RABBIT CEREBELLAR EVENT-RELATED POTENTIALS DURING CLASSICAL NICTITATING MEMBRANE CONDITIONING

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KANIN PIKKUAIVOJEN HERÄTEPOTENTIAALIT KLASSISESSA VILKKULUOMIEHDOLLISTAMISESSA

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Aiemmat tutkimukset ovat osoittaneet pikkuaivojen olevan olennaisesti mukana muistijäljen synnyssä klassisessa vilkkuluomiehdollistamisessa. Moni- ja yksisolumittauksissa on löydetty oppimiseen liittyviä soluaktiivisuuden muutoksia sekä pikkuaivokuorelta että pikkuaivojen syvätumakkeista. Tässä tutkimuksessa mitattiin herätevasteita kanin pikkuaivokuorelta ja interpositus-tumakkeesta oppimisen aikana. Kaneille esitettiin ääni- ja ilmapuhallusärsykkeitä ensin toisiinsa liittymättä ja sen jälkeen pareittain niin, että ääni alkoi 250 ms ennen ilmapuhallusta ja ärsykkeet päättyivät yhtä aikaa. Äänen synnyttämiä herätevasteita verrattiin tilastollisesti eri sessioiden välillä. Herätevasteiden analysointiin käytettiin myös pääkomponenttianalyysiä. Tulokset osoittavat, että oppimiseen liittyviä muutoksia herätevasteissa tapahtuu jo ensimmäisen ehdollistamissession aikana niin, että sekä pikkuaivokuorella että interpositus-tumakkeessa välitön reagointi ehdolliseen ärsykkeeseen, ääneen, muuttuu. Pikkuaivokuorella myös myöhempi aktiivisuus muuttuu jo ehdollistamisen alussa. Interpositus-tumakkeessa syntyy ehdollistamisen aikana positiivinen vaste, joka on yhtäaikainen vilkkuluomen opitun liikkeen kanssa. Tulokset tukevat aiempaa teoriaa, jonka mukaan pikkuaivokuorella on tärkeämpi osuus oppimisen alussa kuin myöhemmässä vaiheessa. Interpositus-tumakkeen aktiivisuus taas liittyy opitun vasteen suorittamiseen.

Rabbit Cerebellar Event-Related Potentials during Classical Nictitating Membrane Conditioning

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Abstract

Cerebellum has proved to be essential in classical conditioning. Multiple and single unit activity recordings from the cerebellar cortex and nuclei have shown that some cells of these regions form time-amplitude models of learned response and some seem to encode CS and US. In this study, event-related potentials recorded in the CS-period from rabbit interpositus nucleus and HVI-lobule of cerebellar cortex were examined instead of unit activity. Comparisons of averaged ERP-waveforms and principal component factor scores show that in both interpositus nucleus and cerebellar cortex CS related early latency activity changes already in the first conditioning session, before any behavioural sign of learning. In the cerebellar cortex also amplitude from 120 to 160 ms decreases at the beginning of the conditioning. During conditioning a deflection reflecting the NM movement before the US is formed in the interpositus nucleus. Supporting the theory based on earlier MUA and single unit studies it is concluded that the cerebellar cortex is more involved in the acquisition than in the retention of the NM response whereas the interpositus nucleus activity is related to acquisition and performance of the CR.

Keywords: Eyeblink classical conditioning, Interpositus nucleus, Cerebellar cortex, Event-related potentials (ERP), Principal component analysis (PCA)

1. Introduction

Rabbit eye blink/nictitating membrane (NM) conditioning has been a widely used paradigm in purpose to determine the neural circuits of basic associative memory (e.g. Steinmetz, 2000; Thompson & Krupa, 1994). In conditioning, an unconditioned stimulus (US), usually an air puff directed to eye or a periorbital shock is paired with neutral conditioned stimulus (CS), usually a tone or a light. The US elicits an unconditioned response (UR) without training: closure of eye and a movement of nictitating membrane. After training, the CS starts to elicit response (conditioned response, CR) as well. In delay conditioning paradigm CS and US are presented temporally overlapping. Essential brain circuits involved in eye blink conditioning include a CS pathway that carries information from auditory nuclei through pontine nuclei to both interpositus nucleus and cerebellar cortex, a US pathway that carries information through inferior olive to both interpositus nucleus and cerebellar cortex and through reticular formation to cranial motor nuclei causing UR. CR pathway runs from interpositus nucleus through red nucleus to cranial motor nuclei causing CR (Lavond, Kim & Thompson, 1993; Steinmetz, 2000; Thompson & Krupa, 1994).

The cerebellum is essential for the formation and storage of the eye blink engram in simple delay conditioning paradigm. Results of earlier studies suggest that the interpositus nucleus activates

brainstem nuclei that generate the CR whereas the cerebellar cortex modulates interpositus excitability changes affecting amplitude and timing of the CR (Gould & Steinmetz, 1996; Perrett, Ruiz & Mauk, 1993). The cerebellar cortex seems to be important in the initial learning of the conditioned response but not in maintaining the memory trace (McCormick & Thompson, 1984; Garcia, Steele & Mauk, 1999; Clark, Zhang & Lavond, 1997). However, the role of the different regions of the cerebellum is by no means clear (e.g. Steinmetz, 2000; Thompson & Krupa, 1994).

The most convincing evidence for the interpositus nucleus of the cerebellum as a primary location of the memory trace comes from lesion and especially reversible lesion studies. Permanent lesion of the interpositus nucleus neurons causes permanent loss of the conditioned eye blink response (Steinmetz, Logue & Steinmetz, 1993; Katz & Steinmetz, 1997). Reversible cooling of the interpositus nucleus prevents learning in naïve animals and in trained animals abolishes learned responses (Clark et al., 1992, 1997). Multiple and single unit studies have shown, that a neural model of the CR is formed in the interpositus nucleus cells during training (McCormick, Clark, Lavond & Thompson, 1982; Clark et al., 1992; Gould & Steinmetz, 1996). Neuronal responses of dentate-interpositus nuclei increase in close relation to the size of the CR (McCormick & Thompson, 1984). In single unit studies cells have been found that respond in relation to either CS, CR or both (Berthier & Moore, 1990; McCormick & Thompson, 1984). During extinction the CS-period interpositus nucleus activity disappears as CRs disappear (Gould & Steinmetz, 1996). Inactivation of the nucleus abolishes also learning related neural activity (Clark et al., 1992).

Also cerebellar cortex lesions have been made but the results are somewhat contradictory. Cerebellar cortex lesions (Perrett et al., 1993) and a pharmacological block of cerebellar cortex output (Garcia & Mauk, 1998) have been reported to disrupt the timing of the CR and reduce the amplitude of the response. Inactivation of only HVI -lobule of the cerebellar cortex has been reported to have no or only little effect on conditioning (Woodruff-Pak, Lavond, Logan, Steinmetz & Thompson, 1993; Garcia et al., 1999), to prevent the acquisition of the CR (Attwell, Rahman & Yeo, 2001) and to retard learning (Lavond & Steinmetz, 1989; Clark et al., 1997). Inactivation of anterior lobe of the cerebellar cortex has been reported to prevent acquisition of the conditioned response, to disrupt earlier learned timing of the CR and to prevent extinction of previously learned responses (Garcia et al., 1999). A neural model of the CR is formed also in the cerebellar cortex (McCormick et al., 1982; McCormick & Thompson, 1984; Gould & Steinmetz, 1996). The onset of the neuronal responses within the cerebellar cortex seems to be related to the onset of the CR (McCormick & Thompson, 1984; Berthier & Moore, 1986). Some cells decrease their activity (Berthier & Moore, 1986). The activity in the HVI-lobule is present even after CRs are extinguished. Activity in the HVI-lobule also changes systematically during backward and unpaired training, which does not happen in the interpositus nucleus (Gould & Steinmetz, 1996). Learning related changes of the cerebellar cortex seem to be dependent on the normal functioning of the interpositus nucleus at least to some extend. Learning related activity occurred in the HVI-lobule of the cerebellar cortex in a disorganized manner when the interpositus nucleus was inactivated (Katz & Steinmetz, 1997). In another study inactivation of the interpositus abolished learned CR-related activity in the cerebellar cortex leaving CS-related activity present (Clark et al., 1997).

Earlier studies have concentrated on multiple and single unit activity. However, learning related changes have also been found in the interpositus nucleus when event-related potentials (ERPs) averaged from electroencephalography (EEG) were studied (Loikkanen & Huovila, 1999). A positive deflection was found at the second half of 250 ms ISI presumably reflecting the learned NM movement. When interpositus nucleus was reversible inactivated this deflection disappeared. During conditioning changes were also found in shorter latency, that were not altered by interpositus inactivation (Loikkanen & Huovila, 1999). Eye blink conditioning paradigm is used also in human study and the cerebellum seems to be involved in learning also in humans (e.g. Schugens, Topka & Daum, 2000). In humans EEG recorded on the surface of the scull can be used

as a research measure. The results gained with EPR research in animals can thus be better compared with human studies in relation to the results of the multiple and single unit studies.

The aim of this study is to further investigate the changes that can be found in rabbit cerebellum ERPs and the role of the interpositus nucleus and the cerebellar cortex in the acquisition of the NM response. Rabbits receive first unpaired presentations of tone CS and air puff US and then the stimuli paired according to the delay paradigm. Usefulness of principal component analysis (PCA) in determining the latencies of variability in the ERPs is evaluated and compared with more traditional ERP research method. It is expected that the results are consistent with the earlier ERP study on the interpositus nucleus (Loikkanen & Huovila, 1999) and unit activity studies on both the interpositus nucleus and the cerebellar cortex (McCormick et al., 1982; Clark et al., 1992; Gould & Steinmetz, 1996; McCormick & Thompson, 1984; Berthier & Moore, 1986, 1990). Some differences between the interpositus nucleus and the cerebellar cortex ERPs can be presumed.

2. Methods

2.1. Subjects

The subjects were eleven male New Zealand albino rabbits, weighing 2.7-3.7 kg at the time of the surgery. The animals were housed individually in metal cages and maintained on 12/12 h light/dark cycles with free access to food and water. All the experiments were done during the light portion of the cycle. The experiments were carried out according to the European Communities Council Directive (86/609/EEC) regarding the care and use of animals in experimental procedures.

2.2. Surgery

Anaesthesia was accomplished and maintained with intramuscular injections of ketamine-xylazine cocktail (2.4 ml ketamine, 50mg/ml; 0.8 ml xylazine, 20mg/ml; 0.8 ml NaCl). After the deep general anaesthesia had been achieved, the animals were placed in a stereotaxic headholder (Kopf Instruments) with bregma 1.5 mm above lambda and implanted with recording electrodes into interpositus nucleus and cerebellar cortex (lobule HVI). The coordinates were (λ +0.5 mm, R 5.0 mm) and (λ -0.5 mm, R 5.0 mm). Electroencephalography was monitored during the implantation procedure and the final depths were determined by observing the characteristic activity of these regions. For additional experimental purposes, the rabbits were also implanted with other recording electrodes, stimulation electrodes and a cooling probe, that were not used in this study.[1] The electrodes were implanted using stereotaxic atlas of the rabbit brain (Shek, Wen & Wisniewski, 1986). The electrodes were connected via wires to a socket cemented to the skull with dental acrylic together with anchoring screws. A nylon loop was sutured into the NM of the right eye. After surgery postoperative analgesics (Temgesic® 0.3 mg/ml) were given if needed and animals were allowed to recover for at least one week before training.

[1] Four of the animals had recording electrodes in lateral pontine nucleus (β +8.0mm, L 2.5mm), CA1 region of hippocampus (β -5.0mm, R 5.0mm); three hypothalamic stimulation electrodes in lateral hypothalamus (β -1.0mm, R 2.0mm), (β +0.0mm, R 2.0mm), (β +1.0mm, R 2.0mm) and a interpositus cooling probe (λ +0.5mm, R 6.5mm)
Five of the animals had recording electrodes in lateral hypothalamus (β -1.0mm, R 2.0mm), (β +0.0mm, R 2.0mm), (β +1.0mm, R 2.0mm), somatosensoral cortex (β -2.0mm, L 5.0mm and somatosensoral skull (β -2.0mm, L 7.0mm) and a interpositus cooling probe (λ +0.5mm, R 5.0mm)
Two of the animals had recording electrodes in hippocampus (β -5.0mm, R 4.0mm), (β -5.0mm, R 5.0mm), (β -5.0mm, R 6,0mm), prefrontal cortex (β +4.0mm, L 0.8mm), (β +5.0mm, L 0.8mm) and in lateral hypothalamus (β -1.0mm, R 2.0mm), (β +0.0mm, R 2.0mm)

2.3. Training procedure

Training occurred in a standard Plexiglas rabbit restrainer (Gormezano, 1966) located in ventilated, sound-attenuated and electrically shielded chamber. Before training rabbits were adapted to the apparatus.

CS was a 350 ms (1 kHz 85 dB) sine wave tone and US was a 100 ms (2.1 N/cm² pressure at the source) air puff directed to the right cornea. The rabbits received first an unpaired control phase that consisted from five to seven daily sessions. Each unpaired session contained 70 + 70 pseudorandomly arranged CS alone and US alone trials. A paired phase of from five to ten daily sessions began after the unpaired phase. Each conditioning session contained 60 CS-US paired trials, 10 CS alone and 10 US alone trials (together 80 trials). In the CS-US paired trials the CS preceded the US by 250 ms, coterminating. Inter-stimulus interval (ISI) was thus 250 ms (onset to onset). The inter-trial interval ITI) varied randomly between 20 – 40 s (M = 30 s) both in the unpaired and paired phases.

The voltage changes were amplified, band pass filtered at 0.1-200 Hz and fed to an A/D converter. Sampling rate was 500 samples per second. The NM movement was measured by linking the NM loop to the wiper arm of a minitorque potentiometer. The extension of the NM was traduced to a voltage change by the potentiometer (1 mm movement equalled 1 V). Any 0.5 mm or greater extension of the NM following CS onset but preceding US onset in CS-US paired trials and following the CS within 250 ms in CS-alone trials was defined as conditioned response.

2.4. Histology

After the experiments, the rabbits were anaesthetised and given intravenously a lethal dose of sodium pentobarbital. Perfusion was done intra-aortically with NaCL followed by 10% formalin. The brains were removed, post-fixed and frozen-sectioned (100 µm slices). The sections were stained with cresyl violet and examined microscopically to determine electrode positions using stereotaxic atlas (Shek et al., 1986).

2.5. Data analysis

The data was gathered by using BRACE© computer program. Statistical tests were performed with SPSS 11.0 or 12.0 for Windows. The first and the last sessions of the unpaired and paired phases were selected to the analysis. For computer technical reasons not all trials were recorded during the unpaired phase with some animals. Therefore the data from the first two and the last two unpaired sessions were used in case of these animals in order to get more data. The baseline period was a 100 ms interval before the CS onset. The mean value of this interval was subtracted from all successive time points. Trials with NM movement greater than 0.5 mm in 100 ms period before the CS onset were defined as bad trials and were extracted from the analysis.

To examine learning on the behavioral level the percentage of trials with CR (CR%) was calculated in each session and changes in percentages were tested with paired t-tests. Also changes in NM maximum amplitude between sessions were examined with paired t-tests.

Averaged ERPs of different animals, electrode locations and sessions were treated as the cases. Thus there were 88 cases altogether (11 x 2 x 4). The behavioral and neural data collected from the beginning of the CS to the beginning of the US were used in this study, leaving time period of 250 ms (125 time points). The number of variables was reduced for principal component analysis (PCA) by calculating means of five successive time points (van Boxtel, 1998). These 25 variables were used as an input to PCA, which was first performed using correlation matrix to determine the number of factors (van Boxtel, 1998). Covariance matrix was then used to extract four principal components. Varimax criterion was used to rotate the initial covariance matrix (van Boxtel, 1998).

In this study, factor loadings greater than 0.7 or smaller than -0.3 were used to identify the region of variability in each of the factors for descriptive purposes. The factor scores extracted from PCA were entered to multivariate analyses of variance (MANOVA) for repeated measures.

In order to compare the results gained with PCA to more traditional ERP research subject averaged ERPs were also analysed by comparing amplitudes between all sessions in each 125 time points with paired t- tests. The level of significance of 0.05 is used in this study, but also results with significance smaller than 0.1 will be reported in order to better compare the results gained with different methods.

3. Results

3.1. Histology

Histology confirmed that the recording electrode tips were located in the HVI-lobule of the cerebellar cortex and in the vicinity of the interpositus nucleus in all of the animals.

3.2. Behavioral results

During the conditioning phase, the percentage of trials with a CR increased from 10 to 86 ($t = 17.68$, $p < 0.001$). The maximum NM extension increased from 0.15 to 2.47 mm ($t = 5.10$, $p < 0.001$). Conditioning generated thus a robust NMR that started about 100 ms after the CS and reached 0.5 mm criterion at 150 ms. During the unpaired phase CR% increased from 1 to 6 ($t = 3.41$, $p < 0.01$) and the maximum NM extension from 0.02 to 0.11 mm ($t = 2.30$, $p < 0.05$). These changes are too small to be considered as learning but rather pseudoconditioning. Averaged NM responses are presented in Figure 1.

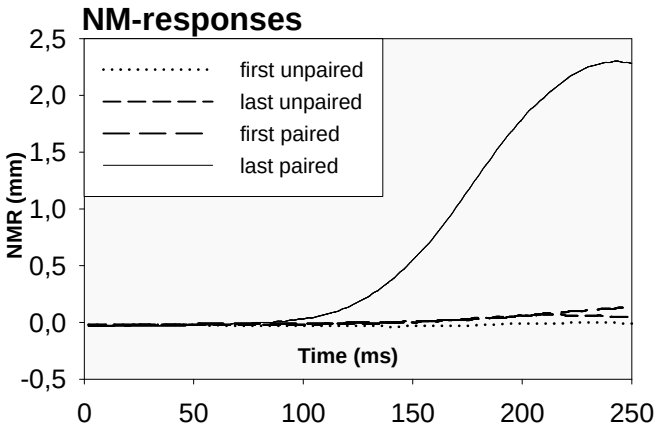

Figure 1. Averaged NM movements in all sessions in time period from the onset of the CS (0 ms) to the onset of the US (250 ms)

3.3. ERP results

3.3.1. T-tests on averaged ERP waveforms

Interpositus nucleus. Averaged ERP waveforms recorded from the interpositus nucleus are presented in the left hand side of Figure 2. A t-test comparison of the 125 time points of the initial ERPs revealed differences between the first and the last paired sessions in time period from 181 to 250 ms (181 – 250 ms: $t = 1.87$ - 10.24 , $p < 0.1$; 192 – 250 ms: $t = 2.57$ - 10.24, $p < 0.05$; 201 – 246 and 249 – 250 ms: $t = 2.88$ - 10.24, $p < 0.01$). No difference was found between the two sessions of the unpaired phase. Between the last unpaired and the last paired sessions differences were found in time period from 187 to 250 ms (187 – 192 and 195 – 250 ms: $t = 1.84 – 3.70$, $p < 0.1$; 201 – 204 and 207 – 250 ms: $t = 2.28$ - 3.70, $p < 0.05$; 217 – 218 ms, 231 – 236 and 239 – 240 ms: $t = 3.29$ - 3.70, $p < 0.01$). Between the first unpaired and the last paired sessions differences were found in time periods from 33 to 36 ms ($t = 1.89$, $p < 0.1$) and from 185 to 250 ms (185 – 250 ms: $t = 1.88 – 3.21$, $p < 0.1$; 201 – 202 and 207 – 250 ms: $t = 2.24 – 3.21$, $p < 0.05$). Between the last unpaired and the first paired sessions changes were found in time period from 18 to 32 ms (17 –

24, 27 – 28 and 31 – 32 ms: t = 1.92 - 2.60, p < 0.1; 19 – 20 ms: t = 2.60, p < 0.05). (See Figure 4 and Table 2 for summary.)

Cerebellar cortex. Averaged ERP waveforms recorded from cerebellar cortex are presented in the right hand side of Figure 2. The first and the last sessions of the paired phase differed marginally in time period from 51 to 56 ms (t = 1.91 – 2.12, p < 0.1). No difference was found between the two sessions of the unpaired phase. Between the last unpaired and the last paired sessions waves differed in time periods from 51 to 64 ms (t = 1.9 – 2.19, p < 0.1) and from 115 to 158 ms (115 – 140, 143 – 154 and 157 – 158 ms: t = 1.82 – 3.45, p < 0.1, 117 – 118, 121 – 136, 139 – 140, 143 – 144 ms: t = 2.29 – 3.45, p < 0.05; 127 – 128 ms: t = 3.45, p < 0.01). Nearly significant changes were found between the first unpaired and the last paired sessions in time period from 53 to 64 ms (53 – 60, 63 – 64 ms: t = 1.94 - 2.2, p < 0.1) and between the last unpaired and the first paired sessions from 123 to 138 ms (123 – 124, 127 – 130, 135 – 138 ms: t = 1.82 - 2.04, p < 0.1). (See Figure 5 and Table 2 for summary.)

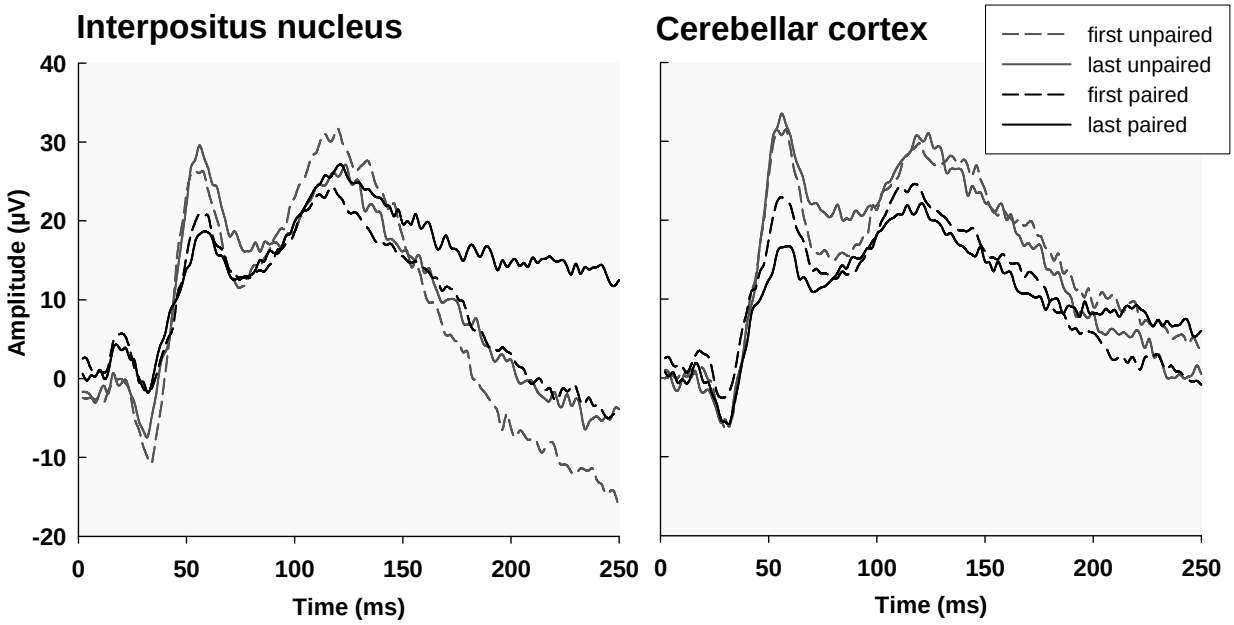

Figure 2. Averaged ERP waveforms in first and last unpaired and first and last paired sessions in time period from the onset of CS (0 ms) to the onset of US (250 ms)

3.3.2. *Principal component analysis*

Four principal components (factors) accounted 93.2 % of the total variance. Factor loadings are presented in Figure 3. Factor latencies were determined as those where factor loadings exceeded 0.7 or went under -0.3. Factor percentages of total variance and factor latencies are presented in Table 1. No significant differences in factor scores were found in MANOVA analyses. However, when factor scores were examined with t-tests similar results were found in the interpositus as in t-tests on initial averaged ERPs. Significant differences were found from the interpositus nucleus between the two sessions of conditioning phase for factor 2 (t = 2.72, p < 0.05) and for factor 4 (t = 2.80, p < 0.05). A nearly significant difference was found for factor 3 (t = 1.91, p < 0.1). Between the two sessions of the unpaired phase no significant changes were found. Between the last unpaired and last paired session differences were found for factor 2 (t = 2.32, p < 0.05) and factor 3 (t = 2.92, p < 0.05). Nearly significant differences were found between the first unpaired and last paired sessions for factor 2 (t = 2.08, p < 0.1) and factor 3 (t = 1.94, p < 0.1). No significant differences were found when examining factor scores concerning cerebellar cortex. (See Figure 4 and Table 2 for summary).

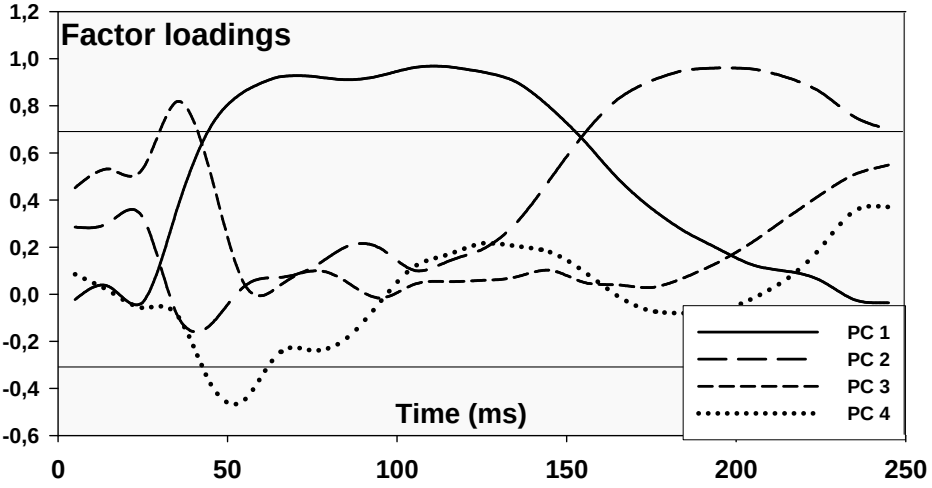

Figure 3. Factor (principal component) loadings. Loadings greater than 0.7 or smaller than -0.3 are used to identify the region of variability in each of the factor

	% **of total variance**	**Latency (max/min latency)**
factor 1	57 %	45 – 155 ms (115 ms)
factor 2	27 %	155 – 250 ms (200 ms)
factor 3	5 %	30 – 40 ms (35 ms)
factor 4	4 %	40 – 60 ms (55 ms)
Total	93 %	

Table 1. Factor percentages of total variance, factor latencies and latencies of maximum/minimum factor loadings. Loadings greater than 0.7 or smaller than -0.3 are used to identify the region of variability in each of the factor

	Interpositus nucleus		**Cerebellar cortex**	
Sessions	**PCA+t-tests**	**t-tests on averaged ERPs**	**PCA+t-tests**	**t-tests on averaged ERPs**
first paired - last paired	(30–40 ms) 40–60 ms 155–250 ms	(181–250 ms) 191–250 ms	-	(51–56 ms)
first unpaired- last unpaired	-	-	-	-
first unpaired - last paired	(30–40 ms) (155–250 ms)	(33–36 ms) (185–250 ms) 207–250 ms	-	(53–64 ms)
first unpaired - first paired	-	-	-	-
last unpaired - last paired	30–40 ms 155–250 ms	(187–250 ms) 207–250 ms	-	(51–64 ms) 117–144 ms (115–158 ms)
last unpaired - first paired	-	(17–18) 19–20 (21–24, 28–29, 32–33 ms)	-	(123–138 ms)

Table 2. Summarizes latencies where significant ($p < 0.05$) or nearly significant ($p < 0.1$) difference can be found between sessions. Nearly significant results in parentheses ( ). Results gained with the two different methods are presented separately in own columns.

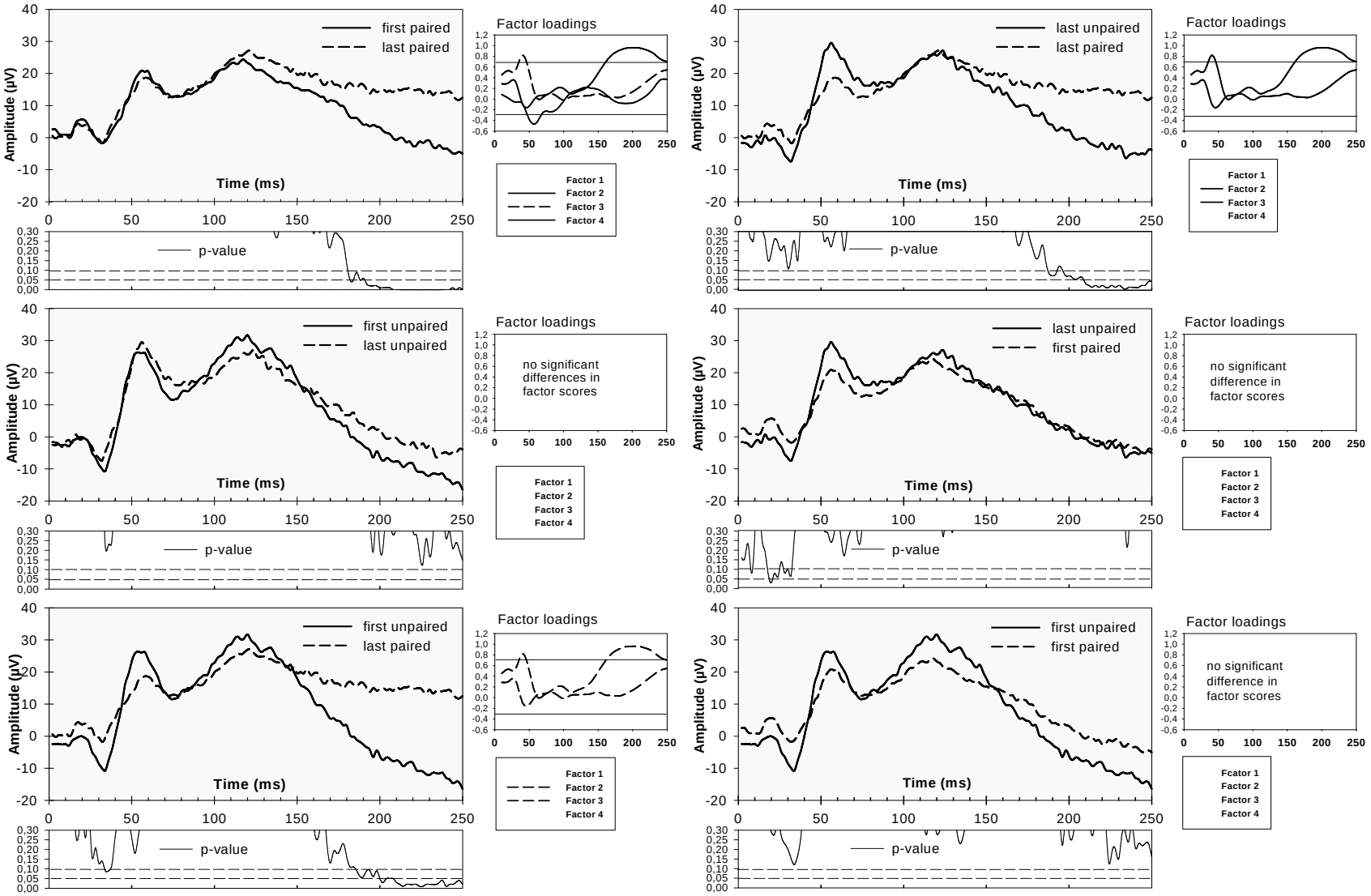

Figure 4. Interpositus nucleus ERPs. Presents interpositus nucleus ERP comparisons of each pair of sessions. Value of significance of t-tests made in every 125 time points are presented below each figure. Significance levels of 0.1 and 0.05 are marked with dash lines. In smaller pictures left to ERP-waves are presented factors (principal components) that indicate difference between sessions. Factors with significant difference ($p < 0.05$) in factor scores between sessions are presented with solid line and factors with nearly significant difference ($p < 0.1$) with dash line. Factor loadings greater than 0.7 or smaller than -0.3 are used to identify the region of variability in each of the factor

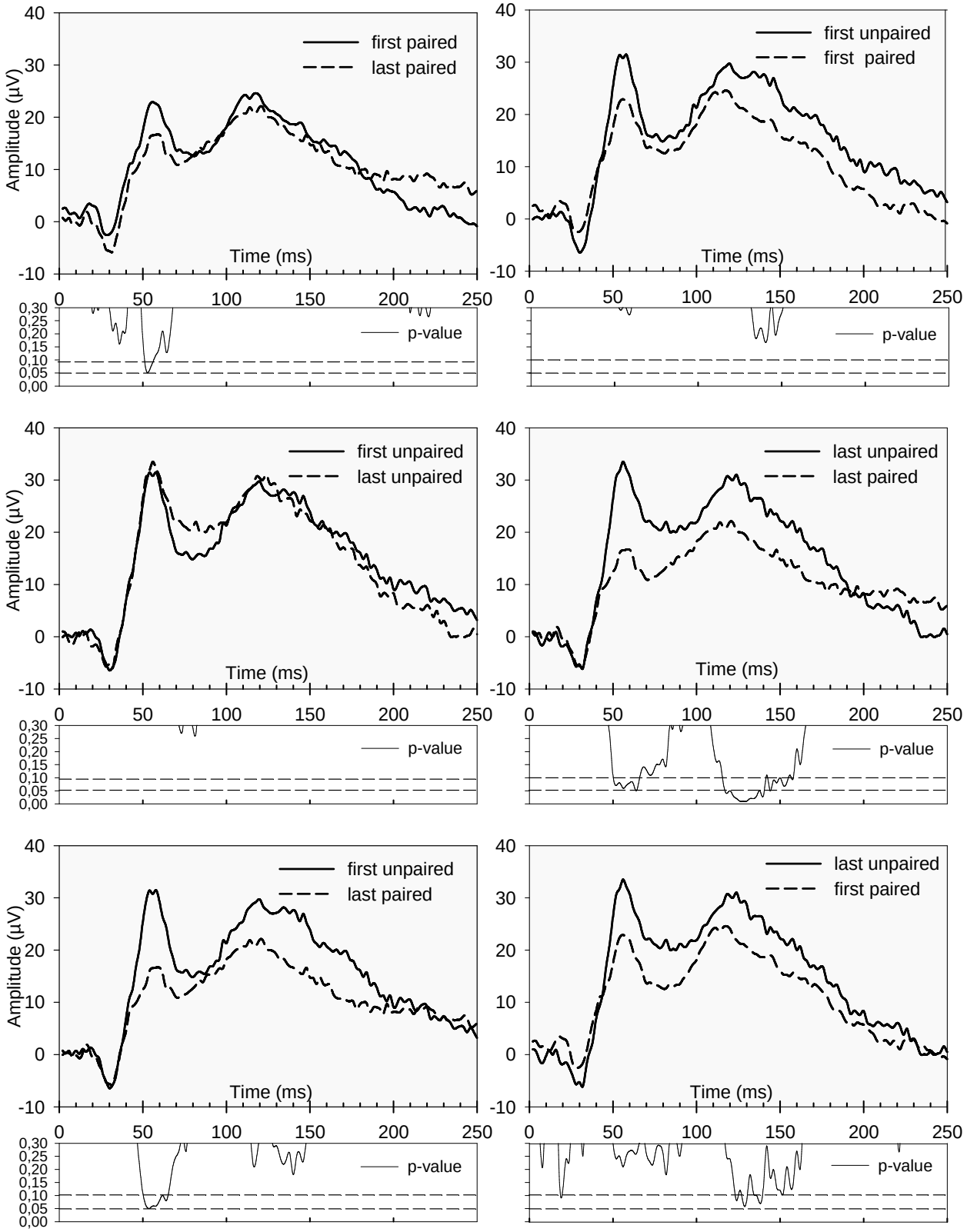

Figure 5. Cerebellar cortex ERPs. Presents cerebellar cortex ERP comparisons of each pair of sessions. Value of significance of t-tests made in every 125 time points are presented below each figure. Significance levels of 0.1 and 0.05 are marked with dash lines.

3.3.3. Differences between the cerebellar cortex and interpositus nucleus

When the two different brain regions were compared, differences were found in the first unpaired session for factor 2 ($t = 2.47$, $p < 0.05$). Similar result was gained with another method as t-tests of the 125 time points of initial ERPs revealed difference in the last 85 ms (165-250, $t = 1.97$-2.85, $p < 0.1$, 169-170, 181-250 ms, $t = 2.25$-2.85, $p < 0.05$). No other differences were found between these two regions.

4. Discussion

The aim of this study was to investigate changes in the rabbit cerebellum ERPs during NM conditioning. Conditioning produced a robust NM response starting about 100 ms after CS onset. Some sensitization could be seen also during the unpaired treatment, but the effect is too small to be considered as learning. The ERP data revealed that in the interpositus nucleus changes at the beginning of the ISI, from 20 to 30 ms, happen already in the first paired session. Changes from 30 to 60 ms start in the beginning of the conditioning phase and continue during further conditioning so that a negative peak around 30 – 40 ms and a positive peak around 50 ms decrease. During conditioning, a positive deflection is formed in the last 100 ms period of the ISI. In the HVI-lobule of the cerebellar cortex main changes in amplitude happen in time periods from 120 to 160 ms and from 50 to 60 ms. Amplitude in time period from 120 to 160 ms decreases already during the first paired session. Decrease of the peak amplitude around 50 – 60 ms starts in the first paired session and continues with further conditioning.

4.1. Methodological discussion

Covariance based, Varimax-rotated principal component waveforms are “typically characterized by unique triangle-shaped, positive factor loadings that are (1) clustered in a narrow time range and (2) lack inverse (negative) or significant secondary loadings at different latencies” (Kayser & Tenke, 2003, p. 2309). This makes it possible to determine the temporal region of variability in the ERP waveforms accounted by one factor. However, principal components extracted in this study are not typical in this sense. All components except the first one account for some variability both at early and late latencies indicating that these changes happen in close relation. Factors 3 and 4 were defined as early latency components but the fact that they have secondary loadings at later latencies may distort the result. The atypical negative component extracted here represents most likely the inverse change in ERP amplitudes at 60 ms time especially in the interpositus nucleus: Over the course of time the amplitude of the 60 ms peak decreases as, on the contrary, at the beginning and at the end of the ISI amplitude increases.

MANOVA analysis of principal component factor scores failed to find any significant differences between sessions. This might be due to the small amount of cases and quite large variability between the subjects. In cerebellar cortex neither t-tests of factor scores revealed significant differences. It is possible that the factors extracted in this study are mostly driven by stronger variability in the interpositus nucleus and thus the comparison of factor scores failed to find any significant differences from the cerebellar cortex. T-tests on the averaged ERPs also show that almost all of the variability in the cerebellar cortex happens at the latencies accounted by factor 1. Factor 1 accounts the most of the variability and may thus represent the general shape of the ERP rather than more precise changes. Not all results gained with PCA were supported by t-tests of averaged ERPs, but it is known that the PCA can reveal features that are not evident in traditional ERP measures. Also, it provided additional information relative to traditional ERP research methods showing that early and late latency changes happen in close relation. PCA has been

criticized for the fact that it has difficulties extracting components with temporal overlap and with dealing the temporal variability of ERPs (van Boxtel, 1998). This is though a problem also in traditional ERP research methods.

Earlier studies on cerebellar activity have been made on multiple and single unit activity. When these methods are compared with ERPs averaged from EEG, it must be remembered that multiple and single unit recording and ERP differ as measures (Buchwald, Holstein & Weber, 1973). Multiple unit activity (MUA) reflects the action potential discharge, as EEG, and thus ERP, is closely related to postsynaptic and afterpotential slow waves. According to Buchwald, Holstein and Weber (1973), multiple unit activity may also be more sensitive to altered responsiveness than the EEG.

4.2. Electrophysiological findings

Multiple unit activity recordings have found similar activation patterns both in the interpositus nucleus and the cerebellar cortex (McCormick et al., 1982; Gould & Steinmetz, 1996). Unit recordings have found cells responding to CS and/or modelling CR (McCormick & Thompson, 1984; McCormick et al., 1982; Gould & Steinmetz, 1996; Berthier & Moore, 1986, 1990). Also ERP-waves of these two regions seem quite similar at the first look. Yet the results of this study show, that conditioning alters these regions differently.

A positive deflection starting approximately 100 ms before the US onset is formed in the interpositus nucleus during conditioning. Although trial by trial changes in latencies of the ERPs and the NM movements were not compared in this study, the grand mean waveforms show that this positive deflection at the end of the ISI is simultaneous with the NM movement. CR-models found in multiple and single unit studies have always preceded the onset of the CR (McCormick & Thompson 1984, McCormick et al., 1982; Gould & Steinmetz, 1996; Berthier & Moore, 1990). So whether this ERP deflection reflects the cause of the NM response or is a reflection of already ongoing movement is not clear. However, as mentioned above, MUA and ERP are not parallel methods. EEG reflects somewhat slower afterpotential waves and this may explain the difference (Buchwald et al., 1973). Factors extracted in this study show, that amplitude changes at the beginning of the ISI and at the end of the ISI happen in relation to each other. Also earlier latency changes seem thus to be relevant in the formation of the learned response.

The deflection at the end of the ISI found in this study is congruent with the finding of Loikkanen and Huovila (1999). They also found out that this deflection disappears when interpositus nucleus is inactivated. This aspect of ERPs is consistent with MUA studies showing that CR-related unit activity disappears when interpositus nucleus is inactivated (Clark et al., 1992). In the study of Loikkanen and Huovila ERPs of paired and unpaired phases were not compared although the same kind of control procedure was used than in this study. However, they did find out that ERP-wave recorded during cooling of the interpositus nucleus was not parallel with the one recorded in the first paired session. In fact, the ERP recorded in their study during cooling looks exactly like the ERP recorded during unpaired sessions in this study. This implies that the effects of cooling should be examined in relation to activity before any paired presentation of stimuli instead of in relation to activity in the first paired session.

The amplitude wave in the cerebellar cortex becomes more flat over the course of training with smaller peaks and to some extent smaller amplitude in general. No such clear reflection of the NM movement can be seen than in the interpositus nucleus. Earlier studies have shown that the cerebellar cortex is important, even though not essential, in the acquisition of the learned response (Lavond & Steinmetz, 1989; Clark et al., 1997; Gould & Steinmetz, 1996). Clark et al. (1997) proposed that the cerebellar cortex is more important in the acquisition than retention of the NM response. Main changes in the cerebellar cortex ERPs do occur at the early stage of the conditioning thus supporting this assumption. Single unit recordings have found Purkinje cells that decrease their

activity during conditioning (Gould & Steinmetz, 1996; Clark et al., 1997; Berthier & Moore, 1986). The decrease of the ERP amplitude might represent this change in the cell activity. Earlier studies have also proposed that the cerebellar cortex modulates the accurate time and amplitude course of the NM movement (Kotani, Kawahara & Kirino, 2003; Berthier & Moore, 1986; Gould & Steinmetz, 1996; Perrett et al., 1993). It has been proposed that the anterior lobe and the HVI-lobule encode different features of the NMR so that anterior lobe modulates timing and HVI-lobule the amplitude of the NMR (Steinmetz, 2000). The decrease in ERP amplitude before and at the time of the onset of the NM movement might be due to this kind of modulatory function. If this is true, the cerebellar cortex codes this information quite fast. This is consistent with the study of Kotani et al. (2003) in which they studied Purkinje cell activity during learning a new timing in decebrate guinea pig. Purkinje cell activity changed already in the first conditioning session when ISI was changed to another (Kotani et al., 2003). It has also been proposed that paired presentation of the conditioning stimuli causes pause in the cerebellar cortex Purkinje cell activity that blocks inhibition to the interpositus nucleus allowing it to generate the CR (Nores, Medina, Steele & Mauk, 2000). No such pause in activity can be seen in the cerebellar cortex ERPs. It can though be proposed that the decrease of the amplitude in the cerebellar cortex from 120 to 160 ms and also at earlier latencies might affect interpositus nucleus activity and the development of the CR.

Recording studies made so far have concentrated on the final outcome of conditioning without examining the changes taking place early in the conditioning phase. In the interpositus nucleus, and to some extent also in the cerebellar cortex, the first alteration of the activity due to conditioning occurs immediately after CS onset. Activity this close to the CS is usually referred as CS or sensory processing related (Berthier & Moore, 1990; Clark et al., 1997; Gould & Steinmetz, 1996). Tracy and Steinmetz (1998) found in their study that more Purkinje cells respond to the CS during paired conditioning than during unpaired presentation of pontine stimulation CS and air puff US (Tracy & Steinmetz, 1998). The results of this study show that processing of the tone in the neural level changes rapidly already in the beginning of the conditioning, before any behavioural sign of learning. It can be seen that the immediate processing of the tone (from 20 to 30 ms) changes first and the later processing (30 to 60 ms) with further paired presentation of the stimuli. The fact that some changes in both the cerebellar cortex and the interpositus nucleus happen already during the first conditioning session strengthens the assumption that both regions are important in initial acquisition of the conditioned response. It also means that some information stays invisible if the effect of conditioning is studied only by comparing activity at the end of the conditioning phase with the activity in the beginning. The finding of this study is thus important and should guide also future studies.

There is also a possibility that the amplitude changes in the first paired session are due to the prior unpaired presentation of the stimuli. Earlier studies have shown that learning is slower when rabbits receive unpaired training before paired (Lavond, Kanzawa, Ivkovich & Clark, 1994). It is thus possible that some neural changes need to happen before normal learning can start. Gould and Steinmetz (1996) found out that cerebellar cortex multiple unit activity is altered also during unpaired presentation of stimuli with ISI as long as 25-35 s. In this study, no significant differences in activity were found between the two unpaired sessions. However, it can not be ruled out that changes due to unpaired presentation might happen already in the first unpaired session. Also, in the first unpaired session there can be seen some not significant difference in the last 60 ms of the 250 ms period in the interpositus nucleus. In the first unpaired session the activity of the two regions differed at the end of the ISI and this difference disappeared during unpaired phase. This indicates that something happened in the between. In no other study changes due to unpaired stimuli presentation could have been found in the interpositus nucleus. On the contrary, the interpositus nucleus is proposed to be able to exhibit excitability changes only when the CS precedes the US (Gould and Steinmetz, 1996).

The effect of the unpaired phase on the early changes of activity should be tested in further studies. This study discussed only CS-period ERPs, no studies have yet been made on the ERPs of the US-period. Multiple and single unit activity studies have shown that also US-period activity changes over the course of training (e.g. Gould & Steinmetz, 1996; Clark et al., 1997). Further studies are also needed to reveal how interpositus and cerebellar cortex EPRs might be related to time and amplitude differences of the CRs between trials and over the course of learning.

4. 3. Conclusion

These results emphasize the importance of neither of the interpositus nucleus or the cerebellar cortex in the acquisition of NMR but rather a functional interaction between them. ERP findings agree with single unit research findings that both the interpositus nucleus and the HVI-lobule of the cerebellar cortex change activity related to both CS and movement of the NM. Neural changes start in both regions already in the first conditioning session and continue with further training. Processing of the tone changes similarly in both regions already in the beginning of the conditioning and the changes continue during further training. The cerebellar cortex is more involved in the early stages of the acquisition than the performance of the already learned CR, whereas the interpositus nucleus activity is related to the performance of the learned response. Future studies should take account of the fact that important changes in activity happen already in the very beginning of the conditioning.

ERP research of animals provides more coherent information with human research than MUA research. This study shows also that event-related potentials can reveal some aspects of learning that are not evident when using multiple or single unit activity measures. Even though EEG is thought to be less sensitive to neural changes than MUA (Buchwald et al., 1973), ERPs appear to be able to reveal more features of activity than MUA in this framework. MUA recordings have ignored the decrease in activation of some neurons in the cerebellar cortex and the clear division of CS- versus CR-related activity both in the cerebellar cortex and the interpositus nucleus found in single unit recordings. ERPs, on the contrary, take account of both of these features.

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