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EVENT-RELATED POTENTIALS OF THE INTERPOSITUS NUCLEUS DURING CLASSICAL NICTITATING MEMBRANE CONDITIONING IN RABBITS

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Aiemmat tutkimukset ovat viitanneet pikkuaivojen ja niihin liittyvien hermokytkentöjen olevan välttämättömiä diskreettien, somaattisten käyttäytymisvasteiden klassiselle ehdollistumiselle; yksi tälläinen käyttäytymisvaste on kanin vilkkuluomirefleksi. Edeltävissä tutkimuksissa on löydetty oppimiseen liittyviä monisoluaktiivisuuden (MUA) muutoksia ehdollistamisen aikana; näitä muutoksia voidaan löytää useilta aivoalueilta. Sekä ehdollistunut vilkkuluomivaste että oppimiseen liittyvä MUA häviävät kun pikkuaivojen interpositustumake (IP) inaktivoidaan jäähdytysmetodia käyttäen. Tässä tutkimuksessa asennettiin pysyvästi jäähdytysputki interpositustumakkeen läheisyyteen palautuvaa inaktivoimista varten, ja solunulkoinen mittauselektrodi asennettiin interpositukseen herätevastemittauksia varten. Vilkkuluomivastetta ja herätevastetta mitattiin klassisen vilkkuluomiehdollistamisen aikana. Kaneille esitettiin ensin seitsemän päivän ajan erillisinä esitettyjä ääni- ja ilmapuhallusärsykkeitä, jonka jälkeen vähintään kymmenen ehdollistamispäivän ajan esitettiin pareittain ehdollisia ääni- ja ehdottomia ilmapuhallusärsykkeitä. Ehdollistamisen jälkeen IP inaktivoitiin ehdollistamissession ajaksi. Ehdollistaminen tuotti ehdollistuneita vilkkuluomivasteita, jotka katosivat interposituksen inaktivoinnin aikana ja palautuivat inaktivoinnin jälkeen. Interposituksen herätevasteita verrattiin eläinten oppimisen eri vaiheissa: ehdollistamisen alussa, ehdollistumisen tapahduttua ja inaktivoinnin aikana. Tässä vertailussa pyrittiin löytämään mahdollisia oppimiseen liittyviä herätevasteiden muotoja, jotka olisivat vastaavia kuin monisoluaktiivisuustutkimuksissa löydetyt. Tilastollisesti merkitseviä muutoksia IP:n herätevasteprofiileissa löydettiin kun edellämainitut vertailut suoritettiin.

Event-related potentials (ERP) of the interpositus nucleus during classical nictitating membrane (NM) conditioning in rabbits

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Previous studies have implicated that the cerebellum and its associated circuitry are essentially involved in classical conditioning of discrete, somatic muscle responses, such as the rabbit's nictitating membrane response. Earlier studies have found changes in the multiple unit activity (MUA) during training that are related to learning; these changes can be found in several brain sites. Both the conditioned NM response and the learning-related MUA are abolished during the cerebellar interpositus nucleus (IP) inactivation by cooling method. In the present study, a cooling probe was cronically implanted into vicinity of the IP to enable reversible inactivation of the IP and an extracellular semimicroelectrode was implanted into the IP to allow the measurement of event-related potentials. Both the NM response and ERP were measured over the course of classical NM conditioning. The rabbits received first seven days of explicitely unpaired stimulus presentations with a tone -conditioned stimulus or airpuff-unconditioned stimulus followed by paired tone-airpuff -presentations for at least ten training days; after training, the IP was inactivated during a training session. The behavioral responses that were used to measure learning and effectiveness of inactivation.The training led to the development of NM conditioned responses (CRs), which were abolished during IP inactivation and reappeared after inactivation. The ERP recordings of the IP in naïve, trained, and trained but inactivated animals were compared in order to reveal possible learning-related patterns in the ERP waveforms corresponding to those found in MUA experiments. Statistically significant changes in the ERP profiles of the IP in naïve and trained, naïve and inactivated, and trained and inactivated animals were detected.

The classical conditioning paradigm of the rabbit eyeblink has been massively investigated and thus the properties of the eyeblink/nictitating membrane response are well-known. Rabbits use both the external eyelids and the NM (also called the third eyelid) to protect themselves from aversive stimuli that might threaten their eyes. Typically, in laboratory experiments, a tone or light is used as an initially neutral stimulus or conditioned stimulus (CS), and a corneal airpuff or periorbital shock as the aversive unconditioned stimulus (UCS), which produces the unconditioned response (UCR) of the external eyelids and the NM (Thompson & Krupa, 1994).

Learning within classical conditioning is based on paired presentations of the CS and the UCS. In basic delay conditioning, the UCS follows the CS immediately or they overlap, thus making this procedure simpler than the trace conditioning, where there is a pause (max. 2 s) between the CS and the UCS.

For delay eyeblink conditioning, converging evidence from numerous studies has made possible to define a neural circuit which includes pathways for the CS, the UCS, the CR and the UCR. In brief, the CS pathway includes sensory relay nuclei, the pontine nuclei, and mossy fiber projections to the cerebellar cortex and interpositus nucleus; the UCS pathway includes trigeminal nuclei, the inferior olive and its climbing fiber projections to the cerebellar cortex and interpositus; the UCR pathway involves the trigeminal nuclei and brain stem motor nuclei. The site of convergence for CS and UCS information, which is essential to produce the learned CR has been localized to the cerebellar interpositus nucleus. The CR pathway is then routed out of the cerebellum to motor nuclei via the red nucleus.

Many studies have given consistent results about the importance of the cerebellar interpositus nucleus in classical conditioning of discrete, somatic muscle responses (Lavond, Kim, & Thompson, 1993). The IP has been suggested to be the locus of the memory trace (engram), since without the IP the animal is unable to learn and if a trained animal's IP is lesioned, it no longer shows learning (Lavond, Logan, Sohn, Garner, & Kanzawa, 1990; Clark, Zhang, & Lavond, 1992). Also the recording and electrical stimulation experiments have supported the hypothesis of IP as the locus of the engram (McCormick & Thompson, 1984).

Earlier studies have shown that in simultaneous recordings of the NM movement and MUA during conditioning, the pattern of increased neural unit activity can be found in the IP as well as in some other brain sites involved in this type of learning (e.g., McCormick & Thompson, 1984). These patterns in IP precede (for approximately 50 ms) and closely parallel the time-amplitude course of the behavioral NM response, and this unit model of the learned response can be seen even when no CRs have yet been developed (Donegan & Thompson, 1991).

The purpose of the present experiment is to find out whether learning-related changes could be detected in the cerebellar IP using the ERP activity measures in addition to the multiple unit activity measures. The experiment was divided into an unpaired phase and a training phase. The rabbits were implanted with recording electrodes, and a cooling probe placed to inactivate the IP. After the unpaired phase, the animals were classically conditioned until well-learned. The reversible inactivation with the cooling probe was used at the end of both the unpaired and the training phase. Electrophysiological recordings were obtained during both phases including the IP inactivation sessions and evaluated in the light of the behavioral learning state of the animal.

METHOD

Subjects

Subjects were five New Zealand albino rabbits (Oryctolagus cuniculus) weighing 2.7-3.7 kg at the time of the surgery. All rabbits were housed individually in a temperature- and humidity-controlled vivarium having a natural day-light cycle (12 h light / 12 h dark) and were given free access to food and water. All behavioral procedures were carried out during the daylight portion of the cycle. The animals were cared for by the experimenters and by the staff and veterinarians of the University of Jyväskylä. The experiment was carried out according to the regulations of the European Union for animal health and care in laboratories.

Surgery

In preparation for surgery, animals were anestetized with intramuscular injections of ketamine-xylazine cocktail (2.4ml Ketalar®, 50mg/ml; 0.8ml Rompun®, 20mg/ml; NaCl 0.8ml) and were positioned in the stereotaxic headholder with bregma 1.5 mm above lambda. The eyes were treated with Oftan® to prevent infections and dryness during the operation.

The rabbits were implanted chronically with a cooling probe (Zhang, Ni, & Harper, 1986) placed to inactivate the cerebellar interpositus nucleus. The cooling probe is able to inactivate tissue in an area of 2.5 mm around the tip. Thus, the cooling probe was placed into the dentate nucleus next to the IP, coordinates (λ+0.5mm, R 6.5mm)[1]. The recording electrodes (figure 1) were implanted into CA1 region of the hippocampus, interpositus nucleus, lateral pontine nucleus, and cerebellar cortex (lobule HVI), of which the interpositus electrode recordings were used in analysis of the present study. The coordinates of the electrodes were (β-5.0mm, R 5.0mm), (λ+0.5mm, R 5.0mm), (λ+8.0mm, L 2.5mm), and (λ-0.5mm, R 5.0mm)[1], respectively. The final depths were determined by observing the characteristic activity on the oscilloscope. For additional experimental purposes, the rabbits were also implanted with three hypothalamic stimulation electrodes, that were not used in this study. The coordinates were (β-1.0mm, R 2.0mm), (β+0.0mm, R 2.0mm), (β+1.0mm, R 2.0mm)[1]. The cooling probe and the electrodes were implanted using stereotaxic atlases of the rabbit brain (Sawyer, Everett, & Green, 1954; Shek, Wen, & Wisniewski, 1986). The electrodes were connected via wires to a socket cemented to the skull with dental acrylic together with anchoring screws. Two interconnected scull screws were used as a reference electrode.

Finally, a small loop of nylon was sutured at the nictitating membrane of the right eye. Postoperative analgetics, intramuscular injections of Temgesic® (0.3 mg/ml), were given as needed after surgery. Animals were allowed to recover for at least one week before training.

1) β stands for bregma, λ for lambda, L/R for left/right and +/- for anterior/posterior in all given coordinates.

Experimental apparatus

The animals were restrained in a standard Plexiglas rabbit restrainer and trained in ventilated, sound-attenuated and electrically shielded conditioning chamber (Gormezano, 1966).

The NM loop was linked by a rigid stainless steel hook (45 mm) to the wiper arm of a minitorque potentiometer to allow the measurement of the NM movement. The extension of NM was transduced to a voltage change by the potentiometer (1mm movement equaled 1V).

Head movements were recorded with a three-dimensional accelerometer made of IC Sensors devices (3021-002P) and trials were recorded on a videotape for the later analysis of the movements. Observation was possible during sessions by means of a video monitor.

A tone generator was placed outside the conditioning chamber and the tone was directed through plastic tubing into the chamber. The airpuff was delivered through another plastic tubing. The apparatus to be attached to the animal's head during training included the potentiometer, the tone tubing, the airjet nozzle, the accelometer, and a multichannel measurement system (Axon) recording the ERPs. Sampling rate was 500 samples per second. The voltage changes were amplified, filtered (band pass 0.1-200Hz) and fed to an A/D converter (Data Translation DT2831G). The data were collected by an IBM compatible computer and the control of the whole experiment was carried out with another computer.

The inner cannula of the cooling probe was attached to a gas delivery system during cooling sessions. The cooling gas used in this experiment was freon-like 1,1,1,2-Tetrafluoroethane (KLEA R-134-A).

CS was a 350 ms, (1 kHz 85dB) sine wave tone directed to the left ear at a distance of 2 cm. UCS was a 100 ms, (2.1 N/cm^2 pressure at the source) air-puff directed to the right cornea at a distance of 1.5 cm.

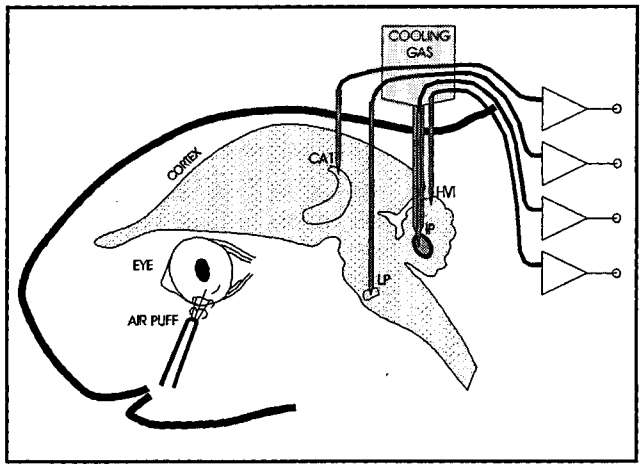

Figure 1: The cooling probe location in the dentate nucleus and the four recording electrodes (CA1 in the hippocampus, lateral pontine nucleus, HVI in the cerebellar cortex and IP in cerebellum). Adapted from Korhonen, Lavond and Arikoski, (1998).

Behavioral training procedures

Prior to training, rabbits were allowed to two adaptation sessions (duration 1 h per session) in the training apparatus. No stimuli were presented on these adaptation days.

Four rabbits (UNPAIRED experimental group) received the unpaired phase, which consisted of seven daily sessions of explicitely unpaired stimulus presentations to rule out pseudoconditioning (Thompson, 1976), prior to the paired training phase. One rabbit (CONTROL animal) did not participate in the unpaired phase but received paired presentations right from the beginning and served as a control animal for the effects of the unpaired stimulus presentation. Every daily unpaired sessions consisted of 70 + 70 pseudorandomly arranged CS-only and UCS-only trials. The intertrial interval (ITI) varied randomly between 20-40s (M = 30s). The same ITI was also used in the paired phase.

The paired phase began after the unpaired phase. Three types of trials were used: type 1 was CS-only trial, type 2 was UCS-only trial and type 3 was CS-UCS paired trial. Each training session consisted of 80 trials (60 x type 3 trials, with 10 x type 1 and type 2 test trials evenly distributed throughout the session), see figure 2.

In the paired trials, the CS preceded the UCS by 250 ms, coterminating, (figure 3). Thus, the interstimulus interval (ISI) was 250 ms (onset to onset).

Conditioned responses were defined as any 0.5 mm or greater extension of the NM following CS onset but preceding UCS onset (on CS-alone trials, a CR was counted as any response ≥ 0.5 mm occurring within 750 ms of CS onset). The UCR was defined as any 0.5 mm or greater extension of the NM within 500 ms following UCS onset.

Bad trials were defined to be those where the NM movement started before the onset of CS (type 1 and type 3 trials) or before the onset of UCS (type 2). In addition to this, trials with excessive head movements were excluded.

The first paired day was considered to represent the untrained animal's performance. Learning was defined as the first time that eight CRs occured in nine, consecutive type 1 or type 3 trials (type 2 trials or bad trials were not counted). To ensure very well learned animals and an asymptotic (achieved maximal) level of learning, at least ten paired sessions were given to the unpaired group and five paired sessions to the control animal. Well-learned session was defined as the session with highest CR% after reaching the learning criteria.

1 3 3 3 3 2 3 3 2 3 3 3 3 1 3 3

Figure 2. The order of the stimuli is presented here in numbers: 1 is CS-only, 2 is UCS-only and 3 is paired trial. This block of 16 trials is repeated five times to give the total number of 80 trials per session.The paired session starts with one CS-only trial, goes on with four paired trials etc.

After ten days of paired training, the cooling inactivation was used with simultaneous paired training. This cooling day consisted of three blocks of 28 trials (3 x type 1, 4 x type 2 and 21 x type 3 trials). This way the session length remained in approximately one hour and was not too exhausting for rabbits. The first of the three blocks was a normal, pre-cooling session (cooling was off), the second block was a cooling inactivation session (cooling was on) and the third was a normal post-cooling session (cooling was off). The blocks followed each other immediately, a little pause was placed between sessions to allow the cooling effect to become full before cooling session and to allow the effect to extinct before the post-cooling session.

Within the inactivation, the neural tissue was cooled with a freon-resembling gas to create a reversible lesion in the IP. The effectiveness of the inactivation was assessed by the decrease of the CR percentages (CR%) during cooling block in animals that had shown a high level of CRs in the pre-cooling block. The recovery from inactivation was assessed by the CR% of the post-cooling block compared to the pre-cooling block CR%. The recovery was re-evaluated the day after the cooling with a control paired session (the NEXT session).

Histology

Following all training, each rabbit was anesthetized intramuscularly and given intravenously a lethal dose of sodium pentobarbital. Perfusion was done intra-aortically with NaCl followed by 10% formalin. The brain was post-fixed and sectioned coronally at 50 μm at the sites of the electrodes with a freezing microtome, stained with cresyl violet and coverslipped for microscopic examination. The sectioning was also videorecorded. The placements of electrode's tips were compared with the coordinates of the stereotaxic atlas of Shek, Wen and Wisniewski (1986) and Sawyer, Everett and Green (1954). The IP electrode tip locations are shown in figure 5. With subject #46, an erroneous wiring with a consequense of false order of recording channel output was found when the brains had been removed from the scull and the electrode connections were verified. Necessary corrections in the channel output orders were made in the data analysis phase for this subject.

Data analysis

The data was collected in 1500 ms periods for every trial us-

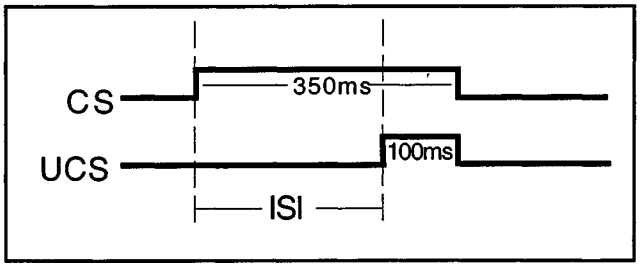

Figure 3. A schematic illustration of the stimulus presentation during paired trials. The ISI is 250 ms.

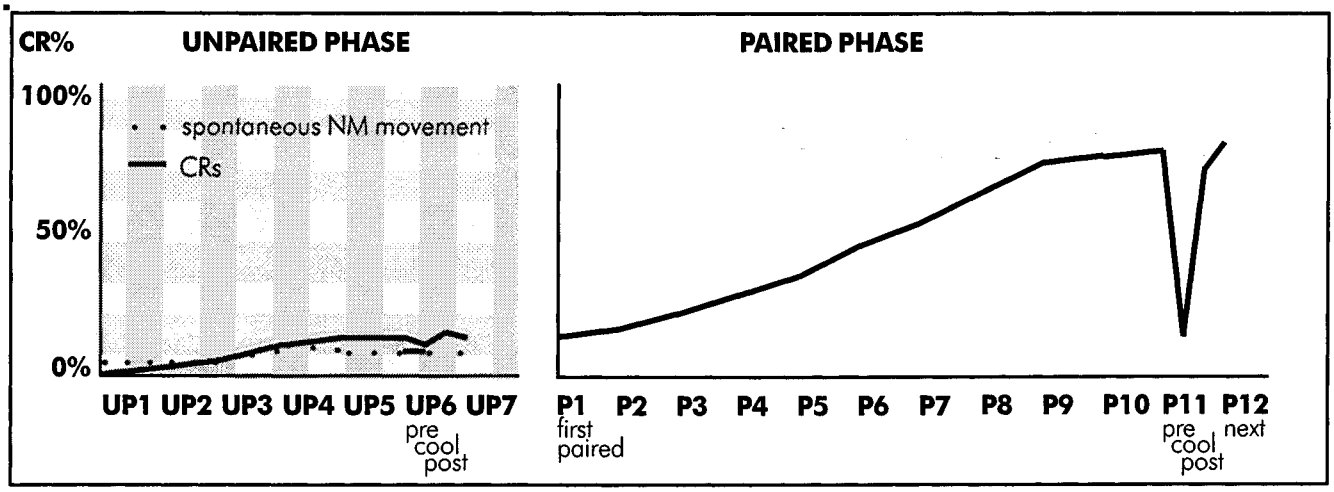

Figure 4. Schematic illustration of the conditioning procedure and the CR% over the course of learning. Pre, pre-cooling session; cool, cooling session; post, post-cooling session; next, the session on the day following the cooling, UP1 to UP7, unpaired days 1 to 7; P1 to P12, paired days 1 to 12.

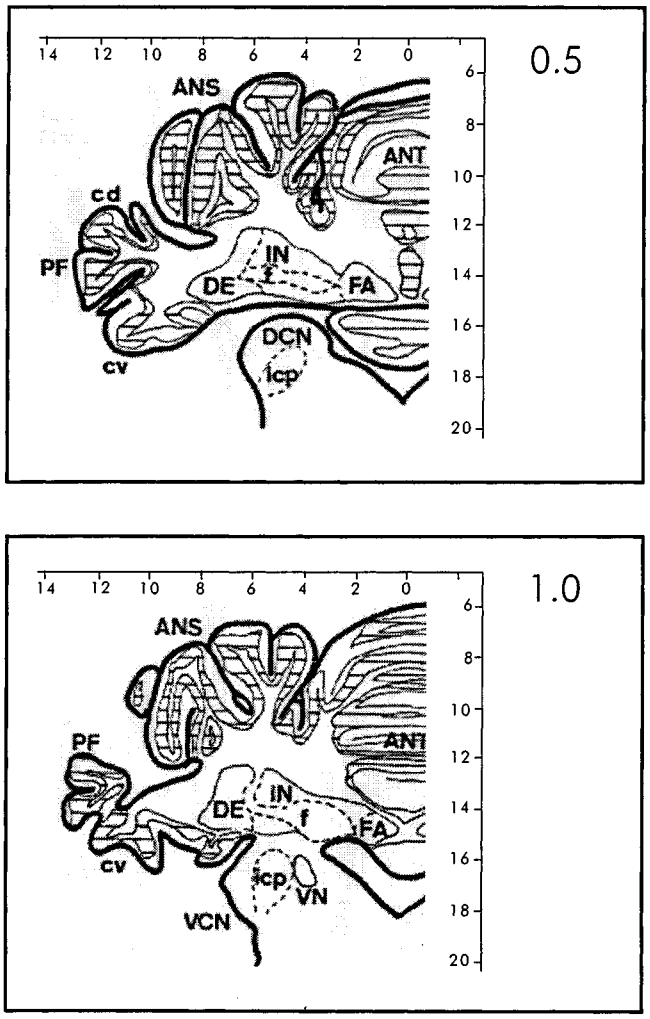

Figure 5. The histological results of the IP electrode tip locations are marked with triangles. The sections are taken from 0.5mm and 1.0mm anterior to lambda. Reprinted from Thompson and McCormick (1980).

ing BRACE© computer program. All analyses were performed with SPSS 8.0 for Windows. The baseline period was 250-500 ms immediately before the onset of the CS, and the average values of this period were used in the baseline correction. The CS period was 500-850 ms and the UCS period was 750-850 ms from the beginning of the data collection. The ISI period was 500-750 ms, respectively.

Behavioral data was collected on trial by trial basis. Average NM movements were calculated for every session and every trial type. The CRs were defined according to predefined criteria and CR% were calculated session per session. In analysis of behavioral data, paired t-tests were used. The following comparisons were made: CR%s of the first and the last unpaired sessions, the untrained (first paired) and cooling sessions, the well-learned and the cooling session, the pre-cooling and post-cooling session and the pre-cooling and next day's session. The effect of training from the first paired to the tenth paired was calculated with MANOVA repeated measures test.

Neural measurement data was collected for every trial and averaged for every session and every trial type over animals. The comparisons were done mainly for the same sessions as for the behavioral data (first paired, well-learned, pre-cooling, cooling and post-cooling sessions). The initial unpaired phase ERP recordings were not included in the analyses of study.

For the MANOVA profile analysis the ISI was divided into twelve successive epochs of 20 ms, 240 ms alltogether. (The first ten ms after the CS onset were left out of the analyses, in order to get twelve even numbers for the epochs.)

In the profile analysis the parallellism between consecutive epochs between compared sessions was tested with multivariate profile analysis (MANOVA). The multivariate analysis was followed by univariate analysis showing the consecutive epochs with significant effect. In case of parallel profiles, the equality of treatment (session) levels was tested with MANOVA.

RESULTS

Behavioral results

The subject's learning was first assessed individually. All of the rabbits learned according to criterion of eight CRs in nine consecutive trials, and reached asymptotical level of performance over 10 days of training. For the paired t-tests, all the data from individual animals were pooled together. All the mentioned CR% -comparisons are presented in Table1.

Rabbit	*A*	*B*	*C*	*D*	*E*
44	*3*	*100*	*0*	*90*	*95*
46	*25*	*71*	*33*	*52*	*71*
47	*24*	*90*	*20*	*81*	*91*
48	*0*	*86*	*0*	*45*	*97*
49	*0*	*83*	*14*	*72*	*87*

Paired t-tests	*t-value*	*significance*
1.unpaired - 7.unpaired	-2.499	ns.
cooling - well-learned	7.111	$p < 0.01$
pre-cooling - post-cooling	-2.992	$p < 0.05$
1. paired - cooling	-0.866	ns.
cooling - post-cooling	4.740	$p < 0.01$
pre-cooling - next day	0.825	ns.
MANOVA repeated measures	*F-value*	*significance*
1.paired to 10. paired day	16.822	$p < 0.001$

Table 1. Above: CR% during 1.paired session (A), Pre-cooling (B), Cooling (C), Post-cooling (D) and Well-learned sessions (E). Rabbit 44 is the control animal without any unpaired-phase. Below the CR% are the t-test result of the paired comparisons of the CR %. Ns., not significant.

The mean (± SE) number of trials required to reach the learning criterion was 293 ± 65.11 trials for the unpaired group and 143 trials for the control animal. MANOVA for repeated measures showed a difference in the CR% from the first paired training day to the tenth (fifth for the control animal) training day, $F = 16.822, p < 0.001$.

The interpositus cooling reduced the CR% significantly compared to the well-learned session (t-test $p<0.001$). In some animals, the first cooling effort was not successful and was repeated. Only the successful cooling sessions were used in the analyses. The cooling effect recovered fully by the next session (pre-cooling block - next days block CR%s were not different), but was still affecting in the post-cooling block, since statistical tests showed a significant difference in the CR% of the pre-cooling and post-cooling blocks ($p < 0.05$).

Electrophysiological results

For the profile analysis (MANOVA), all the IP recordings of the animals were pooled together session by session, excluding the type 2 (UCS-alone) trials. There were no differences in the results when the control animal was included in the analysis compared with the results of the control animal being excluded from the analysis. Also the profiles of the control animal alone were very much similar to those of the unpaired group.

In the present study, we expected that significant learning-related changes in the IP activity would be observed when the ERP recordings made in the first paired sessions and those of the well-learned sessions were compared (trial types 1 and 3). This difference was actually very clear; the multivariate test of parallellism (1.paired vs. well-learned) $F(11,679)$ was 6.24, $p < 0.001$. The univariate test showed differences to be in the epochs 2 to 4 and 7 to 12.

The first paired session and cooling block profiles were not parallel, $F(11,446) = 7.79, p < 0.001$. The univariate test revealed changes in the beginning of the ISI period (epochs 1 to 3 and 4 to 6).

The cooling block and the well-learned session were not parallel, $F(11,445) = 10.39, p < 0.001$. The univariate test localized the differences to all other epochs except the difference between epochs 6 and 7. In the experimental design, the well-learned session is corresponding to the pre-cooling block; in both cases, the animal is well-trained and the IP is in active state. The difference is in the number of the cases in the analysis: there are fewer trials (24 vs. 70 trials / animal and session) and hence fewer cases in the pre-cooling block profile analysis than in the well-learned session. Still, the results were equivalent: the pre-cooling and cooling blocks were not parallel, $F(11,211) = 4.21, p < 0.001$.

The pre- and post-cooling sessions were analysed to find out whether the cooling effect still was detectable on post-cooling sessions, as the behavioral data suggested. According to the profile analyses, the pre- and post-cooling profiles were parallel, $F(11,212) = 1.61$, ns. There were no difference in levels, either, $F(1,222) = 0.26$, ns.

The results of the profile analyses are gathered and the univariate significances marked into figure 6. In addition to the analyses that are in figures mentioned above, we performed some control tests. We expected to find parallel profiles when type 2 trials with a corresponding 250 ms period after the UCS presentation (10 ms excluded, 12x20 ms epochs) would be compared using the same sessions as above. Results confirmed our hypothesis: no significant differences were found. We also used the 250 ms time before the UCS on UCS-alone trials. This was considered to represent a randomly chosen 240 ms with no stimulus presentation immediately before the analysed time period. We wanted to rule out the possibility that an overall change could explain the results. Again, the profiles were parallel with no change in level.

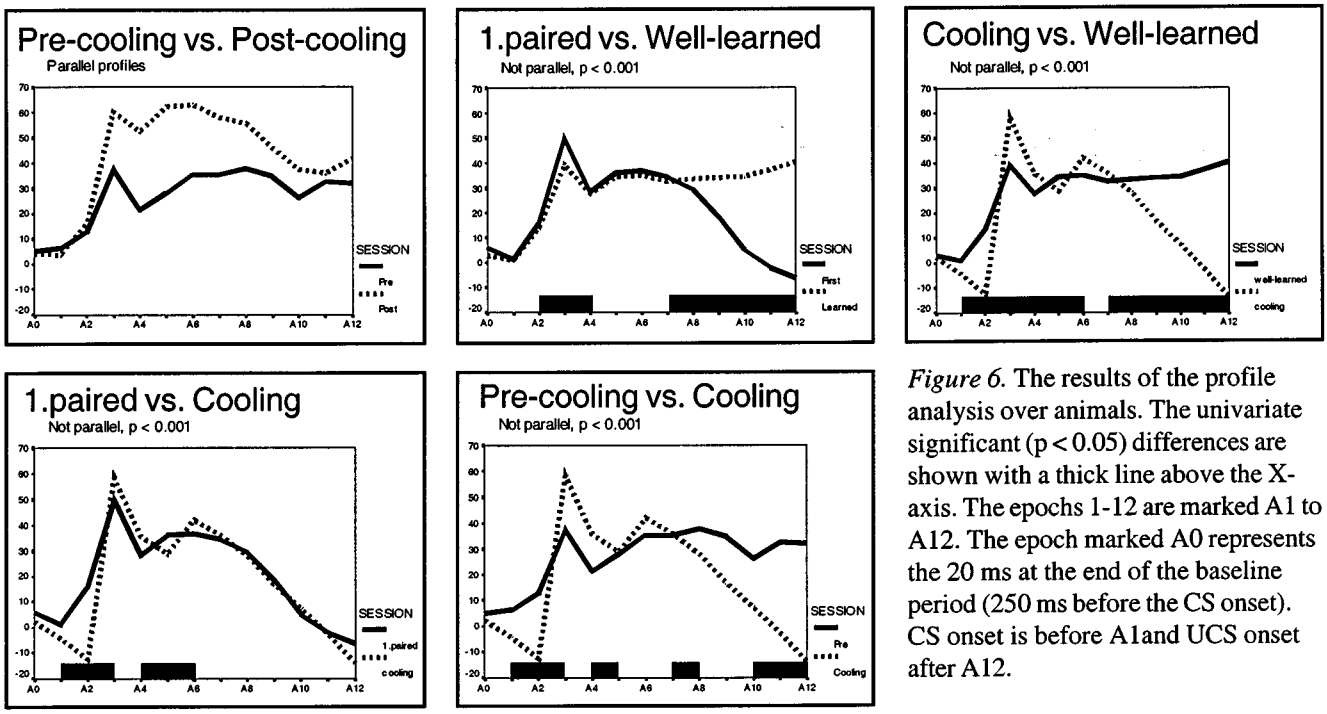

Figure 6. The results of the profile analysis over animals. The univariate significant ($p < 0.05$) differences are shown with a thick line above the X-axis. The epochs 1-12 are marked A1 to A12. The epoch marked A0 represents the 20 ms at the end of the baseline period (250 ms before the CS onset). CS onset is before A1and UCS onset after A12.

Discussion

As far as we know, this is the first study showing that learning-related changes can also be found in the ERPs of the interpositus nucleus. When untrained sessions were compared to the trained, well-learned sessions, a positive deflection was found at the second half of the ISI in trained animals.

In trained but IP inactivated animals the cooling returned the shape of the profile to that of an untrained animal at the second half of the ISI. We assume that this might reflect the fact that the learned response disappears in trained, inactivated rabbit. However, there seems to remain a difference in the ERPs of the first half of the ISI. The changes happen in a short latency (10-120ms), which implies that they are a consequence of sensory processing.

These recordings offer a prospective instrument for evaluating changes in activation levels during behavioral treatments. The IP was a reasonable starting point for this kind of experiments because its involvement in classical eyeblink conditioning is already well established, and thus, it was probable to find learning-related ERP changes in the IP if anywhere. When the special properies of the information obtained with ERP recordings has been clarified, it could be applicable for other brain regions as well, just like the MUA recordings.

Although the number of animals was small in this study, the results of the profile analysis were statistically very significant and the similar results were obtained from all of our subjects. Additional information can be obtained in future by comparing the MUA and ERP recordings that were made simultaneously within same animals. Our raw data includes also the MUA recordings, so this comparison is feasible and will be done in the near future.

The initial unpaired treatment was included as a part of the control procedures to rule out some other possible explanations for the findings (the additional information gathered in the unpaired phase of the experiment will be explained in detail in another paper). Behaviorally the unpaired group reached the learning criterion of eight of nine CRs in a mean (± SE) of 368 ± 55 trials. This is more than in normal training, for example Lavond, Kanzawa, Ivkovich, & Clark (1994) observed that a normal rabbit takes about 140 trials to learn. Reasons for the prolonged learning lie in the initial unpaired treatment, which is known to cause delay in subsequent learning (e.g., Korhonen & Penttonen, 1989). In addition to this, prolonged learning has been observed earlier when a cooling probe has been used (Clark, Zhang, & Lavond, 1992). The implantation of the cooling probe contains a risk of damage to brain sites essential for classical eyeblink conditioning, especially the interpositus nucleus. Without an intact IP, the animal is not able to learn normally, as mentioned in the introduction section. Therefore, animal's ability to obtain learned responses rules out any considerable damage.

To be precise, the trials to criterion observed in this study are comparable to the unpaired group results of the study mentioned above, with 320 ± 74 trials to criterion (Lavond, Kanzawa, Ivkovich, & Clark, 1994). The control animal learned in 145 trials, which comes close to the 140 trials observed by Lavond et al, (1994). This animal that did not receive unpaired stimulus presentations but had the cooling probe provides additional information about the proportional effects of the unpaired sessions and the cooling probe implantation on learning rate in this experiment. Since the learning rate of the unpaired group did not differ from previous studies with an unpaired-paired design, and since the control animal's

learning rate was similar to that of animals without a cooling probe, no significant role for the possible damage to the cerebellar cortex above the cooling site can be inferred.

The cooling was found effective and the ERP recordings as well as the behavioral responses were altered during inactivation by cooling in all five animals. To conclude, these findings show for the first that the ERP recordings indicate changes corresponding to those found in earlier MUA studies.

Together with lesion, recording, and stimulation data collected in previous experiments (Woodruff-Pak, 1997), these results are consistent with the idea that the interpositus nucleus of cerebellum is the site of plasticity associated with learning and memory for this classical CR. These results also imply that changes in ERPs during acquisition and lack of them at the second half of the ISI during inactivation are potentially useful in addition to the information obtained with MUA recordings. Especially in human research, where intracranial recordings are not possible, the ERP changes might be interesting, even though the deep brain structures such as the interpositus nucleus are beyond reach of today's EEG technology.

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THE NEURAL SUBSTRATES INVOLVED IN RABBIT'S NICTITATING MEMBRANE CLASSICAL CONDITIONING

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1 INTRODUCTION TO THE RESEARCH OF LEARNING AND MEMORY

Our purpose is to review the literature on the localization of memory traces within the brain.The main focus of this paper will be the identification of the essential memory trace circuit and localization of the memory trace within that circuit for basic delay classical conditioning of discrete behavioral responses. We will especially concentrate on results acquired with albino rabbits, and we will relate these results to results obtained in humans.

Thompson & Krupa (1994) have suggested that the most fundamental issue in the field of neuronal substrates of learning and memory concerns the physical/biological mechanisms underlying long-term memory formation, storage, and retrieval in the mammalian brain. The knowledge about the neuronal basis of learning and behavior might provide insight into unsolved problems concerning human behavior. A prerequisite to understanding the neural mechanisms by which an organism acquires and retains information is the identification of the site(s) of learning and memory in mammalian systems (Kim & Thompson, 1997). The common terms used in literature in this context are *the engram* or *the memory trace*.

The basic assumption is that the underlying neuronal mechanisms of memory storage and retrieval are the same in all mammals, including humans (Woodruff-Pak, 1997). The main advantage of using animal models is that it allows the study of the basic functional properties of neural network. Models are commonly used because they permit the investigation of certain aspects of what they represent under conditions that are simpler, more easily controlled, and less expensive than the real thing. However, the correspondence between the animal findings and human behavior must always be carefully verified by empirical data (Domjan, 1998).

Considerable progress in understanding the neural bases of learning and memory has recently taken place. One example of this, for instance, are the results concerning the classical conditioning of the rabbit eyelid or nictitating membrane (NM) response, which has been adopted by a number of laboratories as a standard model system for the study of behavioral and neural phenomena associated with conditioning (Steinmetz & Thompson, 1991). Experiments made in these laboratories reveal that the cerebellum is necessary for learning, retention, and expression of classical conditioning of the eyeblink and other discrete responses (Thompson et al., 1997, p.159).

1.1. Brief historical review with some definitions

Human and non-human subjects acquire information about the world through the process of learning, and store that information as a memory trace. Learning is the process of acquiring knowledge about the world

and memory is the retention or storage of that knowledge (Kupfermann, 1991a, p.997). Memory is also a term that is used when an organism's behavior is altered by its previous experience.

The term *engram*, (the site of memory formation), was first used in the 1950s by J.Z.Young (Rose, 1992). In the United States the psychologist Karl Lashley did an extensive series of studies in order to find the so called engrams (Kupfermann, 1991a). Lashley thought that engrams, or memory traces, were stored in the cerebral cortex and his research method, therefore, was lesioning parts of cortex. However, although Lashley and others found that cortical lesions can seriously disrupt learning, they also found that animals can relearn certain tasks even when they are completely decorticated (Kupfermann, 1991a).

Psychologists study learning by exposing animals to information about the environment, usually specified types of controlled sensory experience (Kupfermann, 1991a). Two major procedures are used in these studies, reflecting two major classes of learning: nonassociative and associative. In nonassociative learning (sensitization and habituation), animal is exposed once or repeatedly to a stimulus. As a result, the animal has an opportunity to learn about the properties of a single type of stimulus (Kupfermann, 1991a).

In associative learning, an animal is confronted to the relationship of one stimulus to another, as in classical conditioning, or to the relationship of a stimulus to the organism's behavior, as in operant conditioning (Kupfermann, 1991a). Classical conditioning is the simplest form of associative learning in which animals, including humans, learn relations among events so that their behaviors become better adapted to their environment (Rescorla, 1988). Another term used here is Pavlovian conditioning after Ivan Petrovitch Pavlov, the first researcher to discover this phenomenon. Pavlov's brilliant insight was to combine the concept that learning involves the association between ideas with the concept of the reflex arc (Kupfermann, 1991a). An example of early classical conditioning research was the work carried out by Ernest Hilgard (Hilgard and Campbell, 1936). Hilgard's work established the close correspondence in properties of the conditioned eyeblink response in humans and other animals (Woodruff-Pak, 1997).

Methodologically well conducted classical conditioning research allows reliable control and description of the effective stimuli and the behavioral response. It is essential to have a well-controlled and clearly measurable change in behavior to provide a baseline against which to compare the resulting events. In laboratory experiments, learning can be assessed by providing the subject with repeated learning experiences and observing progressive changes in performance (Kupfermann, 1991a).

Much of the general literature on classical conditioning is based on data collected with the human eyeblink conditioning paradigm, and in the rabbit NM or "third eyelid" paradigm first introduced by Isadore Gormezano (Woodruff-Pak, 1997). Gormezano focused on the eyeblink response because in the absence of special training, rabbits rarely blink. Therefore, if a rabbit blinks after the presentation of a stimulus, it is almost certain that the response is elicited by that stimulus (Domjan, 1998). Since then, classical conditioning of the eyelid closure response in rabbits became a paradigm in which the neural substrates of associative learning and memory of discrete responses can be analyzed (Steinmetz & Thompson, 1991). The most studied brain structures in these experiments have been the cerebellum (e.g. Lavond et al., 1993), the hippocampus and the brain stem structures (e.g., Berger et al., 1983).

2 LOCALIZATION OF ENGRAMS AND MEMORY CIRCUITS

The problem of localizing memory traces has been one of the greatest challenges in analyzing neurobiological substrates of learning and memory in the mammalian brain (Donegan & Thompson, 1991). Most of the progress in these areas has come from habituation -, sensitization -, and classical conditioning studies, both in invertebrates and in simple vertebrates (Kandel, 1991). Similar advances have yet to be made in understanding the mammalian brain (Thompson & Krupa, 1994).

Different forms or aspects of memory critically involve different neural systems in the brain (Thompson & Krupa, 1994). A way of classifying learning and memory is by looking at the way the storing and retrieval of information takes place. As a result, one could consider memory to consist of a declarative and a reflexive modules. Declarative memory includes learning of facts and experiences. Reflexive memory includes forms of perceptual and motor learning that are exhibited by alterations in the performance of tasks (Kupfermann, 1991a, p.1007). The hypothesis that the brain may possess two classes of neural circuits for reflexive and declarative memories, respectively, was supported by Woodruff-Pak & Papka's research (1996). They found that declarative memory was not impaired in cerebellar patients whose nondeclarative memory (as assessed by eyeblink classical conditioning) was severely impaired.

In general, the basic premise is that once we understand a simple form of conditioning, then more complex behaviors will be more tractable to study (Lavond et al., 1993). By restricting learning to basic classical conditioning, recent searches for engrams or memory traces have been successful. At the same time, however, one should be aware of the limitations of the approach and therefore, results do not necessarily account for all forms of learning (Lavond et al., 1993).

The evidence for localization does not depend upon a single technique (Lavond et al., 1993, p.318). In the next section we will illustrate this by including topics like electrophysiological recording of multiple and single units, electrolytic lesions, physical and chemical lesions, physical and chemical reversible lesions, neural stimulation, genetic mutations and pharmacological manipulation.

2.1 Commonly used research techniques in the studies of learning and memory

2.1.1 Lesion techniques

In essence, the lesion technique consists of the removal or lesioning certain parts of the brains of animals to study the changes produced in their behavior (Kolb & Whishaw, 1996). The logic behind the lesion technique is straightforward. By removing tissue the researcher can try to identify brain regions that are

essential for learning and memory of the conditioned eyelid response. For example, lesioning cranial nerve nuclei will abolish CRs and UCRs because motor neurons responsible for eyelid movement are located in the nuclei (Steinmetz & Thompson, 1991).

The lesion approach is not without problems. It is possible that the effects of a lesion are more widespread than is intended; the ablation may disrupt connections to other parts of brain. For instance, it is virtually impossible to remove the entire cerebellar cortex without damaging the underlying interpositus nucleus (Thompson et al., 1997; Chen et al., 1996). In rabbits this is the interpositus nucleus (IP) while in humans this is the globose nucleus. These deep nuclei of cerebellum are part of the essential memory trace circuit in NM conditioning both in humans and rabbits (Thompson, 1991; Woodruff-Pak, 1997; Dudai 1989).

2.1.2 Recording of neuronal activity

In animal subjects, recording electrodes can be placed either on the brain surface, just like in humans, or within the brain (intracranially) offering the opportunity to record directly the activity of small groups of neurons or even of single neurons. The electrophysiological activity can then be related to ongoing behavior (Rosenszweig et al., 1996).

Recording of neuronal activity that changes as a function of learning might result in the identification of structures involved in the formation of the memory. However, such evidence, per se, cannot localize the site(s) of memory formation (Thompson & Krupa, 1994). Electrophysiological evidence can serve to both identify putative sites of storage and rule out other alternative storage sites (Thompson & Krupa, 1994).

2.1.3 Electrical microstimulation

Microstimulation techniques have generally been used to demonstrate that stimulation of a particular brain region is sufficient to produce a behavioral effect (Steinmetz & Thompson, 1991). Electrical microstimulation can substitute for peripheral (sensory) stimulation in order to identify the neuronal circuitry sufficient for a given form of learning (Thompson & Krupa, 1994), e.g., Shinkman, Swain & Thompson (1996) used direct activation of neuronal elements within cerebellum as both UCS and CS, bypassing peripheral structures and pathways.

2.1.4 Anatomical and biochemical changes

Changes in anatomical and biochemical properties, for instance in the number and microstructure of synapses, the number of receptors, the gene expression, etc. can indicate persisting and localized changes (Thompson & Krupa, 1994). For example, in rabbits, it has been possible to count cerebellar Purkinje cells after training in eyeblink classical conditioning; the fewer Purkinje cells a rabbit has, the longer it takes it to condition (Woodruff-Pak et al., 1990). The number of Purkinje cells in cerebellar cortex gives one explanation for some of the individual variation in learning in young rabbits (Woodruff-Pak, 1997).

2.1.5 Genetic mutations

Genes are essential for producing the appropriate neural circuitry of a behavior (Kupfermann 1991b, p.992). By using the modern gene technology, various kinds of mutant, gene knockout, and transgenic animals have been developed. Gain of gene function is created by transgenic technology, and ablation of gene function by using gene knockouts (Arbeit & Hirose, 1999). These animals offer new possibilities in exploring the relationship between cerebellar circuitry, neuronal mechanisms, and behavior (Chen et al., 1996). Eyeblink conditioning is an ideal behavioral model for testing various mutants because the neural circuitry involved in this type of learning has been well described (Lavond et al., 1993; Chen et al., 1996). *The Purkinje cell degeneration (pcd)* mutant mice are born with Purkinje cells, but during the course of development these cells are all lost by the end of the fourth postnatal week (Chen et al., 1996). Thus, the animals exhibit a complete functional decortication of the cerebellum (Thompson et al., 1997). In the absence of Purkinje cells, eyeblink learning still occurs, but it is substantially attenuated and the timing of the learned response is altered (Chen et al., 1996). Another knockout mouse, deficient in the gamma isoform of protein kinase C (PKCgamma knockout) shows impaired motor coordination, but it learns eyeblink conditioning faster than normal mice (Thompson et al., 1998). Thus, transgenic and knockout mouse models are particularly useful for studies of complex neurobiological phenomenon.

2.1.6 Reversible inactivation techniques

A new approach in the research on localizing memory traces in the brain, is the recently developed method of reversible inactivation. Reversible inactivation methods can be carried out in two ways, either by cooling or by the use of drugs, e.g. muscimol and lidocaine (Thompson et al., 1997).

In the cooling method a miniaturized cryoprobe, or cooling probe, is used (Zhang et al., 1986). The cooling probe lowers the temperature of brain tissue around the cryoprobe tip up to a maximum of 1,5 mm (Campeau & Davis, 1990) to 8-10 C degrees. In the drug method, various drugs are used to temporarily inhibit receptors. For instance, *muscimol* is known to temporarily inhibit activity of neurons that express $GABA_A$ (γ-aminobutyric acid) receptors (Krupa, Thompson & Thompson, 1993) whereas *lidocaine* is a sodium channel blocker (Krupa et al., 1996).

Neural regions inactivated by the infusion of lidocaine can be precisely localized, however, due to its short duration it must be infused continuously (Thompson et al., 1997). Another disadvantage in the use of lidocaine is that, in addition of inactivating the target area, it blocks fibers of passage (Clark et al., 1992). These fibers are often traveling to and from other brain structures and unintended blocking of them can effect the functioning of these more distant areas (Lomber, 1999). The cooling probe offers considerable advantages over lidocaine for studying the biology of learning and memory. The cooling probe does not block fibers of passage, except the tissue immediately surrounding the tip of the cooling probe, furthermore it has also proven to be effective in maintaining a constant reliable blockade (Clark et al., 1992). Reversible cooling has the advantage that it can be turned on or off in a matter of seconds (Thompson et al., 1997).

As an example of a well conducted reversible inactivation study we mention the study of Clark

and associates (1992), in which they found that learned NM responses disappeared during cooling of the IP nucleus of the cerebellum, and that the learned behaviors returned when cooling was terminated.

2.2 The use of animal models

The ethical questions concerning the use of animals for research purposes are beyond the scope of this review. With respect to this matter, we refer to the strict laws regulating standards in animal research (Statute No. 1076, 1985; Statute No. 85, 1990; European convention for the protection of vertebrate animals used for experimental and other scientific purposes, 1986). The scientific community strives for the highest quality of animal care and treatment: well-treated animals will provide more reliable scientific results (Foundation for Biomedical Research, 1998).

The main logic in the use of animals is that there are striking similarities between the physiological systems of humans and various species of animals (Foundation for Biomedical Research, 1998). The general structure of the brain seems to be common in all mammals, including humans (Kolb & Whishaw, 1996). On a microlevel, it has also been demonstrated that neurons in the ganglia of primitive organisms and neurons in complex brains are essentially similar in their morphology (Dudai, 1989). Similarities are not confined to morphology only, but also apply to function (Dudai, 1989). In addition, similar chemical substances (neurotransmitters) are used by neurons for communication and regulation in different parts of the nervous system and in different organisms (Dudai, 1989). Nerve cells use similar, often identical, types of molecular mechanisms in their development, maintenance, and function, too. According to Dudai, '*the structural, functional, cellular, and molecular universality of nervous systems supports the "bottom-up" strategy in learning research. If different neurons in different contexts use common signalling systems, why should there not also be some common molecular and cellular mechanisms of learning?*'(Dudai, 1989, p.40).

Animal models permit the investigation of problems that would be otherwise difficult, if not impossible, to investigate directly in human beings, for example using invasive lesions and recording techniques (Domjan, 1998; Woodruff-Pak, 1997). In some medical cases, the researchers have had an opportunity to compare the results of animal lesion studies to lesions in humans, for instance patients with focal brain lesions and those with neurodegenerative diseases. Cerebellar lesion patients, for instance have participated in various classical conditioning experiments, and the results showed that the circuitry for eyeblink classical conditioning is similar in animal and human brains (Woodruff-Pak, 1997, p.344; Bracha et al., 1997).

2.2.1 Rabbits

The similarity between the physiology of rabbits and humans makes the rabbit a good model for the research of human disease (Foundation for Biomedical Research, 1998). The albino rabbit in particular has certain behavioral characteristics that make it better suited for conditioning studies than other species (pigeon, rat, dog and cat) previously conditioned (Gormezano, 1966). The advantageous characteristics are as follows: **(1)** domesticated rabbits are sedentary and tend to sit in one place, even if they are not

restrained (Domjan, 1998), **(2)** the eyelid, NM, and eyeball retraction responses have extremely low rates of spontaneous occurrence (Gormezano, 1966 , p.406). Thus, the NM response of the rabbit provides a discrete, robust, and easily measured behavioral response (Thompson, 1976). **(3)** There is a wealth of data for both humans and other mammals on the behavioral properties of these elementary learned responses (Thompson, 1991).

3 MECHANISM OF LEARNING

Learning is here defined as an enduring change in behavioral mechanisms, triggered by specific stimuli, often resulting in specific responses that are influenced by prior experience (Domjan, 1998). All behavioral changes reflect alterations in the brain produced by learning. Albeit learning can occur in the absence of overt behavior its occurrence can only be inferred from changes in behavior (Kupfermann, 1991a). Given the above definition of learning, the effects of rigid developmental programmes, injury, disease, and drugs are not classified as learning (Dudai, 1989). Since the change in behavior is dependent of past experience, this information needs to be stored in memory systems. Retrieval is the use of memory in neuronal and behavioral operation (Dudai, 1989, p.6).

Dudai has expressed the complexity of the learning situation as follows:

> *One could choose to analyse learning in very complex systems, because they are more 'interesting' and relevant to learning in humans. On the other hand, one might prefer to analyse very simple learning in a very simple nervous system, because it is simpler to dissect and hopefully also to understand. Bridging the gap between these two choices are almost limitless compromises between the complex and the simple, for example analysis of classical conditioning of reflexes in vertebrates.*

3.1 Nonassociative learning: habituation and sensitization

All animals react to both events in their environment (external stimuli) as well as to signs of their internal states (internal stimuli) (Domjan, 1998). Such elicited behavior represents one of the fundamental ways in which the behavior of all animals is adjusted to environmental events (Domjan, 1998; Thompson, 1991). When behavior is altered by experience, it is defined learning.

The simplest form of elicited behavior is reflexive behavior (Domjan, 1998). Reflexes are rapid, stereotyped, and involuntary responses that are usually controlled in a graded way by an eliciting stimulus (Ghez, 1991b). Elicited behavior is per definition not invariant. Even simple reflexes do not always occur in the same way and therefore show plasticity (Domjan, 1998). Learning alters both the structure and function of nerve cells as well as their connections. As a result, these so called plastic changes affect the effectiveness of specific synaptic connections (Kandel, 1991). This makes reflexive behavior interesting for investigators of learning and memory.

Presentation of an eliciting stimulus can cause decreases in responding i.e. habituation, or increases in responding i.e. sensitization. These two basic forms of non-associative learning are so fundamental that they occur in nearly all species (Domjan, 1998).

3.1.1 Habituation

Habituation is a gradual decline of existing responses to a stimulus that is neither rewarding nor noxious (Dudai, 1989; Kupfermann, 1991a). The animal learns to suppress the response after repetitions of the same or very similar stimuli (Dudai, 1989; Domjan, 1998; Kupfermann, 1991a). Response decrement due to sensory adaptation, injury, muscle fatigue, or drugs is excluded (Dudai, 1989; Domjan, 1998).

Habituation as a form of non-associative learning is stimulus-specific (Domjan, 1998). A new stimulus will elicit the previously habituated orienting response, showing that the subject was exclusively habituated to the original stimulus.

3.1.2 Sensitization

Sensitization is a more complex form of nonassociative learning than habituation (Kandel, 1991, p.1012). Sensitization is an augmentation of a response to a stimulus, that usually is strong or noxious. Unlike habituation, sensitization is not highly stimulus-specific; a sensitizing stimulus increases the general responsiveness to different stimuli (Dudai, 1989; Domjan, 1998).

A strong sensitizing stimulus can override the effects of habituation, causing a recovery of the habituated response (Domjan, 1998). This process is called dishabituation. There is controversy about the nature of dishabituation. Dishabituation can be seen as a disruption of habituation, or as an independent, facilitatory process, superimposed on habituation (Dudai, 1989). Dudai (1989) suggests that dishabituation is a special case of sensitization and an independent process.

3.1.3 The Dual-Process Theory

The dual-process theory of habituation and sensitization (Groves & Thompson, 1970) assumes that habituation and sensitization are not mutually exclusive but can be activated at the same time (Domjan, 1998). The response depends on which process is stronger and is supposed to be the net result of both processes (Domjan, 1998). Groves and Thompson (1970) suggest that habituation and sensitization occur in different parts of the nervous system, habituation in the so called *S-R system* and sensitization in what is called the *state system*. The S-R system is the shortest neural pathway between the sense organs stimulated by an eliciting stimulus and the muscles involved in making the elicited response. The state system is a neural structure that determines the general level of responsiveness, or readiness to respond, of the organism (Domjan, 1998).

3.2 Associative learning

In contrast to non-associative learning in which information is gathered about the properties of one stimulus, in associative learning a subject learns about the connection between two stimuli (Kandel, 1991). The two most commonly used procedures for investigating associative learning are classical (Pavlovian) and instrumental (operant) conditioning (Donegan et al., 1991). The distinction between both types of associative learning is based on the experimental procedures being used (Kupfermann, 1991a). Although both classical and instrumental conditioning involve manipulation of stimuli,

responses, and reinforcers, the instrumental conditioning can be regarded as the more complex type of the two types of learning (Dudai, 1989).

3.2.1 Classical conditioning

In classical conditioning an initially weak (neutral) stimulus becomes highly effective in producing a response after it has been repeatedly paired (associated) with a biologically strong stimulus (Kandel, 1991 p.1016). The neutral stimulus, called the conditioned stimulus (CS), is usually a tone or a light. The strong stimulus is called the unconditioned stimulus (UCS), because its effectiveness in eliciting the behavioral response is not dependent on any prior training (Domjan, 1998). One training episode consisting of one pairing of CS and UCS is called a conditioning trial. The time between two successive trials is intertrial interval (ITI) (figure 1). In conditioning experiments, the CS is usually presented in close temporal relationship with the UCS, the CS being prior to the UCS (figure 2). The time from the

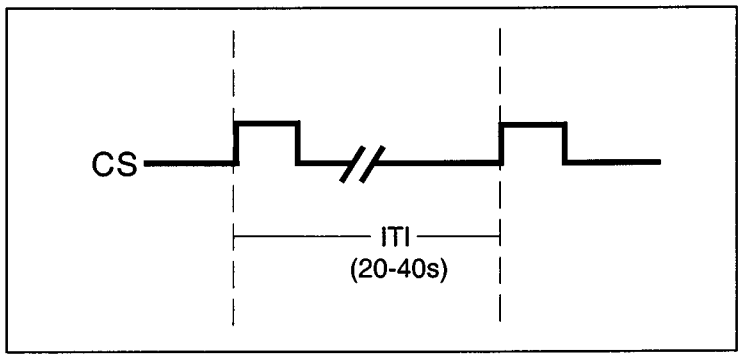

Figure 1. *A schematic illustration of ITI.*

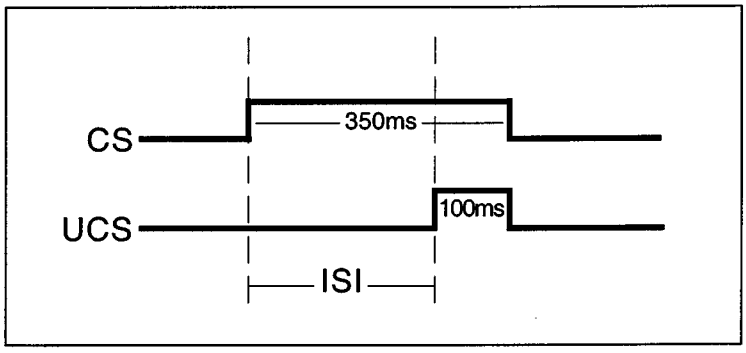

Figure 2. *Basic delay conditioning and an ISI of 250 ms.*

start of the CS to the start of the UCS during a conditioning trial is called the interstimulus interval (ISI) (Domjan, 1998).

The innate behavioral response is called the unconditioned response (UCR), for instance the salivary response of Pavlov's dogs at feeding times. The conditioned response (CR) often mimics

the UCR and starts to appear when the subject learns to associate the CS with the UCS. The behavioral response (CR) can be quantified in three different ways: **(1)** The *magnitude* of the CR, which reveals the size, vigor, or the extent of the response (e.g., the amount of the saliva of Pavlov's dogs) ; **(2)** The *latency* of the CR, which tells about the amount of time delay between the onset of the CS and the occurrence of the CR ; **(3)** The *probability* of the CR occurance (%CRs).

One of the most critical principles in conditioning tasks is that intesity of the CR decreases as a function of the number of CS presentations, when there is no reinforcing presentation of the UCS (Kupfermann, 1991a). This principle is called extinction.

Two types of conditioning tasks, so called *delay* and *trace* tasks can be distinguished on the basis of different temporal arrangements between CS and UCS (Kim et al., 1995). Delay conditioning consists of overlapping CS and UCS, often co-terminating (figure 2). In trace conditioning, there is a "trace" or "gap" between the CS and UCS, hence there is no overlap between CS and UCS (figure 3). The delay procedure is known to produce more rapid and stronger CR learning than the trace procedure (Kim et al., 1995), since trace conditioning requires additional memory capacity due to the interval between the CS and UCS. Clark and Squire (1998) even claim that trace conditioning may require

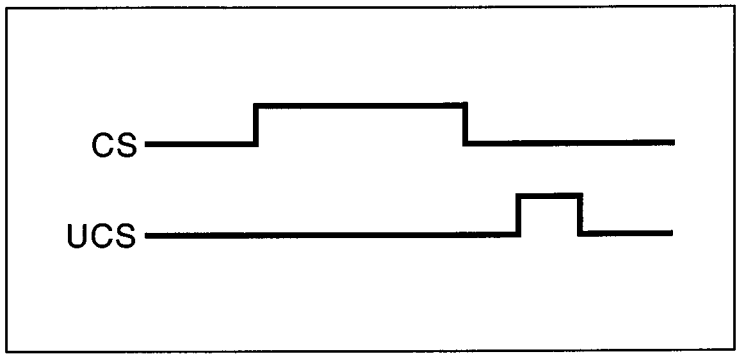

Figure 3. *Schematic illustration of trace conditioning.*

declarative knowledge because the trace interval between the CS and the UCS makes it difficult to process the CS-UCS relationship in an automatic, reflexive way.

The conditioning procedure can also be divided into *aversive* (defensive) and *appetitive* conditioning. This refers to the properties of the UCS: if the UCS is rewarding (e.g., food, water), the conditioning is termed appetitive; and if the UCS is noxious (e.g., electrical shock), the conditioning is aversive or defensive (Kupfermann, 1991a).

In defensive conditioning, the normal CR is an anticipatory response to the aversive UCS (Steinmetz et al., 1992). During training, the onset latency of the CR usually decreases and the peak latency (maximal amplitude) is set around the time of the UCS/UCR (Steinmetz, 1990). Thus, the CR is learned to protect from aversion (Thompson, 1991). Such somatic, often motor responses, for instance eyeblinks in response to airpuffs, are called specific responses since they protect from specific injury (Lavond et al., 1993). Specific responses are differentiated from nonspecific responses, (e.g., heart rate, pupil diameter, skin resistance), related to responsivity of the autonomic nervous system (Lavond et al.,

1993). In view of this, Steinmetz and Thompson describe Prokasy's two-process model as constituting of nonspecific responses that form the first phase and specific responses that form the second phase of conditioning (Steinmetz & Thompson, 1991). The nonspecific and specific response systems appear to be anatomically and functionally dissociable, amygdala for instance being critical to nonspecific responses (or fear-conditioning) and the cerebellum being critical to specific conditioned responses (Lavond et al. 1993).

The Rabbit NM conditioning (basic delay paradigm)

Much of the general literature on classical conditioning is based on data collected with the rabbit eyeblink conditioning paradigm (Woodruff-Pak, 1997). It has widely been used by many laboratories as a model paradigm to study brain substrates of basic associative learning and memory (Thompson & Krupa, 1994).

The rabbit's eyeblink response

The rabbit's eyeblink response (UCR/CR) consists of two discrete, yet highly correlated behaviors: primary eyelid closure and the NM extension (Krupa et al., 1996). Primary, external eyelid closure is mediated by the orbicularis oculi muscles, which are innervated by motor neurons of the facial nucleus via the seventh cranial nerve (Krupa et al., 1996). The NM extension is largely passive and results from contraction of the retractor bulbi muscle, which pulls the eyeball back into the socket, pressing the NM over the eyeball. This process is innervated by the abducens and the accessory abducens nuclei via the sixth cranial nerve in the brainstem (Dudai, 1989). The NM extends only momentarily and rarely passes the midline of the pupil (Dudai, 1989). The NM retraction to its resting position in the canthus of the eye appears to be mediated by the oculomotor (third cranial) nerve, apparently by direct innervation of striated muscle fibers in the NM (Thompson, 1976). The NM response is usually selected because it offers the opportunity to measure the overt behavioral response with greater sensitivity than the external eyelid closure (Dudai, 1989).

Unconditioned and conditioned stimuli: The UCS to cause NM response is a puff of air to the cornea of the rabbit's eye (e.g., duration 100 ms, air pressure 3 psi; equal to 2.1 N/cm^2). The standard training pressure is 3-4 psi (Gormezano, 1966). Another possibility is using a mild periorbital electrical stimulation. The CS can be a tone, a light or tactile stimulation. In the standard procedure, an auditory CS is used (200-400 ms, 85 dB, 1 kHz sine wave tone) (Thompson, 1976).

Conditioning procedure: Rabbits receive basic delay classical conditioning, as shown in figure 2. One training session usually takes one to two hours; a time during which normal, intact, undrugged rabbits will remain virtually motionless in a specific holder developed by Gormezano (Thompson, 1976; Gormezano, 1966). Training session consists of different types of trials depending on the experimental requirements. Typical trials are paired (CS and UCS) and unpaired (CS-only or UCS-only) trials. The durations of the ITI are pseudorandomly varied from 20 to 40 s with an average of 30 s.

A CR can be defined as a NM extension greater than 0.5 mm during the ISI (after CS onset but before UCS onset). The rabbit's spontaneous blinking rate is about 1 to 3 responses per

hour (Gormezano, 1966), or 2-3 % of trials when a 500 ms CS period is used (Steinmetz et al., 1992). The UCR can be defined as a NM extension after the onset of UCS (Ryou et al., 1998). The extension of NM is transduced to a voltage change by potentiometer, and the voltage changes are fed to an A/D converter and analyzed and stored by computers. During training the CR amplitude develops until it is as large as the UCR amplitude (Thompson, 1976).

There is some variation in the criteria for rejecting a CR (bad trials). One way to eliminate a bad trial is to exclude NM movements (0.7 mm or more) in the baseline period or eyelid closure in the first 25 ms of the CS period (Lavond et al., 1990; Clark et al., 1992). Trials can also be rejected if there are too many head or body movements during trial, to prevent contamination of CR recordings by artifact.

In some experiments, recording electrodes are implanted within the brain (intracranially) to

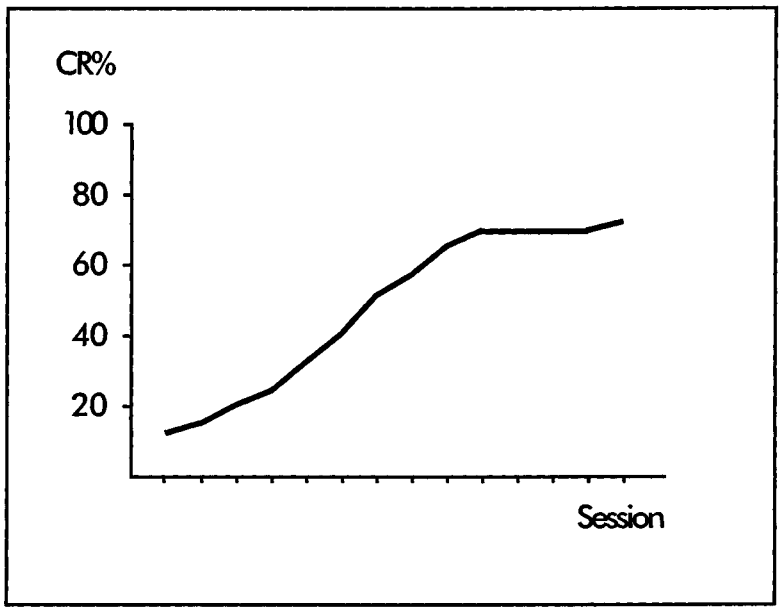

Figure 4: *A learning curve that reaches the asymptotic level of 70% CRs.*

record the activity of small groups of neurons or even of single neurons. The measured activity can then be related to ongoing experience or learning-induced changes in behavior (Thompson & Krupa, 1994, Rosenzweig et al., 1996).

Training procedures: In surgery to prepare the animal for the experiment, a small loop of surgical suture is placed into the NM to allow the NM movement measurements. Animals are allowed to recover from surgery before training. They are habituated to the training apparatus. This actually means that a rabbit is placed in a plexiglas restraining box in a sound-attenuating training chamber for 1 hour in two days in order to become adapted (Clark et al., 1992; Lavond et al., 1990; Ryou et al., 1998).

Training for classical conditioning can only start after finishing habituation to the training apparatus. One actual daily experimental session consists of about 100 trials per day, which are presented in a predefined order. Most trials are paired trials,where the CS presentation starts e.g., 250 ms before UCS presentation (ISI), both continuing together for 100 ms to coterminate (see figure 2). USC-alone

trials can be used to define the response to the USC without interfering effect of CS. The effect of CS per se and the development of learning are inferred from the CS-only trials.

Often used criterion to learning is the first time that eight CRs occurred in nine, consecutive trials (possible USC-alone trials excluded). Behavioral criterion of two successive 10-trial blocks with eight or more CRs is also used (Akase & Alkon, 1989); because at this point, rabbits are just beginning to show a marked increment learning curve (figure 4, p.13) but have not reached behavioral asymptote (Disterhoft et al., 1977). Another criterion is based on the percentage of CRs during a session / part of a session (block of e.g., 9 trials). Well-trained animals, those at the behavioral asymptote, are defined as those that respond at greater than 70 % (or 80%) of CRs on two consecutive days following learning (Clark & Lavond, 1996; Akase & Alkon, 1989). CR amplitude is said to be the most sensitive and reliable measure of performance of the NM response (Ivkovich et al., 1993). After training, retention can be tested and compared to earlier learning phase results to see the amount of extinction.

The experimental procedures include endless amount of variation, e.g. in the nature of stimuli or their timing, the age of subjects, lesion size and location, context etc. The standard procedure was described here.

3.2.2 Instrumental conditioning

Another major form of associational learning, discovered by E. Thorndike and studied by B.F.Skinner, is instrumental conditioning (or operational conditioning, trial-and-error learning). If classical conditioning is the formation of a predictive relationship between two stimuli (the CS and the UCS), instrumental conditioning can be considered to consist of the formation of a predictive relationship between a stimulus and a response (Kupfermann, 1991a, p.1000). We will describe shortly the main properties of instrumental conditioning and its correspondence with the classical conditioning.

In instrumental conditioning, one specific feature in a subjects behavior is critical in producing a significant outcome (Domjan, 1998). In principal instrumental learning starts from random behavior. Certain feature within this behavior might, on being systematically reinforced, develop from a random response to a systematic response, a so called instrumental response. Hence, the key feature in instrumental conditioning is response reinforcement, as a result of which the probability of this response increases. Reinforcing is a term that refers to strengthening or increased rate of responding (Domjan, 1998). The general observation is that behaviors that are rewarded tend to be repeated at the expense of behaviors that are not, whereas behaviors followed by punishment will be less likely to recur; a principle called the law of effect (Thorndike, 1911). Animals generally learn to associate stimuli that are biologically important to their survival; still, there is enormous variation between the effectiveness of different reinforcers. Thorndike's idea of belongingness explains these differences by the organism's evolutionary history that predisposes the brains of that species to learn certain associations more readily than others (Kupfermann, 1991a; Thorndike, 1932).

The laws that govern instrumental and classical conditioning are quite similar, suggesting that they may be manifestations of a set of common underlying neural mechanisms (Kupfermann, 1991a).

In both forms of conditioning, there are optimal time settings for creating optimal conditioning. Classical and instrumental conditioning are clearly distinguishable conceptually and procedurally, but in practice most conditioning procedures involve instrumental as well as classical conditioning. For example in rabbit eyeblink conditioning, the conditioned response also helps the subject at least partly to protect himself from an airpuff, if the response is correct in timing. In this way the rabbit's behavior can also be interpreted as exemplifying instrumental avoidance. However, isolating the effects of these common components can be difficult and is sometimes impossible (Domjan, 1998).

4 PARTS OF THE BRAIN INVOLVED IN CLASSICAL CONDITIONING

4.1. Background

The great complexity of the vertebrate brain makes finding engrams or memory traces a true challenge. Short-term memory formations might be maintained by ongoing neural activity, but long-term memory is more likely related to plastic rather than dynamic changes and, hence, involves physical changes in the brain (Kupfermann, 1991).

4.1.1 Sensory and motor pathways

As mentioned before, elicited behavior occurs in reaction to specific environmental stimuli (Domjan, 1998), and the knowledge about these stimuli is acquired through a variety of sensory receptors. In vertebrates the sensory information is carried to the central nervous system via neural sensory pathways and transformed by the brain into perceptions and/or into commands for movement (Domjan, 1998; Kandel et al., 1995). The neuronal information concerning the movement or motor output is relayed via these motor pathways. The circuit of neurons that is responsible for producing a reflex, the *reflex arc* , represents smallest number of neural connections necessary for reflex action at the level of the spinal cord (Domjan, 1998; Kalat, 1995). It consists of a sensory (or afferent) neuron activated by the stimulus; an interneuron in the spinal cord, relaying the sensory message further; and the motor neuron, which receives stimulation from the interneuron. The motor (or efferent) neuron activates the muscles involved in movement.

4.1.2 Learning and modification of behavior

The sensory messages may be relayed to the brain by additional afferent neural structures and through that circuitry the reflex reaction may be modified (Domjan, 1998). This modification includes synaptic changes underlying learning (Kandel et al., 1995). Often the only way to tell whether or not learning has occured are the changes in behavior. Our definition of learning presumes, however, that the change occurs in the underlying mechanisms of learned behavior, not only in the overt behavior itself, since behavior can be altered for many reasons in addition to learning, e.g., motivational states (Domjan, 1998). This is why behavior, or *performance*, is separated from learning (Domjan, 1998).

In many types of learned behavior the sites of plasticity are more centrally located than the primary sensory systems or motor neurons (*sensory-motor circuit*) (Thompson, 1976). The whole neural

circuitry essential for the development and expression of a particular form of learning is called *the memory trace circuit*, including both the sensory-motor circuit and *the memory trace (engram)*, i.e. the subset of neural elements that exhibit the learning-induced plasticity associated with learned behavior (Thompson, 1976). Memory is often localized in different places throughout the nervous system (Kandel et al., 1995). Lavond and associates (1993) describe the essential and nonessential memory traces or engrams as follows: '*Essential memory traces represent the circuitry responsible for forming the association in classical conditioning. Nonessential memory traces are important for facilitating, adapting, and modifying the final performance of the learned behavior* '(Lavond et al., 1993).

4.1.3 Localization

The reflexive memory for a particular task is considered to be tied to the activity of the correspondent sensory and motor systems involved in learning that task (Kandel et al., 1995). As a result, reflexive memory can be studied in reflex systems in both vertebrates and invertebrates.

4.2 Essential Pathways in the Rabbit NM Conditioning

In this section, we will concentrate on the examination of the essential memory circuits involved in the rabbit NM conditioning, using an auditory CS and an airpuff UCS.

The study of the anatomical engrams in relatively simple conditioning tasks starts from the identification of the neuronal systems essential for the relevant behavior (Dudai, 1989). After this, the *loci of plasticity* in the essential systems are attempted to identify, as well as in brain regions known to connect with them (Dudai, 1989). The reasonable start point in identifying engrams of relatively simple conditioning in the vertebrate brain is the input pathway for the CS (Dudai, 1989).

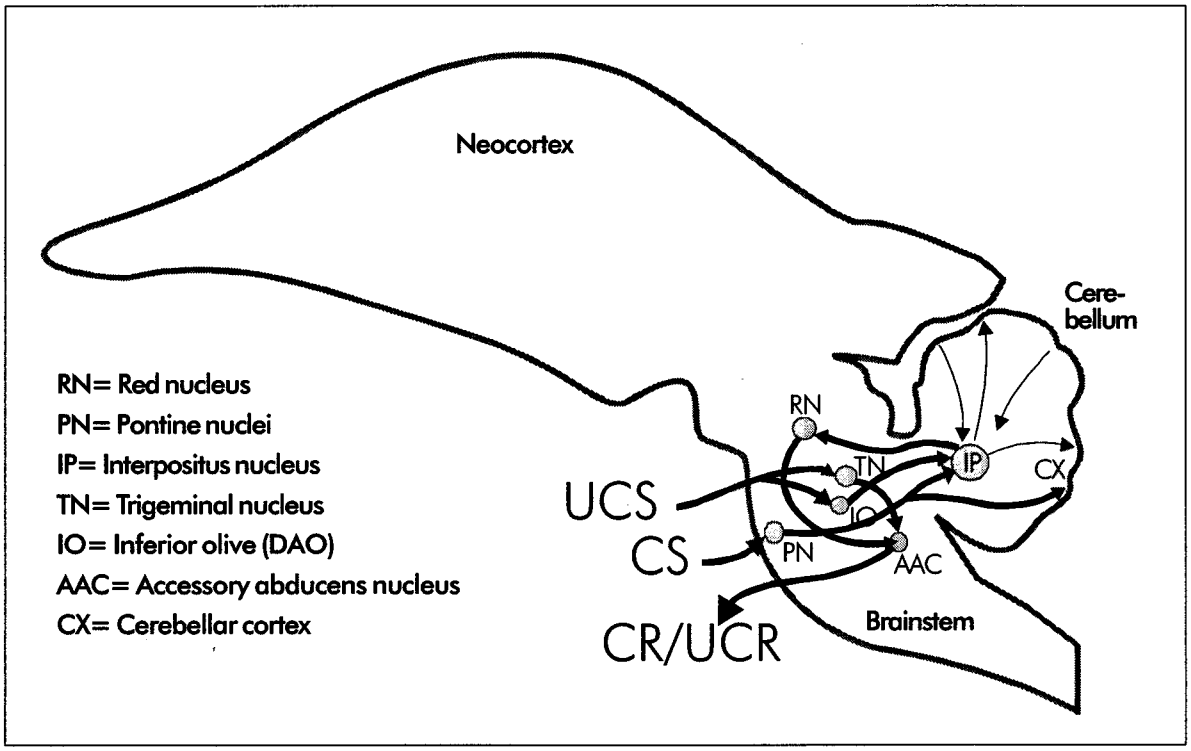

Figure 5. *CS/UCS- and CR/UCR-pathways involved in NM conditioning (Pinel et al., 1996)*

4.2.1 CS-pathway: the route of the auditory stimulus

The main route of auditory impulses is from the receptors in the ear to the auditory cortex. In the human brain, the cochlear nucleus receives input from the ipsilateral ear and sends impulses on to superior olive nucleus. The superior olive nuclei (and all the later stages) receive input originating from both ears (Kalat, 1995). The route then goes to the inferior colliculus in the midbrain, and next to the medial geniculate nucleus in the thalamus and from there finally to the auditory cortex.

However, there are other routes in addition to the main auditory route, one route particularly important in view or this paper is the route going to the cerebellum. It forms the CS pathway in the classical conditioning of rabbit's NM. The ventral cochlear nucleus projects to the lateral region of the pontine nuclei in the brain stem (Gould et al., 1993). The pontine nuclei then send axons (so called

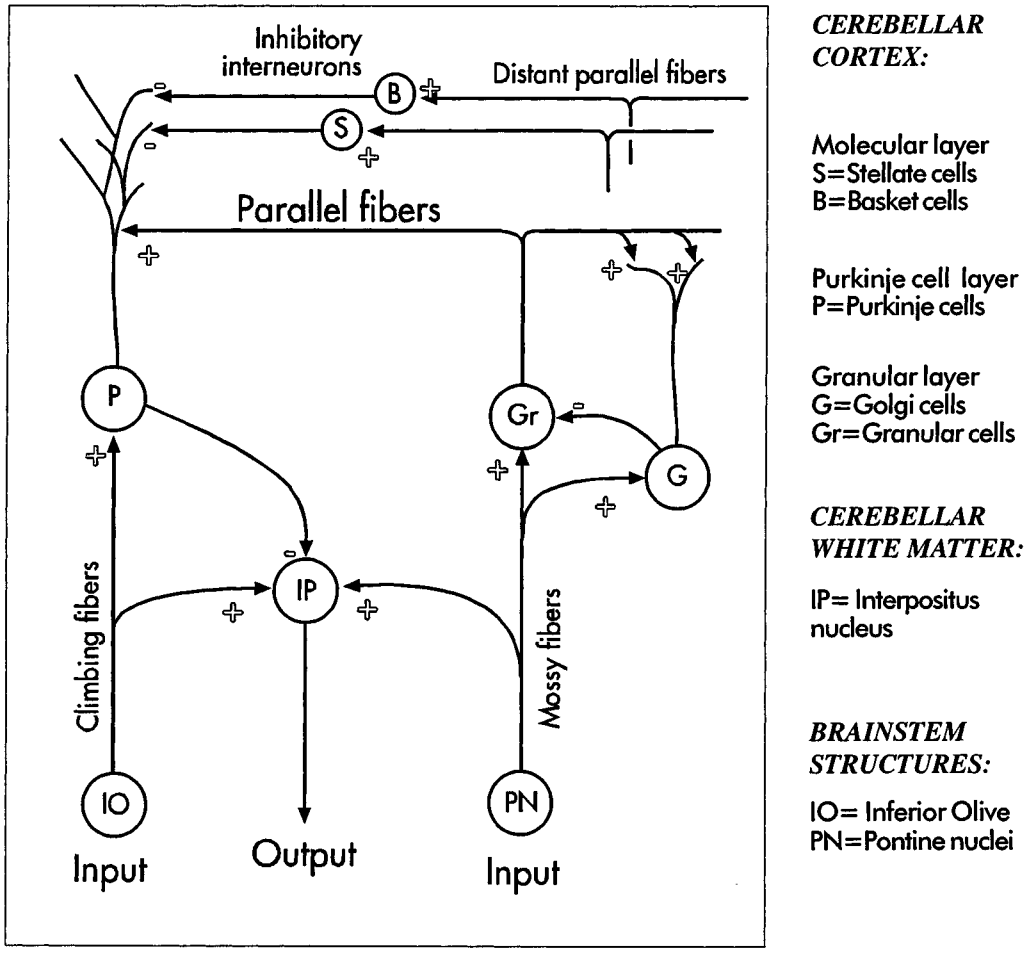

Figure 6. *The structure of the Cerebellar cortex*

mossy fibers) to two parts of the cerebellum: the cerebellar cortex and the IP nucleus. The mossy fibers constitute the major afferent input to the cerebellar cortex; they synapse on the granule cells of the granular layer (Kandel et al., 1995).

4.2.2 US-pathway: the inferior olive-climbing fiber system

Information about movement errors and aversive UCSs is provided by the *inferior olive-climbing fiber system* (Thompson et al., 1998). The UCS pathway consists of somatosensory projections via the trigeminal nucleus to the dorsal accessory portion of the inferior olive (DAO) and from there as climbing fiber projections to the cerebellum (Clark & Lavond, 1996). Climbing fibers go to the deep cerebellar nuclei (e.g. interpositus nucleus) and to the cells of the cerebellar cortex (granule cells and Purkinje cells) (Rosenzweig et al., 1996).

4.2.3 Site of convergence

Signalling between neurons takes place at electrical or chemical synapses (Kandel et al., 1995). Another important principle in the organization of the brain is the divergence and convergence of neuronal connections (Kandel et al., 1995). Divergent neurons usually branch out and make multiple connections with target neurons that represent the second stage of information processing; subsequent connections will diverge even more (Gordon, 1991, p. 583). In the situation of convergence, a target cell receives information from several presynaptic cells, hence such a neuron is target for progressively converging connections (Kandel et al., 1995). By receiving signals from multiple neurons, the target cell is able to integrate diverse information (Kandel et al., 1995). Convergent pathways permit a given brain region to integrate the input it receives from various sensory systems (Kandel et al., 1995).

Convergence is an important mechanism by which higher centers can control the expression of reflex behavior, especially the spatial organization of reflexes, determining which sensory sensory inputs are enhanced or suppressed and which muscles contract or relax (Gordon, 1991, p.583). The convergent inputs onto a neuron may include for example sensory inputs from the periphery, e.g., information related to the CS and UCS in conditioning tasks (Gordon, 1991). In classical conditioning, the learning or association of CS and UCS has to occur at the brain sites where the two forms of information converge (Kim & Thompson, 1997). In the rabbit eyeblink conditioning, both the IP and the cerebellar cortex are hypothesized sites of UCS-CS convergence (Kim & Thompson, 1997; Dudai, 1989). We will take a closer look at these hypotheses later in this chapter.

4.2.4 UCR-pathway

The unconditioned motor response is mediated by the neurons of the trigeminal nuclei. These nuclei, in turn, excite motor neurons in the accessory abducens nucleus, and from there the sixth cranial nerve relays to the retractor bulbi muscle (Pinel, 1990). Contraction of this muscle pulls the eyeball back into the socket, which finally leads to the passive extension of the NM across the eye (Krupa et al., 1996).

4.2.5 CR-pathway

The CR pathway appears to consist of fibers starting from the interpositus nucleus ipsilateral to the trained side of the body in the superior cerebellar peduncle, then crossing to relay in the contralateral magnocellular division of the red nucleus, and crossing back to descend in the rubral pathway to act

ultimately on motor neurons (Donegan & Thompson, 1991).

4.3 Localized engrams in eyeblink conditioning

With respect to basic delay conditioning, converging evidence from lesion, stimulation, and recording studies allowed the construction of a relatively complete theoretical circuit including neural pathways for the CS, UCS, CR and UCR (Clark et al., 1997). Our description of the participating brain substrates will follow the pathways described above.

4.3.1 Lateral pontine nuclei

The pontine nuclei are located in the pons. Their axons cross the midline and run to the contralateral half of the cerebellum, and thus they are involved in the cerebellar control of movement and posture (Kelly, 1991; Tracy et al., 1998). Pontine nuclei are a site of convergence for sensory input obtained from various peripheral sources (Knowlton & Thompson, 1992), hence it is a possible site of learning or association between CS and UCS. Recent work (Cartford et al., 1997) has provided evidence that learning-related unit activity which has been observed in the pons, is mainly dependent on the IP and thus, the pons is not a site of essential plasticity for learned response.

4.3.2 Trigeminal complex

The trigeminal complex is located in the brain stem and consists of the spinal, principal, motor, and mesencephalic trigeminal nuclei and the surrounding reticular formation (Clark & Lavond, 1996; Dodd & Kelly, 1991, p. 701). The spinal trigeminal nucleus is divided into three subnuclei, pars caudalis, pars interpolaris and pars oralis (Clark & Lavond, 1996). Like most cranial nerves, the trigeminal (5^{th} cranial) nerve includes both sensory and motor components (Kalat, 1995). In the rabbit NM conditioning, the trigeminal nerve conveys sensory information about the corneal airpuff UCS, and the neurons of the trigeminal nuclei (pars oralis) in turn excite motor neurons in the accessory abducens nucleus, which project via the 6^{th} cranial nerve to the retractor bulbi muscle causing the eyeball retraction, and the nictitating membrane response (Pinel, 1990).

The trigeminal complex is of interest to researchers of the conditioned eyeblink response for two reasons: **(a)** it is part of the essential circuitry for eyeblink conditioning (see figure 5) **(b)** because the trigeminal complex has access to CS and UCS information as well as motor systems for driving the CR, it might participate in the generation of the behavioral CR or even be a site of plasticity for the learned response (Clark & Lavond, 1996). However, the results obtained by Clark & Lavond (1996) show that the trigeminal complex is not the site of essential plasticity, but this region normally plays an important role in eyeblink conditioning.

4.3.3 Inferior olive

In untrained animals, the neuronal unit activity recorded in the inferior olive (IO) shows no responses to the CS, instead, it shows a clear evoked increase in unit activity at the onset of the UCS, which is

consistent with the hypothesis that IO is part of the UCS pathway (Lavond et al., 1993). The UCS pathway acts as the reinforcing or teaching input to the cerebellum for the learning of movements (Thompson et al., 1998). Lesions of the inferior olive, especially those located in dorsal accessory olive (DAO) region of the inferior olive (contralateral to the trained eye) cause an inability to acquire conditioned NM responses. The UCS is still aversive to the animals after the DAO lesion; the difference is that they are unable to learn or remember the CR (Thompson et al., 1998). If the DAO is lesioned after learning, animals show gradual extinction of the CR (Thompson et al., 1998). Extinction demonstrates that the memory trace cannot be in the IO (Lavond et al., 1993).

4.3.4 Cerebellum

In humans, the cerebellum is a large hindbrain structure (Kalat, 1995). It occupies most of the posterior cranial fossa and is composed of the cerebellar cortex, internal white matter, and three pairs of *deep nuclei*: the fastigial, the interpositus (itself containing two nuclei, the globose and emboliform), and the dentate nuclei (Ghez, 1991a).

The cerebellum receives input from the periphery and from all levels of the central nervous system and the output is primarily transmitted to the motor regions of the cerebral cortex and the brain stem (Kandel et al., 1995). The input and output connections of the cerebellum run through three symmetrical pairs of tracts (cerebellar peduncles) that connect the cerebellum to the brain stem (Dudai, 1989). Afferent pathways convey information to the cerebellum via *the inferior cerebellar peduncle* and *the middle cerebellar peduncle* (Glickstein & Yeo, 1990). *The superior cerebellar peduncle* is the primary efferent peduncle of the cerebellum via the deep cerebellar nuclei.

Anatomically the cerebellum is divided into three lobes (*anterior, posterior and flocculonodular lobe*) and subdivided into ten lobules (Ghez, 1991a). The surface of the lobules is covered with convolutions called folia. Furthermore, the cerebellum can be divided longitudinally into *vermis* in the midline, and the left and right *cerebellar hemispheres* (Ghez, 1991a). Each hemisphere is composed of an intermediate and lateral part (Ghez, 1991a).

Functional divisions corresponds roughly with the anatomical subdivisions (Kandel et al., 1995). The flocculonodular lobe forms the *vestibulocerebellum* connecting the vestibular nuclei in the medulla. The vermis projecting to the fastigial nucleus and the intermediate hemispheres projecting to the interpositus nucleus form the *spinocerebellum.* The *cerebrocerebellum* is the lateral part of the cerebellar hemisphere connected to the pontine nuclei (input) and the dentate nucleus (output) and further to the thalamus and the motor and premotor cortices (Kandel et al., 1995).

The cerebellar cortex is a relatively structure consisting of three layers (figure 6): the molecular, the Purkinje cell, and the Golgi granular cell layers (Ghez, 1991a). These layers contain five types of neurons: stellate, basket, Purkinje, Golgi, and granule cells, all arranged in a highly regular manner (Ghez, 1991a). The top layer called *molecular layer* is mainly composed of the axons of the granule cells. These axons split, and from them parallel fibers emerge (Kalat, 1995). The parallel fibers run perpendicular to the extensive dendritic trees of Purkinje cells supplying excitatory input to the Purkinje

cells (Kalat, 1995). The other excitatory input to the Purkinje cells, the climbing fibers, originate in the IO and their axons wrap around the Purkinje neurons forming powerful synaptic connections (Ghez, 1991a). Stellate and basket cells are inhibitory interneurons of the molecular layer; they modulate the activity of the Purkinje neurons (Kandel et al., 1995). The next layer, the *Purkinje cell layer*, contains the cell bodies of the Purkinje neurons, which are the sole output of the cerebellar cortex; this output is inhibitory and uses γ-aminobutyric acid (GABA) as a neurotransmitter (Ghez, 1991a; Dudai, 1989). The *granular layer* contains a vast number of densely packed small neurons, mostly small granule cells (Ghez, 1991a). Also a few inhibitory Golgi cells are found in this layer (Kalat, 1995). The granular cells form complex synaptic contacts with the afferent mossy fibers (Ghez, 1991a). Mossy fibers influence Purkinje neurons indirectly through these synapses with granular cells (Ghez, 1991a).

The traditional view of the cerebellum is that it coordinates gait and voluntary movement, is responsible for balance and posture, and is important in speech and control of gaze (J.D. Schmachmann in Cerebellum and cognition, p.xxvii). It also seems that the cerebellum plays a role in cognitive processing (Bloedel & Bracha, 1997, p. 629). For any behavior in which it is involved, the cerebellum participates both in regulating its execution and in modifying its characteristics to optimize its performance in a specific context (Bloedel & Bracha, 1997, p. 630).

Cerebellar interpositus-nuclei: A number of studies have reported that the essential memory traces involved in basic delay conditioning are formed in the cerebellar interpositus nucleus and/or the cerebellar cortex (Ryou et al., 1998). The CS relayd by mossy fibers from the pontine nuclei and the UCS relayd by the climbing fibers from the IO converge in the cerebellum (Steinmetz and Thompson, 1991; Yeo et al., 1985a; Yeo et al., 1985b).

The IP is one of the deep nuclei in the cerebellum. It receives its input mainly from the lateral pontine nucleus, the IO and the cerebellar cortex and its major output projects to the red nucleus and then to the motorneurons in the facial, abducens, and accessory abducens nuclei (Dudai, 1989).

Strong evidence from lesion, recording, and stimulation studies supports the hypothesis that the neuronal plasticity responsible for classical conditioning resides in the interpositus nucleus of the cerebellum or in its afferents (Lavond et al., 1993; Lavond et al., 1990).

Steinmetz and associates point out, that at least two experimental criteria must be met for a brain region to qualify as a putative essential site for the acquisition and storage of neural plasticity associated with learning: (**a**)Neural recordings should show a learning-induced pattern of firing and (**b**) Destruction of the region must prevent learning and abolish retention without affecting the reflex UCR (Steinmetz et al., 1992). The IP lesion (as small as 1 mm^3) both prevent acquisition and abolish retention of the learned behavioral eyeblink response (CR) without otherwise impairing the reflex response (UCR) or learning on contralateral side of the lesion (Steinmetz et al., 1992; Thompson, 1991). The conclusions drawn from these results are **(1)** that the ipsilateral IP is necessary for the learning of the eyeblink response, **(2)** that it is also needed for retention of the learned response, and **(3)** that the ablation of the IP does not prevent the motor response. The effects of unilateral IP lesions do not carry over to the contralateral side; the learning on the unlesioned side procedes quite as in normal, intact animals (Lavond et al., 1994).

The observation that the lesion does not affect the reflex UCR or impair learning on the contralateral side of the body is important, as these findings indicate that the effect of the lesion is not due to a general performance deficit or due to alterations in nonspecific systems like arousal or motivation (Steinmetz & Thompson, 1991). Recordings from the IP during eyeblink conditioning revealed discharging cell populations when the conditioning stimuli (CSs) were presented. Further, these recordings showed cells that also discharged as a result of training just prior to execution of the classically conditioned response (Steinmetz et al., 1992); in other words, the IP neurons showed learning-induced firing patterns. Thus, the IP fulfills both criteria set for the putative essential sites of neural plasticity mentioned earlier.

Other results give additional support to the role of interpositus nucleus in this type of learning. For example, stimulation of the IP evoked a discrete eyeblink before any conditioning occurred (McCormick & Thompson, 1984), which requires that the site of plasticity be hard wired and that conditioning modify the threshold for the CS in order to evoke the motor response (Lavond et al.,1993, p.326). In recent work, reversible lesioning created by cooling has shown that when the IP is cooled during 5 days of acquisition training, the animal exhibits no behavioral learned responses and no learning-related unit activity occurs in the red nucleus (the major cerebellar projections target). When cooling is terminated and training continues, the subjects acquire learning as if they were completely naïve. (Lavond et al., 1993)

Cerebellar cortex: Although cerebellar lesions that also affect the interpositus nucleus abolish CRs completely and permanently, the effects of lesions of the cerebellar cortex in itself are less clear (Kim & Thompson, 1997). Researchers have consistently found CRs after large cerebellar cortical lesions when the IP remains intact (R.E. Clark et al., 1990; Lavond et al., 1993). The same results have, however, been interpreted in different ways, as described here by Lavond and Thompson:

> *We (R.F. Thompson and D. G. Lavond) agree that the cerebellum and its associated circuitry are essential (necessary and sufficient) for classical conditioning of discrete, adaptive, somatic, conditioned responses to aversive stimuli and that the cerebellar cortex plays an extremely important role in classical conditioning. Thompson believes that the engram is represented in the interpositus and multiply in cerebellar cortex. Lavond believes that cortex is important for some aspects of this learning but not essential for the basic association (Lavond et al., 1993).*

Thompson (1991) stresses that the organization of the cerebellar cortex with its abundance of neuronal machinery compared to the interpositus nucleus makes it more suitable for forming memory traces. The number of Purkinje cells correlates highly with the amount of eyeblinking; in the absence of Purkinje cells, eyeblink conditioning is reduced significantly, but not completely blocked as with IP lesions (Kim & Thompson, 1997). In recordings, the cells in the interpositus increase their activity while Purkinje cells in the cortex decrease their activity, which is consistent with the inhibitory projection from cortex to the IP (Lavond et al., 1993).Because of the reciprocal connections between the IP and the cerebellar cortex, the latter structure cannot be ruled out as playing a key role (Thompson et al., 1998). Yeo et al. (1984) also reported that small lesions of the cerebellar cortex in the hemispheral portion of lobule VI, with sparing of the underlying nuclei, abolish the ipsilateral conditioned NM response and prevent its reacquisition.

The evidence supports an important role for the cerebellar cortex in amplifying and in timing of the conditioned response. After cerebellar cortical lesions, conditioned responses take much longer to acquire and they are small and inconsistent, while unconditioned responses are larger than in the intact animal. Most likely the cerebellar cortex is critically involved in facilitating acquisition and in improving the size, shape, and timing of conditioned responses. (i.e. the cerebellar cortex learns something). However, The majority of the studies has found that the cerebellar cortex does not appear to be essential for basic classical conditioning (see also: Lavond et al., 1993).

Arguments against the role of the cerebellum in NM conditioning. The lesion and recording studies have generally been cited as evidence that plasticity in the cerebellum is critically involved in the learning and memorizing of classically conditioned responses. Welsh & Harvey (1989) and Kelly et al. (1990) have challenged the cerebellar hypothesis. These researchers claim that cerebellar lesions simply produce a performance deficit that affects the execution of CRs initiated by plasticity present outside the cerebellum (Steinmetz et al., 1992a; Steinmetz et al., 1992b). We will next present some of the controversial results and conclusions of these investigators and the main criticism on their conclusions, as well as some alternative interpretations regarding the localization of the engram in rabbits NM conditioning.

As said earlier, a number of studies have reported that lesions of the cerebellar interpositus nucleus (IP) completely and permanently abolish the nictitating membrane CR but have no effect on the UCR. In contrast, Welsh and Harvey (1989) claim that lesions of the IP do affect the UCR (Ivkovich et al., 1993). Many factors can influence the UCR (e.g., individual animal differences); so in order to demonstrate effects of lesions on the UCR it is essential to compare UCRs in the same animals before and after lesion, which Welsh & Harvey did not do (Thompson & Krupa, 1994 p.528).

Welsh & Harvey (1989a) also question the cerebellar hypothesis because they report that conditioned responses still occur with cerebellar lesions; however, many of their lesions are incomplete and in the few cases of complete lesions, their animals did not show CRs (Lavond et al. 1993 p. 332).

Welsh & Harvey (1989b, 1991) reported conditioning despite IP anesthesia acquired by lidocaine injections. The reversible lesioning experiments by Clark et al. (1992), Chapman et al. (1990) and Krupa et al. (1996) however contradict this observation. Cannula location and drug dose / concentration are critically important in maintaining the inactivation state; this might explain the results obtained by Welsh & Harvey (Lavond et al., 1993; Clark et al., 1992).

Kelly & Bloedel (1990) also tried to find evidence that conditioning of the nictitating membrane/ eyeblink reflex in the rabbit could occur in the decerebrate-decerebellate preparation (Kelly et al., 1990). However, they reported results from only a very small subset of their animal population, and used measurement methods that would count spontaneous responses as CRs. Moreover, they did not use any of the control groups, which are considered to be essential to rule out nonassociative processes such as sensitization or pseudoconditioning. Finally, they did not use standard training procedure (e.g., their 9 s intertrial interval instead of 20-40 s) (Lavond et al., 1993, p. 333). In fact, Mauk & Thompson (1987) showed that normal animals, trained and then decerebrated, retained the eyeblink CRs only if the

cerebellum and the red nucleus (part of the CR pathway) *were not damaged* (Lavond et al., 1993).

Kelly & Bloedel (1990) also doubted the permanence of the disappearence of the eyeblink CR after IP lesioning, and argued that the recovery of the eyeblink CR following IP lesions was not seen in earlier studies because the effects of these lesions were examined after only a period of days following the lesions. However, the impact of extensive post-lesion training has been carefully examined (Steinmetz et al., 1992; Lavond et al., 1984), and the animals never showed any signs of recovery of the CRs (Steinmetz et al., 1992).

Krupa and associates (1996) used reversible inactivation with muscimol to determine whether brainstem structures (accessory abducens nucleus, ACC; facial nucleus, FN; and surrounding reticular formation, RF) are critically involved in the acquisition of the rabbit conditioned eyeblink response. During inactivation training, no CRs or UCRs were seen, however, when training was continued without inactivation, the subjects performed the CR at asymptotic levels right from the start of the training without inactivation (Krupa et al., 1996). The rabbits had fully learned the CR during inactivation of the brainstem nuclei, despite showing no CRs or UCRs during inactivation, a result which rules out any critical role for the ACC, FN, or surrounding RF in the acquisition of the classically conditioned eyeblink response (Krupa et al., 1996).

The existing evidence strongly favors the hypothesis that the essential memory trace circuit necessary for the NM conditioning includes the cerebellum and its associated brain stem circuitry and that the memory traces are formed and stored in the cerebellum (Thompson et al., 1997).

4.3.5 Red Nucleus

As mentioned earlier in defining the conditioned response pathway, the main hypothesis regarding the red nucleus (RN) is that it acts as a relay for motor commands from the cerebellum, and that plasticity generating CR occurs in the cerebellum or an afferent structure of it (Clark & Lavond, 1993). In addition to this, the results of Ryou and associates (1998) suggest that conditioning-related inputs from the IP via RN may critically affect the conditioning-related neuronal activities in the hippocampus as well as the behavioral CR.

4.3.6 Hippocampus

In a recent review, Ryou et al. (1998) concluded, that the role of hippocampus in conditioning is still unclear. In summary, they showed that **(1)**. Hippocampus participates in representation of the temporal relations between CS and UCS during conditioning. **(2)**. Hippocampus is important in encoding the context (Penick & Solomon, 1991). **(3)**. The hippocampus participates in the modulation of temporal or topographical characteristics of learned responses, especially when anonoptimal ISI is used. **(4)**. For more complex paradigms such as trace conditioning, the hippocampus is important. However, it is not essential for standard delay conditioning, since the hippocampal lesions have not prevented conditioning under these circumstances (Ivkovich & Thompson, 1997; Ryou et al., 1998).

The property of the hippocampal neurons to preferentially respond to stimuli that are temporally

paired (in such a way as to facilitate conditioning), has made it an interesting target for research in this area (Weiss et al., 1996). Thompson (1991), also emphasizes that the engagement of the hippocampus in learning is not limited to classical conditioning but appears to occur in most forms of learning. Several hypotheses have been advanced to explain the paradox between the recording and lesion data of hippocampus (Penick & Solomon, 1991). The hippocampus could possibly be one of several parallel systems involved in the elaboration of the CR (Thompson et al., 1976), or hippocampus simply serves as a motor feedback or reefference system (Moore, 1979). A final possibility is that the hippocampus participates in a variety of learning functions but is only essential in certain conditions (Penick & Solomon, 1991).

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