean responses stay close to zero mm. The results from the following sessions are also comparable to the results found by Arikoski et al. (1997). During the third day of post-trial treatments there can be seen an increase in the NM responses which continues to the fifth day. When reversing the treatment to pre-trial procedure, on the first day the responses stay at the same level as on the last post-trial treatment day and the responses decline on the third and fifth training days. There does not seem to be large differences between the behavioral results in these two studies despite the different conditioning procedures.

The multi-unit activity measures showed that during the post-trial sessions the MUA grew rapidly in the post-trial HS sessions. Arikoski et al. (1997) study shows the same result. In the present study the MUA was recorded also following reversal of the post-trial HS treatment to pre-trial HS treatment. The results showed that after reversal the multi-unit activity decreased.

How the brain stimulation to lateral hypothalamic area might have an effect on hippocampal learning? Gasanov, Kasimov and Bagirova (1989) have tested the hippocampal neuronal activity using electrical, cholinergic and monoaminergic hypothalamus stimulation (Gasanov et al, 1989). They found that the hippocampal theta-rhythm caused by the hypothalamic stimulation is completely and irreversibly blocked when the stria terminalis is lesioned (Gasanov et al., 1989). They suggest that there are interrelations from the amygdala and hypothalamus to the hippocampal theta activity (Gasanov et al., 1989). Thus the hypothalamic stimulation might have an effect to the hippocampus activity through these connections.

Anyway, in the present study the hypothalamic stimulation seemed to have an effect on learning. It would be interesting to compare the results to a control group that only receives the trace conditioning treatment without any hypothalamic stimulation. This
arrangement makes it possible to evaluate how effectively the post HS stimulation facilitates learning using trace conditioning paradigm.
REFERENCES


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The Mesolimbic Dopamine System: From Motivation to Action, John Wiley & Sons Ltd., USA
Figure 3. Placement of hypothalamic stimulation electrodes.
Figure 4. Placement of hippocampus recording electrodes.
APPENDIX 3.

Figure 9. MUA from first post HS trial. Tone CS is delivered at 250 ms and airpuff UCS at 500 ms.

Figure 10. MUA from last post HS trial. Tone CS is delivered at 250 ms and airpuff UCS at 500 ms.