Specific and Nonspecific Associations in Eyeblink Conditioning in Rabbits

Licentiate's thesis

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Introduction

The purpose of this work is to set up theoretical and practical guidelines for a research for determining the emotional memory engram associated with learning of a specific event. More specifically, the memory trace formation for a discrete conditioned eyeblink response (Gormezano, 1966) is a well defined process but little is known about the locations and processes which are involved in dealing with the emotional content of the unconditioned stimulus, i.e., about the motivation to learn such a task. In their review, Thompson & al (1998) make an initial distinction between reinforcement and motivation in eyeblink conditioning, mainly concentrating to the former. However, as they point out, in certain circumstances the subject can be taught the task without any aversive expression whatsoever. Specifically, they note that if the training is restricted only to the cerebellum (by electrical brain stimulation) the subject learns the task but do not seem to be experiencing any aversion related to the defensive reflex-eliciting stimuli, which normally occurs of the stimulus is presented peripherically (airpuff towards the cornea is a standard procedure). Stanton (2000) reviews the literature of Pavlovian conditioning and states that the more than a century of work on the field implies that conditioning, at least in mammalian species, involves three basic classes of associations: sensorimotor, affective and cognitive; he further asserts that the structures basically involved in these associations are, respectively, the cerebellum, amygdala and hippocampus. The relative involvement of these basic associations (and the structures behind them) varies depending on the task the subject faces. In classical eyeblink conditioning, the sensorimotor system is probably the most essential, and therefore, most widely studied. However, during the eyeblink training, other kinds of associations can be seen, although not as striking as in fear conditioning, for instance.

When the eyeblink conditioning is done with natural stimuli (tone CS and airpuff US) the subject does not only learn to perform the defensive eyeblink response to the CS. Long before the emergence of the conditioned response the animal's reflex is modified in a robust and specific manner to the CS (Donegan & Wagner, 1987). This modification of the unconditioned response in this context is called reflex facilitation for the UR is elicited more vigorously both in amplitude and latency (Wikgren, Ruusuvirta & Korhonen, 2000). Only a part of this modification is related to the performance of the discrete conditioned response. It is well known that by inactivating or lesioning the anterior part of the interpositus nucleus in the cerebellum, the conditioned responding ceases. This, however, does not affect the conditioned reflex facilitation (Wikgren et al, 2000). Therefore, it is likely that some learning specific activity is found during blocking of the discrete and well-timed learned response. The structures where this kind of activity could be found, would probably involve the very same regions that are associated with emotions. Thus, reflex facilitation during the IPN inactivation might serve as a useful tool in understanding the emotional content of the memory trace formation.

Two classes of associations are proposed during the eyeblink conditioning. These have been described by the word-pairs like diffuse-discrete, preparatory-

consummatory, autonomic-somatic (Brandon & Wagner, 1991; Konorski, 1967). The rationale has been that in any conditioning procedure that involves emotionally significant US, there is a dual process of learning consisting of two classes of representation of the US, emotive and sensory (Brandon & Wagner, 1991). These representations are supposed to be relatively independent processes. Further it is proposed that the emotive representations affect the formation of the somatic ones. Brandon & Wagner (1991) nicely showed this is a series of experiments where the context CS (the emotive CS) became to potentiate learning of the eyeblink reflex to a new CS.

One has to bear in mind that terms used to refer to emotional and cognitive aspects of learning are generally anthropomorphic ones. Here, for the most part of the text I will consider associations formed during the eyeblink conditioning as belonging to either one of the two categories: discrete and nonspecific. Therefore, much cannot be said about whether *conditioned fear,* for instance, refers more to the actual emotion or conscious interpretation of such a state. For my purposes, the conditioned fear consists of the actual movement to avoid such a condition as opposed to all the other conditioned aspects of fear (autonomic responses, arousal, etc).

General methodology in eyeblink conditioning experiments

Conditioning procedures

The simplest case of conditioning is the standard *delayed classical conditioning* (upper left in Figure 1). Here the CS starts before the US and, at the end, temporally overlaps with it. This is a procedure, which results in the fastest learning rate. When the stimuli are presented in reverse order the procedure is generally named *backward conditioning* (lower left in Figure 1). It is notable that this procedure does not result in overt responses even though the two stimuli are just as contiguous in time as they are in delayed conditioning. This is supposed to be due to the properties of the CS and US

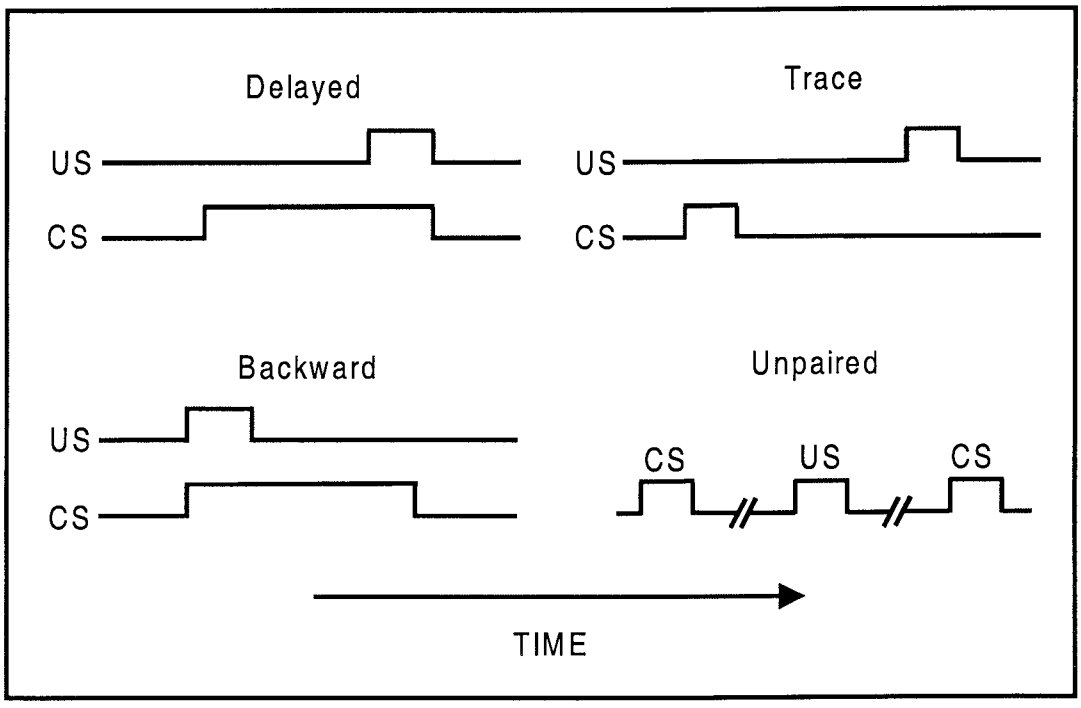

Figure 1. The basic training paradigms used in conditioning experiments

by themselves, that is, there are qualitative differences between the stimuli that could serve as either a CS or a US. This qualitative difference probably stems from biological significance of the stimuli. *Trace conditioning* is a procedure somewhat more demanding than simple delayed conditioning (upper right in Figure 1). Here, the CS and the US are separated from each other by a temporal gap of no stimuli (trace interval). Characteristic to the trace conditioning is that the learning takes more time and involves higher structures in the brain. It is supposed that trace conditioning is a more cognitive task for it requires something we would call a short-term memory, that is, a delayed representation of the recently presented CS for the association to be formed (Pavlov, 1927).

Common control procedures in conditioning experiments involve *unpaired treatment* and *discrimination training*. Unpaired treatment (lower right in Figure 1) is used to make a distinction between learning-related activity and activity that happens as a process of repeating the stimuli. In unpaired treatment the stimuli to be used later in training are presented without temporal contiguity to each other. The unpaired treatment retards learning rate when the subject is later trained with the same stimuli, this is probably caused by latent inhibition. In other words, the subject originally learns that there is *no* biologically significant consequence to the CS. In discrimination training the subject is taught not to make the UR in the presence of one stimulus (CS-) but elicit UR to another (CS+). These stimuli could be, for example, tones of different frequency. In a sense, to learn not to respond is an associative process in contrast to the unpaired treatment. But the advantage is that by using discrimination training the effect of the US on the CS period can be defined in detail in contrast to the standard classical conditioning, which, inevitably, is a sum of non-associative and associative processes.

Methods for registering neural activity

Event-related potentials (ERPs) are time-locked electric fields generated by synchronous neural activity within specific brain areas engaged in neurosensory or cognitive processing. ERPs are obtained from the averaged EEG as a response to a temporally constant event or stimulus. In humans various components of ERPs (relatively constant temporal features, like peaks and deflections in the averaged curve) are linked to different sensory or attentive processes (Hugdahl, 1995). Unlike in human studies, in animal studies the ERPs are usually measured from chronically implanted intracranial electrodes (e.g., Ehlers, Somes, Lopez & Robledo, 1998). Intracranial event-related potentials (ERPs) reflect changes in extracellular activity in a certain area with a much greater spatial accuracy than in human studies. The need for the use of ERPs in animal models usually stems from this. It is next to impossible to locate the specific brain areas behind the generation of a certain feature in human studies. Animal model can offer a method of determining this. This viewpoint that has been taken, for example, to model the mismatch negativity in animals (Astikainen, Ruusuvirta & Korhonen, 2000).

In animal studies, in a partial contrast to event-related potentials, *multiple unit activity* (MUA) is usually recorded. MUA refers to the measurement of action potentials of the cells nearby the tip of the recording electrode. Therefore, the measure is not about the synchronous extracellular electric field activity, but a measure of what the cells around the electrode tip are actually doing. While intracranial ERPs tell us about functioning of probably thousands or tens of thousands of cells, the MUA is about summed action potentials of tens to hundreds of neurons. A further step down in spatial accuracy is to measure spike activity of a single cell. As the nervous tissue

is not constructed of homogenous masses of cells, it is interesting to know how different types of cells contribute to a task or a happening the subject (or sometimes a mere slide of tissue) is facing.

Electrical brain stimulation

Electrical brain stimulation (EBS) is used to artificially produce activity in a certain area. It is known that this kind of stimulation produces sensations even at psychological level in humans. That is, patients in brain surgery (during which they are awake) report even experiencing certain happenings from the past when a mild electric shock is administered to the cortex. In animals, hypothalamic stimulation, for instance, can be used to produce emotional states in the animal ranging from rage to pleasure. In the case of the rabbit eyeblink conditioning, EBS can be used as both the CS and the US. This could be very useful in determining the critical circuits of learning. For example, direct stimulation of the pontine nuclei (which is a site conveying sensory information to the cerebellum) could be used as a CS instead of the natural tone CS. By this method certain other brain areas can be bypassed and then it can be inferred that those areas were not absolutely critical for the actual memory trace formation. In the same manner, various motor nuclei stimulation can be used as a US. Stimulation of some of the motor nuclei that produce eyeblink reflex as a US supports conditioning while others do not. We will come to these findings later.

Reversible inactivation of the neural tissue

Reversible lesions have certain advantages compared to the traditional lesion by destroying tissue permanently (ablation, aspiration). Reversible inactivation methods include local administration of anesthetics or other chemical agents that block neural functioning (lidocaine, tetrodotoxin, muscimol) and cooling of the tissue. The local cooling has at least two important advantages over use of the local anesthetics or other chemical agents. First, the effect of cooling is very rapidly induced and wears off just as rapidly. Second, the cooling does not affect on fibers of passage and its effect is more specific than that of lidocaine, for instance. The construct of a cooling device was initially presented by Skinner (1968) and further modified by Zhang et al (1986). Lavond et al (1992) have determined the functioning of the cold probe in a real situation implanted in the IPN of the rabbit. Additionally, they defined the temperature gradient in neural tissue. Under 20° C the neural tissue becomes inactive. This fact can be exploited in the rabbit that has learned the task of the eyeblink conditioning, by lowering the temperature in the cold probe tip near 0° C, the cooling results in inactivating an area of 2 mm in diameter. Cooling does not have any permanent effects and can be switched on and off as required by the experiment. Lavond et al (1992) have reported that as little as 30 s is enough to release the inactivation after the coolant flow is stopped. Normally, 2 min is used (e.g., Wikgren & Korhonen 2000). The use of reversible inactivation makes it possible to study effects of a certain region within subjects, and thus, to simplify the design of the experiment.

Learning-related neural activity

To define something as learning-related is not a particularly simple thing to do. Here, it is useful to make a distinction between learning-related and experience-related neural activity. Experience-related neural activity would be something that can be recorded simply due to proceeding of the experiment, that is, the changes in neural activity would reflect nonassociative properties of learning. It is known that the

behavior of the subject changes even in the absence of any experimental stimuli when pre- and post-experiment behaviors are compared (Schreurs & al, 1995). Learning-related neural acitivity can be determined by comparing the conditioning group to an explicitly unpaired control group. Anything that happens in addition to the UP group could the be dubbed learning-related. Further, by inactivating the IPN, it could be determined whether what we call learning-related, disappears. If not, it could be inferred that that learning-related activity is not involved in execution of a discrete movement.

The issue of localization

Methods of reversible inactivation and electrical brain stimulation have been described so far. However, nothing has been said about the use of these techniques in combination for the actual localization. As a matter of fact, the combination of reversible lesions and recording of behavioral and neural activity is a recently developed method of localization (Thompson & al., 1997). For example, inactivation of either the red nucleus or interpositus nucleus completely prevent expression of the behavioral CRs in eyeblink conditioning (when it comes to the discrete somatic response). Additionally, when active, learning-related activity can be recorded from both nuclei in well-trained rabbits. However, inactivation of these structures cause different consequencies to learning-related activity in the other. That is, when the red nucleus is inactivated, the conditioned responses cease but learning-related activity can still be seen in the interpositus nucleus (case B in Figure 2). In contrast, when the interpositus nucleus is inactivated all learning-related activity disappears from the red nucleus as well as the conditioned response (case A in Figure 2). From this data it can be concluded that the memory trace is projected from the interpositus to the red nucleus, not vice versa. (Chapman, Steinmetz, Sears & Thompson, 1990; Clark & Lavond, 1993).

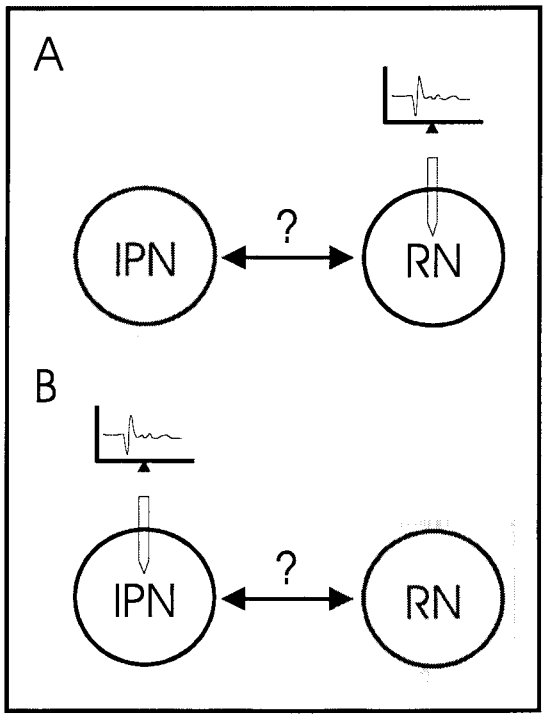

Figure 2. Principle of localizing with reversible lesion and neural recording.

Further, inactivation of these structures during training causes very different outcomes. Namely, when the red nucleus is inactivated during acquisition the rabbit shows no signs of learning, even though it is completely able to perform the eyeblink reflex properly as a response to an airpuff towards the cornea. However, from the first trial after the inactivation is stopped the rabbit shows CRs. So, the memory trace had to be formed somewhere before the red nucleus and the inactivation of the red nucleus merely prevented performance of the CR. This is not the case when the interpositus nucleus is inactivated during training. When the inactivation stops the rabbits learns the task as if naïve, that is, the training had no effect at all. This argues strongly for that the interpositus nucleus is a good candidate to look for plasticity during eyeblink conditioning.

Neural substrates for learning the discrete conditioned response

In this section, *essential* circuitry for acquisition and maintenance of the conditioned eyeblink response is presented. It has been shown that removal of the tissue rostral to the red nucleus, including the hippocampus and cerebral cortex, does not prevent acquisition or retention of the conditioned eyeblink response in the delayed conditioning paradigm. Therefore the essential circuitry for eyeblink conditioning lies within the regions of the brainstem and cerebellum. In brief, this circuitry involves three components: 1) conditioned stimulus pathway that consists of sensory relay nuclei, the pontine nuclei and mossy fiber connections to the cerebellum, 2) unconditioned stimulus pathway which includes somatic sensory relay nuclei, the inferior olive and its climbing fiber connections to the cerebellum, and 3) the conditioned response pathway which includes the cerebellum, its projections from the interpositus nucleus via the superior cerebellar peduncle to the red nucleus and red nucleus projections to premotor and motor nuclei (for a review, see e.g., Anderson &

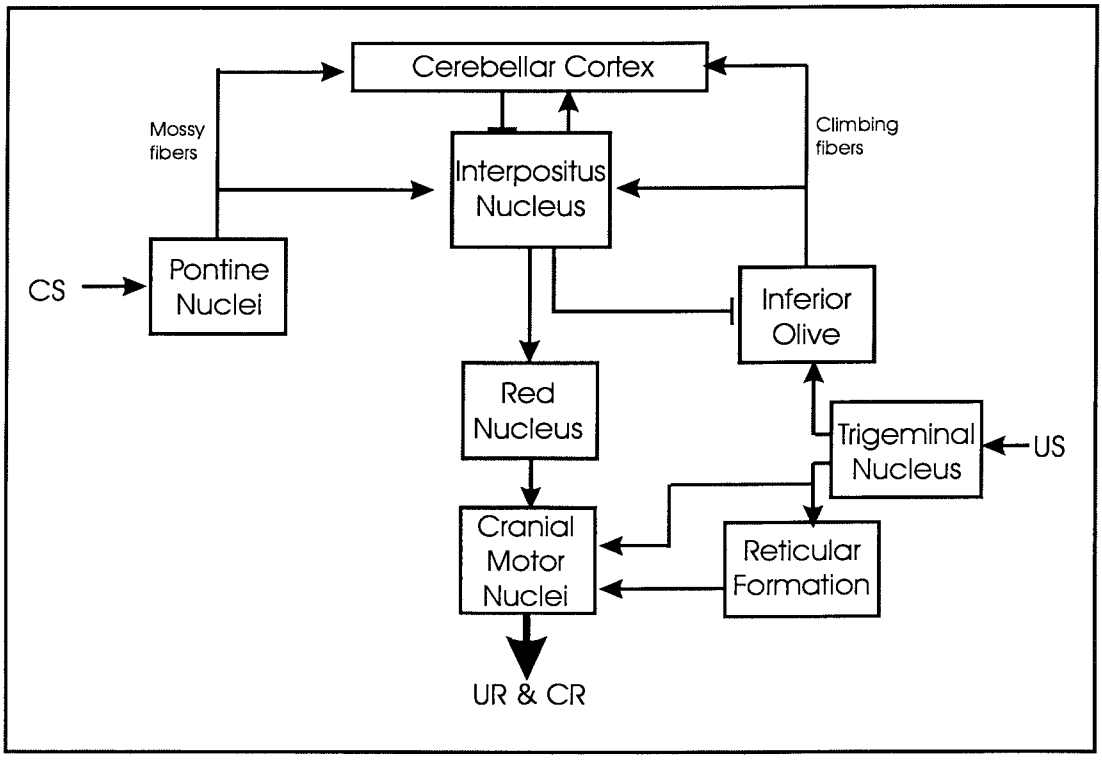

Figure 3. A simplified scheme of the areas involved in learning the somatic defensive eyeblink conditioning. Laterality is not shown.

Steinmetz, 1994). It should be noted that areas outside of this circuitry by no means are inactive or futile for this kind of learning but are not critically necessary for the task. On the contrary, the animal with the circuitry presented in the following is capable of learning the conditioned eyeblink response but the rate of learning, as the timing of the response is compromised. Further, with this circuitry alone the animal does not experience aversiveness of the unconditioned stimulus, nor is capable to learn if the situation is made even a bit more complex than is the case in classical delayed conditioning (e.g., trace conditioning).

The US/UR pathway

Classical conditioning always involves conditioning of an existing reflex response. Therefore it is appropriate to discuss the circuits that are responsible for emission of the hardwired reflex. The notion 'eyeblink reflex' refers actually to a wide range of reflexive movements (Berthier, Desmond & Moore, 1987). The whole eyeblink response consists of eyeball retraction and eyelid movements in addition to the nictitating membrane movement, which is normally the dependent variable of interest. It should be noted that the results presented in the following refer to the whole range of these reflexive movements. This notion is important for the reason that the airpuff-induced UR reflex is consisted of all of these elements in natural proportions but this is not necessarily the case if the electrical stimulation US (a mild shock in adjacency of the eye) is used. In fact, electrical stimulation of the skin in the facial area readily elicits other kinds of involuntary responses as well.

Initially, the airpuff to the cornea causes the rabbit to emit a defensive eyeblink reflex. Therefore, the activity measured in certain areas of the brain is either involved in sensing the stimulus or making a motor response. The trigeminal nuclei in the brain stem are the primary sites for making such a response. In fact, this reflex can be initiated in a decorticated and decerebellated animal. The tactile information from the cornea is initially relayed to the ventral half of the pars oralis of the trigeminal sensory complex. Information is then projected to the accessory abducens which project to the retractor bulbi muscle via the VIth nerve. This is, however, only one part of the reflex circuit, presumed to be involved with the initial, short-latency component of the reflexive movement (Anderson & Steinmetz, 1994). The other circuit involves projections from the pars oralis to the area of the reticular formation caudal to the accessory abducens. These reticular formation cells then project to the accessory abducens thus completing the circuit (see Figure 3). This circuit is thought to control the longer latency components of the UR

The aforementioned circuits then accomplish the reflexive movements. But, as noted earlier, the critical plasticity in eyeblink conditioning occurs probably in the cerebellar structures. Therefore, there must be connections through which the information about the reflex-eliciting stimulus is carried to the cerebellum. Studies have shown that trigeminal nucleus projects to the cerebellum via at least three routes. The most essential route in the case of eyeblink conditioning is that the principal trigeminal nucleus, trigeminal interpolaris and pars caudalis project to the inferior olive, which in turn is the source of climbing fibers to the cerebellum. Trigeminal complex also projects to the cerebellum via the mossy fibers originating from the pontine nuclei. It is notable that the cerebellum is considered a processing site rather than a mere relay site in reflexive movement. These processes are discussed in more detail below.

The CS pathway

The tone evokes spike discharges in the cells in the cochlear nucleus, which is the primary nucleus for auditory modality. The cochlear nucleus, in turn, projects primarily to the inferior colliculus, superior olive and higher auditory structures (Anderson & Steinmetz, 1994). Of interest here is that the cochlear nucleus projects also to the lateral pontine nuclei, which in turn are the source of the mossy fibers to the cerebellum. The studies in well-trained animals concerning the latencies of tone evoked activity within the lateral pontine nucleus suggests that it represents two aspects of activity. First, acoustic-related activity occurs within the first 20-40 msec after the tone onset and, second, later activity that correlate with the conditioned response. However, the latencies correlating with the learned response are more or less the same as the behavioral responses, making it unlikely that this region actually drives the CRs. Furthermore, during interpositus nucleus inactivation the learning-related activity, but not the acoustic related activity was abolished in the pontine nuclei.

The pontine nuclei relay information from all sensory modalities to the cerebellum via the mossy fibers. The qualitative differences between the climbing and mossy fibers are most important for theories of learning. These will be considered below.

Learning related changes in neural activity

As the above mentioned stimuli are given in a rigid temporal order (see Figure 1) the animal learns to respond to the initially meaningless stimulus by making the defensive reflex. In other words, the animal has been conditioned to the weak stimulus. In the case of the simple delayed eyeblink conditioning in the rabbit, the CS is usually a tone given before the airpuff. No anticipatory behavior could happen without plasticity in the brain. The crucial question therefore is, which site in the brain causes the learning-related activity in others, that is, which site is critical for this kind of learning to occur.

The most prominent feature of learning-related neural activity concerning the conditioned eyeblink response is that not only can one measure activity occurring during presentation of the CS but that the neural activity measured from variety of regions actually forms a template of conditioned response. That is, histograms of multiple unit activity have the same shape as the conditioned response. This is particularly true in the hippocampus, interpositus nucleus, red nucleus and other brainstem motor nuclei (McCormick, Lavond & Thompson, 1983). Strikingly, this pattern of activity develops earlier than the first CR is seen and within a trial the template precedes the behavioral response for about 30 msec. However, in every location studied to date, the inactivation of the interpositus nucleus causes abolition of such a template (Sears & Steinmetz, 1990; Clark & Lavond, 1996; Thompson & al, 1997; Ryou, Cho & Kim, 1998). By no means this alone is enough for evidence that the interpositus nucleus is the only site that is absolutely critical for learning the CR, for at least two reasons. First, a body of other regions is still not investigated, and second, some structures of the brain are too diffuse or wide to be studied by lesioning techniques known today. For instance, there has been a vigorous debate over the issue whether the cerebellar cortex is the most critical site in this kind of conditioning (for cerebellar cortex theory, see Hesslow, 1999). Lesions to the cerebellar cortex are very difficult to perform without damaging the underlying structures like the deep nuclei. And on the other hand, lesions to the deep nuclei (like interpositus nucleus) are not easily done without damaging the cortex, at least to a small extent. On the other hand, there is no doubt that even a very small lesion to the anterior part of the interpositus

nucleus would completely and permanently prevent expression and acquisition of the eyeblink CR. Steinmetz and Logue (1992) gave paired eyeblink training for months to interpositus lesioned rabbits without even a sign of learning.

The cerebellar cortex and interpositus nucleus may have different roles in the eyeblink conditioning. It is known that learning related activity can be recorded from both areas, but the cerebellar cortex shows learning–related activity to backward conditioning also, which is not the case in the interpositus nucleus (Gould & Steinmetz, 1996). Gould and Steinmetz (1996) conclude that it seems possible that the interpositus nucleus is only able to exhibit changes in excitability when mossy fiber activation (CS) precedes climbing fiber activation (US). Therefore, even if the cerebellar cortex is able to form learning-related activity in backward conditioning, the association is not enough to drive a conditioned response (it is commonly known that backward conditioning does not result in overt behavioral responses). Further, lesions to these areas have different outcomes in eyeblink conditioning. Lesions of the relevant portion of the cerebellar cortex (Larsell's lobule HVI) retard the CR acquisition but does not prevent it and affect only temporarily performance of previously learned CRs (Lavond, Steinmetz, Yokaitis & Thompson, 1987; Lavond & Steinmetz, 1989). Further, subjects of a mutant strain of mice, which lack of Purkinje cells (the only output) of the cerebellar cortex can be eyeblink conditioned, although the rate of acquisition is severely retarded (Chen, Bao, Lockard, Kim & Thompson, 1996).

Pathway for a conditioned response

During inactivation of the critical motor nuclei (accessory abducens nucleus, facial nucleus and surrounding reticular formation) the subject is completely enable to show CRs or URs. However, if these areas are inactivated in a naïve rabbit and eyeblink conditioning administered, asymptotic learning is perceived right from the termination of the inactivation (Krupa, Weng & Thompson, 1996). Same kind of effect results from temporal inactivation of the red nucleus with one important exception. During the red nucleus inactivation, the rabbit is able to perform the UR to the US as it normally would, but it can not exhibit conditioned responses. Therefore, the red nucleus is considered to be the primary motor output of the CR. As noted earlier (see The issue of localization), the red nucleus inactivation in itself does not prevent learning. Furthermore, electrical stimulation of the red nucleus produces eyeblinks, but such brain stimulation does not support conditioning when used as a US (Chapman, Steinmetz & Thompson, 1988). The site of the critical plasticity in eyeblink conditioning must therefore be afferent to the red nucleus.

The superior cerebellar peduncle is the primary output pathway of the cerebellum. The cerebellar deep nuclei project fibers through the superior cerebellar peduncle to the red nucleus and thalamus. The red nucleus projects to the motor neurons in the accessory abducens and the facial nucleus, the brain stem nuclei involved in the unconditioned response, thus making the conditioned contribution to the reflexive movement.

Possible locations of the emotional memory trace

In sum, the interpositus nucleus and, possibly, the overlying cerebellar cortex is the location for the primary sensorimotor memory trace in eyeblink conditioning. There is no such an instance where the discrete conditioned responding survives the lesion or inactivation of this area. This holds in every modality of the CS (Wikgren & Korhonen, in press) and in different paradigms, critically involving higher brain areas,

as is the case in trace conditioning. However, while interpositus inactivation in naïve rabbits prevents acquisition of the CR and in conditioned rabbits abolishes the CR, some learning-related changes can still be seen. These learning-related changes involve autonomic responses (conditioned heart-rate response) and nonspecific responses (conditioned reflex facilitation; Wikgren, Ruusuvirta & Korhonen, 2000) in the presence of the CS.

Autonomic associations during eyeblink conditioning

The corneal airpuff as a US is sufficient to support conditioned bradycardia if the intensity used is strong enough (McEchron, McCabe, Green, Llabre & Schneiderman, 1991). In the case of acoustical heart rate conditioning with the corneal airpuff as a US, it has been shown that auditory synaptic inputs to the medial subnucleus of the medial geniculate nucleus neurons increase in strength as a result of classical conditioning (McEchron, Green, Winters, Nolen, Schneiderman & McCabe, 1996). Further, lesions to this area disrupts learning when auditory stimulus is used as a CS (LeDoux, Iwata, Pearl & Reis, 1986; McCabe, McEchron, Green & Schneiderman, 1993). Fear-conditioned acoustic stimulus induces changes also in dorsal lateral geniculate nucleus (Cain, Kapp & Puryear, 2000), this is further associated with the 'stand-by' state of neurons in the neocortex, which causes low voltage fast activity, a typical EEG pattern in mildly anxious, fearful or hyperaroused subjects.

Lesions to or reversible inactivation of the amygdala results in reduced conditionability to emotional stimuli. Projections of the amygdalar central nucleus to the lateral tegmental field in the thalamus, which project on a variety of cranial motor nuclei, may form the substrate by which the ACe contributes to conditioned modulation of various reflexes, such as the nictitating membrane reflex (Whalen & Kapp, 1991; Kapp, Supple & Whalen, 1994). One way known to researchers how the amygdala contributes to modulation of the reflex is the reflex facilitation (see below), lesion to the amygdala does not prevent learning of the discrete behavior but retards the rate of acquisition and prevents the reflex facilitation (Weisz & Harden, 1992)

McCaugh & al (1993) have considered the role of the amygdala in aversive learning. They suggest that the amygdala serves to modulate memory storage in other brain regions. It is known that epinephrine given post-training enhances memory consolidation in aversive tasks, but this not the case when the amygdala is lesioned (Cahill & McCaugh, 1991). Emotionally arousing stimulation may influence memory through effects involving the release of norepinephrine within the amygdala.

Using single-unit measures from various nuclei in the amygdalar complex, Richardson and Thompson (1984) were not able to find evidence for learning related activity within the amygdala. More recently, as the amygdala has been a subject for more extensive investigations, its role in fear conditioning has been clarified. Romanski, Clugnet, Bordi and LeDoux (1992) were able to register single units within the lateral nucleus of the amygdala, which showed responses to both auditory and aversive somatosensory (footshock) stimuli. Further, the response latencies for these units were > 30 msec, which suggests that the amygdalar lateral nucleus may have a role in forming the association between neutral and aversive stimuli. This opposes the findings of Richardson & Thompson (1984) for they claimed that the response latencies of the amygdalar units were too long to have an effect within the time course of the standard eyeblink conditioning in rabbits.

Given that animals with amygdalar lesion are compromised in learning the aversive conditioning and exhibiting reflex facilitation in eyeblink conditioning

(Weisz, Harden & Xiang, 1992), the assumption of the amygdalar involvement in learning the aversive aspects of eyeblink conditioning is supported.

The role of the dorsal hippocampus, basolateral amygdala and perirhinal cortex was assessed by Sacchetti, Ambrogi Lorenzini, Baldi, Tassoni and Bucherelli (1999). They suggest that the hippocampus is involved in early consolidation of the context memory and amygdala and perirhinal cortex in CS processing

Further evidence about the role of amygdala, as well as other limbic structures, in memory consolidation stems from the finding that amygdaloid learning-related activity increases rapidly at the outset of conditioning but ceases, as the conditioned responding becomes asymptotic. This decrease in neural activity within the limbic system can be seen even if the subject, after initial training, does not receive paired trials for several days. In other words, limbic activity can be seen only as related to the consolidation process (Maren & al, 1991; Quirck & al, 1997; Poremba & Gabriel, 1999). Amygdalar lesions prevent acquisition of the learning-related neural activity within the thalamic nuclei and in related areas of the cingulate cortex (Poremba & Gabriel, 1997), which is shown to be essential for discriminative avoidance learning (Gabriel, 1993). This suggests that the memory trace for such an event is not located in the amygdala but the structure is needed in initiation and consolidation of the memory trace. In amygdala-lesioned rats, the long-latency learning-related activity in the auditory cortex is diminished, when a rat is fear-conditioned to a tone (Armony, Quirk & LeDoux, 1998). This line of evidence suggests that the amygdala has a critical role not only in expression of the learned response, but in the formation of the learning-relevant neural plasticity. However, this also indicates that there are yet unidentified areas of the brain that are capable of modified learned behavior and memory no longer served by the limbic circuit (Poremba & Gabriel, 1999). The cerebral cortex may well be a site for the long-term plasticity in fear conditioning.

Evidence for 'cognitive' aspects of eyeblink conditioning

In theories of brain substrates of learning, the hippocampus (together with the rest of the limbic structures) is generally linked with the explicit forms of a memory. In animal models the extent of explicitness of a memory is not very straightforwardly defended, but certain aspects of learning has been possible to show to be dependent on the hippocampal functioning. For starters, a template of learning can be recorded from the hippocampus during conditioning. That is, the multiple-unit activity predicting the temporal pattern of the CR to be can be recorded before the presence of the actual CR. This pattern of activity is dependent on the functioning of the interpositus nucleus, as is shown in the experiment where the reversible inactivation of the IPN blocks the learning related activity in the hippocampus. This offers evidence that the hippocampus is not itself the site of the memory trace but it is possible that it links the learned specific event with other kind of information available to the organism, as it is known that the hippocampus deals with more complex aspects of learning (Squire, 1992).

In basic delayed classical conditioning the hippocampus is not a necessary structure (Schmaltz & Theios, 1972) even though learning-related activity can be measured from there (Berger, Alger & Thompson, 1976). But, if the conditioning task is made even a bit more difficult, like is the case in trace conditioning, the hippocampus seems to be a critical structure. In trace conditioning, there is a temporal interval between the CS and the US, thus, the subject must temporally delay the representation of the CS if it is to learn the connection between the CS and the US (Pavlov, 1927). Hippocampus-lesioned subjects are strongly retarded or not able to

learn this task (Solomon, Vander Schaaf, Thompson & Weisz, 1986; Moyer, Deyo & Disterhoft, 1990; for contradicting evidence, see Port, Romano, Steinmetz, Mikhail & Patterson, 1986). The hippocampal slice studies in trace-conditioned rabbits show that the synaptic transmission between the hippocampal areas CA1 and CA3 is enhanced when recorded one hour from the last training session. However, this elevation of transmission could not be seen 24 h after the last training session (Moyer, Thompson & Disterhoft, 1996; Power, Thompson, Moyer & Disterhoft, 1997). This, like the other studies on limbic areas in memory formation, shows that hippocampus is involved in memory trace formation, but not in maintenance. Stanton (2000) notes that the cognitive system (hippocampus) is not a critical component in simple delayed conditioning - it merely represents the episode and receives an 'efferent copy' from the other systems. This is in marked contrast with 'higher order' conditioning which is critically dependent on the cognitive aspects of learning. Thus, basic delayed eyeblink conditioning involves relatively little cognitive aspects.

It is notable that stimulation of the lateral septum (which is efferent to the hippocampus) serves as an efficient CS in eyeblink conditioning while stimulation of the medial aspect of the septum (afferent to the hippocampus) does not (Knowlton & Thompson, 1989). Knowlton and Thompson suggest that the reason for this may be that stimulation of the lateral septum, a hippocampal afferent, does not result in information that can be appropriately processed to act as a CS. That is, hippocampal processing is needed in transforming the stimulation, which happens in the case of stimulating the hippocampal efferent. Given the location and physiological properties of the hippocampal formation, higher structures may modulate the cerebellar input as shown by Knowlton, Thompson & Thompson (1993) by hippocampal processing. Auditory cortex also shows learning-related activity and therefore may reflect modulation of the conditioning task (Kraus & Disterhoft, 1982). It is also shown that two types of hippocampal cells exhibit the opposite activity patterns in different stages of trace conditioning (McEchron & Disterhoft, 1997). This may be the basis for temporal representation of the US onset, and therefore, the mechanism by which the hippocampus deals with the cognitive demand of the trace-conditioning task.

It is notable that the amygdala and hippocampus interact in contextual fear conditioning. Both lesions in the relevant aspects of the hippocampus and amygdala prevent fear conditioning (Maren & Fanselow, 1995). It may be concluded that in these studies the hippocampus lesion prevented the processing the contextual CS and the amygdala lesion prevented the fear US processing (not just exhibition).

Conditioned reflex modification

Conditioned reflex modification in the rabbit eyeblink conditioning preparation is, at a first glance, a unique phenomenon in one sense. Namely, throughout species and conditioning procedures the CS is likely to modify the unconditioned response in a manner of diminution, that is, the UR is diminished if it is preceded by a conditioned stimulus as compared to US-alone trials (Donegan & Wagner, 1987). However, what happens in the eyeblink conditioning in rabbits, is that the opposite occurs. As stated previously, the eyeblink reflex is modified long before the occurrence of the CR proper. More specifically, the amplitude and peak latency of the UR changes very rapidly when the conditioning training is administered. The presence of the CS is a critical factor; when the CS precedes the US, the animal exhibits more vigorous and rapid eyeblink reflexes than if the US is presented alone. This reflex facilitation can be observed very quickly during training, but it is notable that in the very outset of the training, the CS does not have an effect (Wikgren, Ruusuvirta & Korhonen, 2000).

This, along observations in humans (Marcos & Redondo, 1999), suggests that the process is associative and therefore must be learned. It is known that the interpositus lesion does not interfere with conditioned reflex facilitation even though the lesion prevents acquisition of the CR proper (Weisz & LoTurco, 1988).

When studied in more detail, the reflex modification in the eyeblink conditioning possibly occurs in accordance with other subject populations and conditioning procedures. Specifically, when a stronger US is used, the reflex modification occurs in the form of conditioned diminution (Donegan & Wagner, 1987; Canli, Detmer & Donegan, 1992). There is also a negative correlation between the amount of reflex diminution and conditioned responding. That is, after the extinction phase of the experiment, the CS does not have an effect on the UR anymore (Morrow, 1966).

Amygdala in the limbic system has traditionally been linked to all kinds of emotional memory. It has even been proposed that amygdala drives a 'hot' memory system as opposed to hippocampus-driven 'cool' memory system. The current opinion is that whereas the hippocampus is involved in memory tasks that are not emotionally strong, the amygdala deals with all kinds of emotionally challenging situations such as fear conditioning etc (LeDoux, 1993; Stanton, 2000). According to this view it should be unlikely to find emotional regulation of this kind of task from the amygdala, since the aversive stimulus used in eyeblink conditioning (the airpuff) is hardly aversive enough to arouse a serious emotional response.

The conditioned modulation of the UR is not only of interest on its own right but may be representative of a larger class of phenomena (Donegan & Wagner, 1987). Therefore, classification of the types of reflex facilitation may serve as a useful basis for identifying the locations for nonspecific plasticity in the nervous system. Given that the reflex facilitation remains even in the absence of the learned specific response, as is the case during inactivation of the IPN, there should also be some learning-related activity that it correlates with. The identified learning-related activity so far in every studied location has been shown to cease during the IPN inactivation. However, these studies have mostly been restricted to multi-unit activity, which is an index of discharges rather than changes in extracellular potentials in a given site. It is quite possible that at some frequency, studying the event-related potentials as local field potentials instead of spike activity could identify learning-related activity.

Donegan & Wagner (1987) cite earlier work of Wagner (1979) who proposed that signaled US (preceded by a CS) generally results in modified UR (UR'). Then the UR' will be added to, or subtracted from, by the CR. This would mean that on CS+US trials, the measure of responding following the US will be a combination of a conditioned response and a modified unconditioned response (CS+US → CR+UR', as they put it). In the light of our recent work this, however, does not hold. Namely, from this classical interpretation it would follow that if the memory trace for the CR is blocked (as is the case in interpositus nucleus inactivation), the UR' should give way to the UR proper. But, on the contrary, during inactivation of the interpositus nucleus, the discrete conditioned response abolishes, but the CS still affects to the UR; the reflex facilitation is conditioned but it does not depend on the same structures than the conditioned eyeblink response (Wikgren & al., resubmitted manuscript). Nor is the conditioned response a sum of the CR and the UR as proposed by (Clark, Zhang & Lavond, 1992). If it were so, the UR to the signaled US would change during IPN inactivation, which is not the case.

Discussion

Conclusions

The use of the reversible inactivation, neural recordings and electrical brain stimulation tends to make physiological psychology of memory a vast logical puzzle. As the sensorimotor aspects of eyeblink conditioning (the cerebellar/brainstem contribution) have gained significant clarity over the 30 years of research, little is still known about the other aspects in this memory trace formation. The research into these other aspects (cognitive, motivational, affective, etc.) is important in respect of binding this relatively simple form of learning with a wider picture of how the nervous system produces adaptive behavior in organisms. This knowledge is no less than essential in understanding the very nature of all representations needed in the brain to cope with the everyday world. Distant as this goal may seem, there is no reason for pessimism, because the modern research methods are probably sophisticated enough for allowing very accurate reasoning about the brain functions correlating with the psychological phenomena. The literature sited here possibly hides even more information than a single person or research team can make out of it. Therefore the limits of the modern physiological psychology stem from our own ability or inability to reveal logical inferences from the vast amount of observations available even by today.

In sum, it may be concluded that different aspects of conditioning are indeed processed by separate structures in the brain. So, in any learning there are multiple tasks to be performed by the brain, and multiple representations to be formed about the happenings during such tasks. In eyeblink conditioning, the cerebellum is the most critical area for making the discrete conditioned response, that is, the memory trace is formed and maintained there. This learning is affected by emotional and, possibly to some extent, by cognitive processes. Analyzing the associations formed during eyeblink conditioning in detail is important because the search for an emotional (for example) memory engram is not possible if the content of a conditioned emotion is not known. That is, the conditioned emotion cannot be operationalized if it is not known in a very detailed manner. So far it is known that the amygdala plays a crucial role in formation of this kind of memory trace, but it deals with a wide range of phenomena whereas different association might be stored in several areas. This has been the major rationale in trying to define different aspects of reflex facilitation in this paradigm (Wikgren, Ruusuvirta & Korhonen, submitted). Demonstration that some types of this phenomenon are not dependent on cerebellum as others are, offers a starting point for localization the critical area for conditioned, CS-mediated reflex facilitation. Hopefully in the future, it is possible to gain similar breakthrough in localization as was the case when the critical area for the conditioned NM-reflex was found by Thompson's group.

Suggestions and predictions for future research

The research concentrating on the amygdalar learning-related activity is of importance in the case of eyeblink conditioning. The first step is to define, whether the amygdala indicates learning-related activity during blocking of the discrete CR, during interpositus nucleus inactivation, that is. The amygdala is critically involved in conditioned fear, therefore it is likely that some learning-related activity would be exhibited there or in some of its efferent structures as a response to the CS during IPN inactivation. Because it is known that conditioned nonspecific behavior and autonomic responses are seen during IPN inactivation, it is only reasonable to assume

that some structure of the brain shows plasticity in the task. Whether the airpuff in eyeblink conditioning causes conditioned *fear* is a more controversial issue. In fear conditioning, the association is formed extremely quickly (sometimes one paired trial in enough), which is not the case in eyeblink conditioning. *Conditioned annoyance* could be a more appropriate term to describe the experience of the rabbit in eyeblink conditioning. Nevertheless, this conditioned annoyance could be a minor case of conditioned fear and therefore the research in amygdaloid activity in eyeblink conditioning becomes the primary issue of interest. Amygdalar stimulation causes EEG-desynchronization (Kapp, Supple & Whalen, 1994). Therefore it could be expected that in well trained animal there should be both amygdalar learning-related spike activity as well as EEG-desynchronization as a response to the CS.

On behavioral level, the work of Lang and his associates suggest that emotional responses are founded on two basic motive systems, aversive and appetitive. In their studies in human and animal subjects (Lang, Bradley & Cuthbert, 1998; Greenwald, Bradley, Cuthbert & Lang, 1998), primed with either negative or positive reinforcement, different outcomes are observed to the reflex probes presented after these primed stimuli. This would be of interest to show in eyeblink conditioning also. Specifically, two tones of different pitch, reinforced with appetitive or aversive stimuli, should have different outcomes on reflex facilitation when used as CSs in eyeblink conditioning. The CS previously reinforced with aversive US should facilitate the reflex whereas the appetitively reinforced CS should result in diminution of the reflex.

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INACTIVATION OF THE INTERPOSITUS NUCLEUS BLOCKS THE CONDITIONED RESPONSE ACQUIRED BY A SOMATOSENSORY CONDITIONED STIMULUS IN RABBIT EYEBLINK CONDITIONING

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Abstract

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1. Earlier studies suggest that the memory trace for the conditioned eyeblink reflex is formed and maintained in the interpositus nucleus (IPN) in the deep cerebellar nuclei when either an auditory or visual stimulus is used as a conditioned stimulus (CS).
2. In the present study, the eyeblink reflex of the rabbit was conditioned to a somatosensory CS (an airpuff onto the back).
3. In well-trained animals, the IPN was reversibly inactivated by local cooling and the existence of the learned responses to the CS was then tested.
4. The reversible IPN inactivation blocked the memory trace to the somatosensory CS. The finding further supports the view that IPN-mediated memory trace formation is not dependent on the modality of the CS.

Keywords: cerebellum, classical conditioning, interpositus nucleus, reversible inactivation

Abbreviations: conditioned response (CR), conditioned stimulus (CS), interpositus nucleus (IPN), intertrial interval (ITI), nictitating membrane response (NMR), unconditioned stimulus (US).

Introduction

In the rabbit nictitating membrane response (NMR) conditioning (Gormezano 1966), the conditioned stimulus (CS; usually a tone or a light) is paired with the eyeblink reflex-eliciting unconditioned stimulus (US; usually an airpuff towards the eye or an electrical stimulation of the skin nearby). This results in a conditioned response (CR), i.e., the rabbit starts to blink its nictitating membrane (NM) in response to the presentation of the CS alone. Thus far, those CSs successfully used have included auditory, visual

and somatosensory stimuli (Lewis et al 1987). It seems that the elicitation of the conditioned NM response is independent of the modality of the CS.

The neural circuits for the formation of the memory trace are well known (Thompson et al 1997; Anderson and Steinmetz 1994). The interpositus nucleus (IPN) has been shown to be the critical site for the formation and maintenance of the memory trace (Clark et al 1992). It has been shown that temporary inactivation of the IPN blocks both the acquisition and retention of the memory trace if the CS is auditory or visual (Clark et al 1992). However, despite the ability of the somatosensory stimuli to serve successfully as a CS in this paradigm, its dependence on IPN functioning has not, to our knowledge, been shown. Current assumption is that the IPN-mediated memory trace formation is CS-modality nonspecific (Thompson et al 1997). If the association between the CS and US occurs in the IPN, then its inactivation should abolish learning to any conditioned stimulus. We tested this hypothesis by training rabbits in the standard delayed NMR conditioning paradigm with a somatosensory CS and then reversibly inactivating the IPN by use of a cold probe. We assumed that the CR would be blocked in the same way as in the case of the other CS modalities.

Materials and Methods

Animals

Nine adult New Zealand albino rabbits weighing 3.5 - 3.7 kg at the time of surgery served as subjects. The animals were individually housed in metal cages on a 12:12 hr light-dark cycle with free access to food and water. All experimental procedures were performed during the light portion of the cycle. The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures. The study was permitted by the ethics committee for animal research of the University of Jyväskylä.

Surgery

The animals were anesthetised with i.m. injections of a ketamine-xylazine cocktail (Ketaminol, 50 mg/ml, 5.6 ml; Rompun, 20 mg/ml, 2.2 ml; physiological saline 2.2 ml). The initial dosage was 4 ml and the anesthesia was maintained by additional injections of 2 ml every 20 - 40 min. After a deep general anesthesia had been achieved, the animals were placed in a stereotaxic instrument (Kopf Instruments) with bregma 1.5 mm above lambda. A

longitudinal incision was made to reveal the skull onto which the headstage designed to hold the minitorque potentiometer was cemented with dental acrylic using four stainless steel anchoring screws. A nylon loop was sutured into, but not through, the NM of the right eye. The cold probe implantation followed the guidelines concerning recording-electrode implantation reported in Korhonen (1991). The cold probe was implanted in the IPN using the coordinates 0.5 mm anterior and 5.0 mm lateral to the lambda. Analgesics (Temgesic 0.3 mg/ml) were provided 2 hours after the surgery and additionally if needed.

Apparatus

During the experiments, the NM loop was linked by a rigid stainless steel hook to the wiper arm of the minitorque potentiometer for measuring NM movement. The extension of the NM was transduced to voltage by the potentiometer (1 mm equalled 1 V).

The construction of the cold probe was modified after that presented in Zhang et al (1986). The shaft of the probe was not warmed, as it has been shown that lesioning the cerebellar lobule HVI, through which the probe was penetrated, does not prevent the retention of a conditioned NMR (Lavond et al 1987). In short, the cold probe consists of two stainless steel tubes one inside the other. The inner tube delivers the coolant inside the outer tube, 1 mm from the tip, which is sealed by solder. The coolant exits through a plastic tube attached to the outer cannula at a Y-shaped junction. The coolant used was freon-like 1,1,1,2-Tetrafluoroethane (KLEA R-134-A).

Procedure

The subjects were given at least one week to recover after surgery before the actual experimental procedures. On the first day, the animals were adapted to the experimental situation by placing them in a Plexiglass restraining box (Gormezano 1966) located in a soundproof conditioning chamber. Training consisted of six daily consecutive sessions. The CS was an airpuff administered at the back of the rabbit through a hole in the lid of the restraining box (2.1 N/cm^2 source pressure, 350 ms, ~15 cm posterior to neck). The US was an airpuff towards the cornea (2.1 N/cm^2 source pressure, 100 ms). The training session consisted of 60 paired trials, where the CS was followed by the US, 10 CS-alone and 10 US-alone test trials in a pseudorandom order. The paired trials were presented in a delayed fashion where the stimuli coterminated. The intertrial interval (ITI) varied between 30 and 50

s (mean ITI = 40 s). In order to prevent the rabbits from hearing the sound of the airpuff, white noise (20 - 20 000 Hz, 78dB) was continuously presented through miniature earphones. After five training sessions, the rabbits were subjected to a cooling session. This session was divided into three phases: pre-cooling, cooling and post-cooling. Each phase consisted of 30 trials (4 US alone, 4 CS alone and 22 paired trials). After the first phase, the cold probe was activated and the gas flow was adjusted so that the temperature in the cold probe fell below 5 °C. This took about two minutes. After the cooling phase, the experiment was again interrupted for about two minutes in order to allow the temperature to return to the normal level.

Histology

After the experiments, the animals were anesthetised with i.m. injections of ketamine-xylazine cocktail and then overdosed by an i.v. injection of pentobarbital. The rabbits were then perfused via the ascending aorta with saline followed by 10 % formalin. The brain was removed and fixed in formalin-sucrose solution for at least one week. Frozen coronal sections of 100 µm were taken from the site of the cold probe. Slices were mounted on gelatinised slides and stained with cresyl violet. The locations of the cold probe tips were determined according to the stereotaxic atlas (Shek et al 1986).

Data Analysis

Included in the analysis were the peak response amplitudes of the CS and US periods (0 - 250 ms after the CS and 0 - 250 ms after the US respectively) from the paired trials and the CR percentages in each session. Extension of the NM exceeding 0.5 mm was counted as a response. Trials with NM movement exceeding 0.5 mm during a period of 100 ms prior to the stimuli were rejected. All statistical analyses were carried out as analyses of variance (ANOVA) for repeated measures.

Results

Histology

The locations of the cold probe tips are presented in Fig 1. Animals with a cold probe tip located further than 2 mm away from the target (N = 2) were excluded from the analysis.

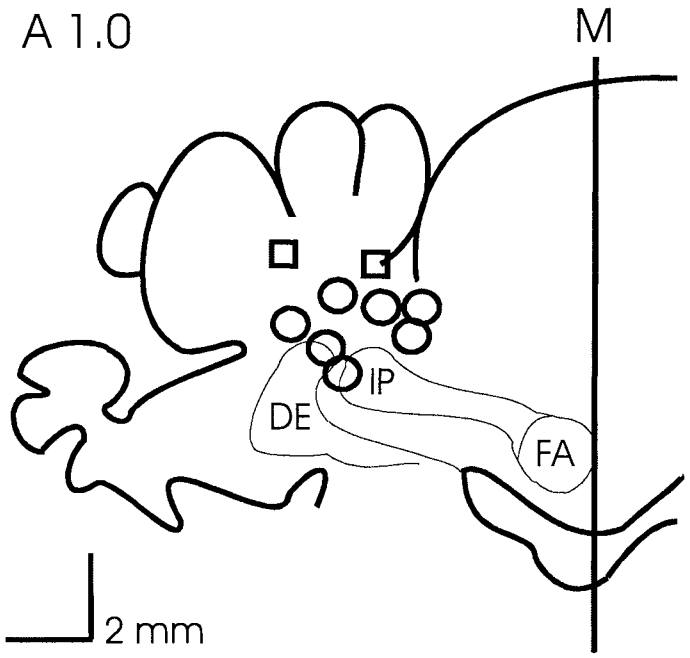

Fig 1. Locations of the cold probe tips (anterior view). Circles indicate the locations in those rabbits with successful CR abolition, squares stand for cases where cooling had no significant effect on the CR. Key: M, midline; DE, dentate nucleus; IP, interpositus nucleus; FA, fastigial nucleus

Conditioned Responding

The results for the CR percentages are shown in Fig 2. The CR percentage increased as a function of the training sessions [1 X 5 design; $F(4,24) = 8.78$, $p < 0.001$] and varied as a function of the cooling phases [1 X 3 design; $F(2,12) = 24.51$, $p < 0.001$]. The results for the response amplitudes to both CSs and USs are depicted in Fig 3. The CR amplitudes increased as a function of the training sessions [$F(4,24) = 3.73$, $p < 0.001$]. Once well trained, the animals' CR amplitude varied as a function of the pre-cooling, cooling and post-cooling phases [$F(2,12) = 5.94$, $p < 0.01$]. Conditioned responding was also analyzed for the two rabbits with the cold probe tip locating in the white matter between the cerebellar cortex and the deep nuclei (indicated by squares in Fig. 1). In these rabbits, the CRs diminished neither in amplitude nor in percentage during the inactivation.

Unconditioned Responding

To ensure that performance during inactivation was not compromised in general, especially because of the unwarmed shaft of the cold probe used, the

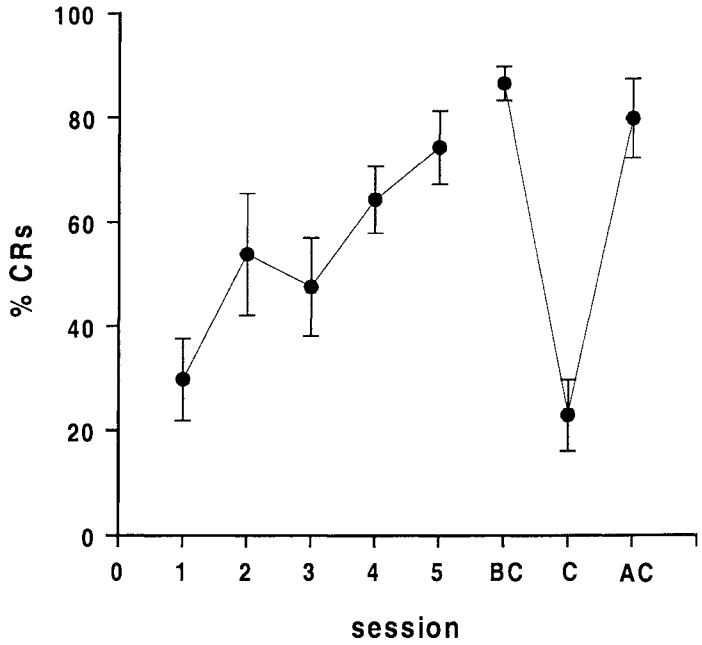

Fig 2. Percentages of the conditioned responses as a function of the training session. Key: BC, before cooling; C, during cooling; AC, after cooling

unconditioned response amplitudes were also examined. Unconditioned response amplitudes increased during conditioning [$F(4,24) = 8.08$, $p < 0.001$] but after reaching the asymptotic level by the end of the training, they were not found to vary as a function of the cooling phases. Thus, the reduction in the CR percentage and amplitudes during cooling was not due to the animals' lack of ability to blink.

Discussion

Local cooling of the IPN blocked the conditioned response acquired with a somatosensory CS, but had no influence on the UR. In line with previous findings, this favours the hypothesis that an IPN lesion abolishes the CR to all CS modalities. The cerebellar nuclei hypothesis (Lavond et al 1993) is that the information about the CS arrives at the cerebellum via mossy fibers from the pontine nuclei. Information about the US, in turn, is carried by the climbing fibers from the inferior olive nuclei in the brain stem (Anderson and Steinmetz 1994). Both connections have collaterals to deep cerebellar nuclei, including the IPN. The association formed in the IPN would then be projected onto the red nucleus which is the motor output of the CR. Another

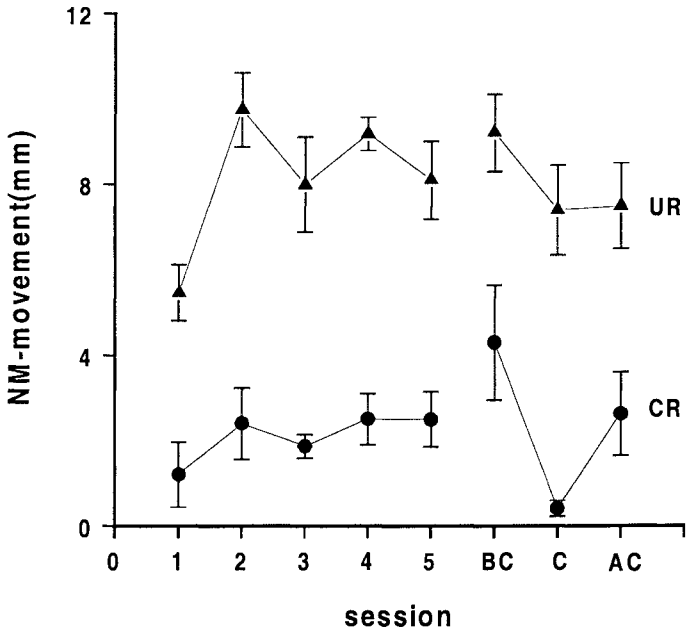

Fig 3. Amplitudes of the CRs and URs as a function of the training sessions (1 - 5) and the cooling phases. Conventions are as in Fig 2.

view is that the memory trace is formed as a result of interaction of the inputs from the CS and US in the cerebellar Purkinje cells (Yeo and Hardiman 1992). Yet another view holds that critical plasticity occurs in both the cerebellar cortex and the deep nuclei (Mauk and Donegan, 1997; Steinmetz, 2000). Our results favor theories which involve a critical role for the IPN. This is because the CR was diminished only in those subjects where the cold probe tip was located within a 2 mm range from the IPN (the inactivation of the overlying white matter and cortex, as was the case in misses in implantation, did not affect the CR amplitude or frequency whatsoever).

Pontine nuclei are shown to be selective to the CS modality (Steinmetz et al 1987), but their projections carry information concerning every modality to the cerebellum (Brodal and Bjaalie 1992). The cells in the IPN are probably not specific to the modality of the CS, but respond to sensory information as such (mossy fiber connections) and to the information about reflex-eliciting stimuli (climbing fiber connections), thus creating a connection between them. Despite the assumption that the somatosensory information may be carried to the cerebellum via the inferior cerebellar peduncle in addition to the medial cerebellar peduncle, which is known to carry somatosensory, visual and auditory information (Blodel and Courville 1981), the acquisition and maintenance of the CR seems to be mediated by the

same critical site. This is the IPN and possibly, the overlying cerebellar cortex.

Conclusion

The IPN-mediated memory trace seems to be independent of the CS-modality. This is consistent with the view that a hard-wired connection (such as a reflex) can be associated virtually to any initially meaningless stimulus. Thus, an organism's hard-wired patterns of behavior may serve, through modification, as the basis of acquiring new skills.

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Prog. Neuro-Psychopharmacol. & Biol. Psychiat.
J. Wikgren and T. Korhonen
Running title: Somatosensory CS and interpositus nucleus

Running head: REFLEX FACILITATION IN EYEBLINK CONDITIONING

Reflex facilitation during eyeblink conditioning and subsequent dentate-interpositus inactivation in rabbit

Jan Wikgren, Timo Ruusuvirta and Tapani Korhonen
University of Jyväskylä, Finland

Abstract

The subtypes of reflex facilitation in the rabbit eyeblink conditioning were determined. In Experiment 1, reflex facilitation in three conditions were assessed. It was found that the presence of the conditioned stimulus is a critical factor in reflex facilitation during an early phase in conditioning. Experiment 2 was designed to determine whether any type of reflex facilitation is dependent on the functioning of the dentate-interpositus nucleus (DIPN). Although the inactivation of the DIPN resulted in abolition of the conditioned responding, the presence of the CS still caused facilitation of the UR. Furthermore, the inactivation reduced the peak amplitude in the US-alone trials. The possible processes contributing to subtypes of reflex facilitation are discussed.

Reflex facilitation during eyeblink conditioning and subsequent dentate-interpositus inactivation in rabbit

Classical conditioning of nictitating membrane response (NMR) in rabbits (Gormezano, 1962) involves pairing a conditioned stimulus (CS; e.g., a tone) with an unconditioned stimulus (US; e.g., an airpuff towards the cornea). Repetitious temporal pairing of these stimuli results in acquisition of a conditioned response (CR), movement of the nictitating membrane (NM) as a response to the CS. In addition to the elicitation of the CR, pairing of the stimuli results in modification of the unconditioned responses (URs; Harvey, Gormezano, & Cool-Hauser, 1985; Weisz & LoTurco, 1988; Weisz & McInerney, 1990; Weisz & Waltz, 1990). This reflex modification is perceived as a change in latency and/or amplitude of the UR. In the case of NMR conditioning with relatively short interstimulus interval (ISI; less than 1 sec), modification in the form of reflex facilitation occurs (Scheurs, Oh, Hirashima, & Alkon, 1995).

Empirical findings suggest at least three aspects in reflex facilitation. First, mere exposition to the experimental setting results in more vigorous eyeblink responses as shown by Schreurs et al. (1995). This could be referred to as nonassociative reflex facilitation. Second, the presence of the CS in the delayed conditioning paradigm causes greater URs in training trials compared to US-alone trials, even though learning as measured by the presence of CRs has not yet occurred (e.g., Weisz & LoTurco, 1988). This could be called CS-mediated reflex facilitation. Third, a robust level of CRs correlates with permanent facilitation of the UR when US-alone trials were compared before and after extensive training (Schreurs & al, 1995). This could be called CR-related reflex facilitation.

Parameters in conditioning (duration and intensity of stimuli) or pharmacological treatment usually affect both reflex facilitation and learning (Harvey & al., 1985), that is, they are highly correlated phenomena, and so being, it is probable that their processing is mediated by overlapping structures in the nervous system. On the other hand, it has been shown that conditioned responding is dependent on the dentate-interpositus nucleus (DIPN) in the cerebellum (e.g., Anderson & Steinmetz, 1994; Steinmetz, Lavond, Ivkovich, Logan, & Thompson, 1992). However, lesion to this area does not prevent the ability of the CS to facilitate the UR (Weisz & LoTurco, 1988), while the reflex facilitation is inhibited by lesion in the amygdala, with no influence on learning per se (Weisz, Harden, & Xiang, 1992).

In experiment 1, we studied development of reflex facilitation over the time course of conditioning, and compared it with reflex facilitation found during explicitly unpaired treatment. We predicted the following: 1) The facilitation of the UR can be seen in all cases as a function of experience (nonassociative reflex facilitation); 2) the URs in the paired trials will be facilitated most rapidly (CS-mediated reflex facilitation), and 3) after robust level of CRs in the paired group, the URs in US-alone trials will be facilitated more than in the unpaired treatment (CR-related reflex facilitation). In Experiment 2, the DIPN in well-trained rabbits was reversibly inactivated to assess whether any of these types of reflex facilitation is dependent on DIPN functioning, which has been shown to be essential for CS-CR conditioning.

Experiment 1

Method

Subjects.

Subjects were 21 adult New Zealand albino rabbits weighing 2.5 - 3.7 kg at the time of the surgery. The animals were individually housed in metal cages on a 12:12 hr light-dark cycle with free access to food and water. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) regarding the care and use of animals for experimental procedures. The study was permitted by the animal research ethics committee of the University of Jyväskylä.

Surgery.

The animals were anesthetised with i.m. injections of a ketamine-xylazine cocktail (Ketaminol, 50 mg/ml, 5.6 ml; Rompun, 20 mg/ml, 2.2 ml; physiological saline 2.2 ml). The initial dosage was 3 - 4 ml and the anesthesia was maintained by additional injections of 2 ml every 20 - 40 min. After a deep general anesthesia had been achieved, the animals were placed in a stereotaxic instrument (Kopf Instruments) with bregma 1.5 mm above lambda. A longitudinal incision was made to reveal the skull onto which the headstage (designed to hold the minitorque potentiometer, an airpuff delivery nozzle and tone tubing), was cemented using four stainless steel anchoring screws.

Cold probes were implanted for the unpaired group (they served as subjects in Experiment II). The construction of the cold probe was modified after that presented in (Zhang, Ni & Harper, 1986). The shaft of the probe was not warmed, as it has been shown that lesioning the cerebellar lobule HVI, through which the probe was penetrated, does not interfere with the acquisition of conditioned NMR (Clark, Zhang, &

Lavond, 1992). In short, the cold probe consists of two stainless steel tubes one inside the other. The inner tube delivers the coolant inside the outer tube, 1 mm from the tip, which is sealed by solder. The coolant exits through a plastic tube attached to the outer cannula at a Y-shaped junction. The coolant used was freon-like 1,1,1,2-Tetrafluoroethane (KLEA R-134-A). The implantation followed the guidelines concerning recording-electrode implantation reported by Korhonen (1991). The cold probe was implanted near the DIPN using the coordinates 0.5 mm anterior and 5.0 mm lateral to the lambda. Analgesics (Temgesic 0.3 mg/ml) were provided 2 hours after the surgery and additionally if needed.

Experimental procedure.

The subjects were given at least one week to recover after surgery before the actual experimental procedures. On the first day, the animals were adapted to the experimental situation by placing them in the plexiglas restraining box in the sound proof conditioning chamber. Rabbits were divided in two groups: the unpaired (UP) group (n = 9) and the classical conditioning (CC) group (n = 11). The UP session consisted of 70 presentations of both tones (1000 Hz, 85 dB, 350 ms) and airpuffs (2.1 N/cm^2 source pressure, 100 ms) given in a pseudorandom order with intertrial interval (ITI) varying between 30 and 50 s (mean ITI = 40 s). The CC session consisted of 60 CC trials, where the tone was followed by the airpuff, 10 CS alone and 10 US alone test trials in pseudorandom order. The paired trials were presented in the delayed fashion where the stimuli coterminated. The ITI was the same as in the UP treatment. The subjects were treated for five subsequent daily sessions. Peak amplitude values for CRs and URs were defined from a period of 250 ms after CS and US, respectively. Any movement of the NM exceeding 0.5 mm was counted as a response. Excluded from the analysis were trials where NM movement exceeding 0.3 mm occurring during a period of 100 ms prior to the trial was observed.

Statistical procedures.

Included in the analysis of the unconditioned responses were three types of trials [US trials in unpaired group (US/UP), US period in conditioning trials (CS+US/CC) in the paired group and US-alone trials in paired group (US/CC)] at three different stages of experience (the first trial in the first session, the last trial in the first session, the last trial in the last session). Experience has shown that 60 paired trials are usually not enough for the development of the conditioned responses in our setting. Therefore, by the last trial of

the first session the URs should not yet be influenced by learned response. Analysis of variance (ANOVA) for repeated measures was used. When the experimental groups were compared, the Treatment served as a between subjects factor in a mixed model. Further, t-tests were used to assess differences between groups or trial types in a certain training phase.

Histology.

After the experiments, the animals were anesthetised with an im. injection of ketamine-xylazine coctail and then overdosed by an iv. injection of pentobarbital. The rabbits were then perfused via the ascending aorta with saline followed by 10 % formalin. The brains were removed and fixed in formalin-sucrose solution for at least one week. Frozen coronal sections of 100 µm were taken from the site of the cold probe. Slices were mounted on gelatinised slides and stained with cresyl violet. The locations of the cold probes were determined according to the stereotaxic atlas by Shek, Wen and Wisniewski (1986).

Results

Conditioned responding.

As expected, no CRs were observed in any of the conditions during the first session. The analyses of the URs, therefore, are free of contamination by learned overt motor responses. By the fifth session, the mean CR percentage in the paired group was 77.4 % ± 7.2 (SEM). No CRs were observed in the unpaired group.

Unconditioned responding.

Figure 1 depicts the development of the UR-peak amplitude over the course of treatment. They were analyzed in a pairwise fashion. As seen, common to all treatments and trial types is the UR's tendency to increase as a function of experience, as indicated by significant main effects of Training Phase in both groups [in the CC group $F(2,20) = 25.25$, $p < 0.001$; in the UP group $F(2,16) = 13.06$, $p < 0.001$].

Within the CC group, URs in CS+US trials grew more rapidly than in US-alone trials as indicated by significant interaction of Training Phase X Trial Type [$F(2,20) = 18.57$, $p < 0.05$]. Paired samples t-test revealed that the difference was significant only for the last trial of the first session of training (left side in Figure 1). Further, there was no significant difference in the last trial of the first session between US/CC and US/UP conditions indicating that the presence of the CS is a necessary factor in this phase of training to maintain reflex facilitation.

Comparison between US-alone trials in the experimental groups is shown in the middle of Figure 1. Interaction of Training Phase X

Treatment was significant [$F(2,36) = 2.88$, $p < 0.05$]. Independent samples t-test revealed significant difference for the last trial in the last session [$t(18) = 2.24$, $p < 0.05$)] in which the CC group had already acquired robust level of conditioned responding. Taken together, this confirms the existence of facilitation of the UR to US-alone trials in the CC group.

Further evidence for the effect of an associative process on reflex facilitation is that when the CS+US/CC and US/UP trials were compared (on the right in Figure 1), the difference was significant both for the last trial of the first session [$t(18) = 2.24$, $p < 0.05$] and for the last trial of the last session [$t(18) = 2.05$, $p < 0.05$], but not for the first trial of the first session, where the subject had not yet experienced paired presentation of the stimuli in the CC group.

Discussion

These results suggested the occurrence of all three types of reflex facilitation, and that it is possible to dissociate them by taking samples from appropriate phases in conditioning. The fact that the later URs were larger in amplitude in all conditions is an indication of nonassociative reflex facilitation. Existence of CS-mediated reflex facilitation can be concluded from the finding that the UR in the CC group was facilitated more in a condition where the US was preceded by the CS, and that the initial level of responding was the same in all conditions, that is, the CS did not have any effect on the UR at the beginning. US-alone condition in the CC group did not elicit more vigorous responses in the absence of the CR, when compared to the US-alone condition in the UP group - a finding indicating that the US/UR-circuit had not been yet modified. However, in the end of training the URs to US-alone trials in the CC group were significantly larger than those in the UP group. This indicates the presence of CR-related reflex facilitation and presumably relatively permanent modification of the US/UR-circuit.

These results are in accordance with those of Scheurs et al. (1995), who showed conditioning-related facilitation in the US-alone trials when a robust level of CRs was achieved, but not when the extent of conditioning was low (17% CRs after the first day of conditioning in their data). Our data suggests that CS-mediated reflex facilitation, at this point in the conditioning process, reflects a conditioned activational state in the organism, in which the CS functions as a specific signal for the forecoming event.

To investigate whether reflex facilitation is dependent on the functioning of the DIPN, we set up Experiment 2, in which the aim was

to test whether the amount of reflex facilitation is altered when the CR is blocked by inactivation of the DIPN. Since the DIPN is essential for forming and maintaining the association between the CS and the US, Experiment 2 will show whether any type of reflex facilitation is influenced by learning.

Experiment 2

Method

Subjects and surgery.

Subjects were the same 9 adult female New Zealand albino rabbits used in the unpaired group in Experiment 1.

Procedure.

After the unpaired treatment, the rabbits were trained in the same manner as the CC group in Experiment I. After reaching the learning criterion of 8 out of 9 consecutive conditioned responses and being overtrained one session, the rabbits underwent a session which involved cooling of the dentate-interpositus nucleus. The cooling session was divided in three blocks: pre-cooling, cooling and post-cooling. Each block consisted of 30 trials (4 CS only, 4 US only and 22 CS+US trials). CR and UR amplitudes and latencies were detemined as averaged measurements of US alone trials and CS+US trials. After the first block, the cold probe was activated and the gas flow was adjusted so that the temperature in the cold probe fell below 5 °C. This took about two minutes. After the cooling block, the experiment was again interrupted for about two minutes in order to allow the temperature return to the normal level

Results

Histology.

In 6 out of 9 subjects the cold probe was correctly located near the dentate-interpositus nucleus and their data were included for the analyses. The remaining 3 misses were excluded from the analyses. The locations of the coldprobe tips are shown in Figure 2.

Conditioned responding.

Figure 3 illustrates the amplitudes of CRs before, during and after cooling. Repeated measures ANOVA revealed significant main effect of Cooling [$F(2,10) = 3.23$, $p < 0.05$].

Unconditioned responding.

Peak amplitudes and peak latencies of the URs in both US alone and CS+US trials in all three phases of cooling session are shown in Figures 4 and 5, respectively. Main effect of Cooling on peak amplitude in repeated measures ANOVA was significant [$F(2,10) = 8.47$, $p < 0.05$] as well as the main effect of Trial type [$F(1,5) = 7.86$]. In

contrast, interaction of Cooling x Trial type was not significant [$F(2,10) = 1.73$, $p = 0.38$]. For peak latency, the main effect of Cooling was not significant [$F(2,10) = 0.29$, $p = 0.755$], contrary to the main effect of Trial type, which was significant [$F(1,5) = 25.58$, $p < 0.01$]. Interaction of Cooling and Trial type was not significant either [$F(2,10) = 0.88$, $p = 0.396$].

The significant main effect of cooling on amplitude was rather surprising as a body of earlier research indicates that reversible inactivation or complete lesioning of the DIPN has no effect on unconditioned responses (e.g., Clark, McCormick, Lavond, & Thompson, 1984; Lavond, Hembree, & Thompson, 1985; Yeo, Hardiman, & Glickstein, 1985; Steinmetz, Lavond, Ivkovich, Logan, & Thompson RF, 1992; Ivkovich, Lockard, & Thompson, 1993; Clark, Zhang, & Lavond, 1992). As seen in Figure 4, the amplitude in US-alone condition was greatly reduced. An additional ANOVA for repeated measures was performed with both trial types separately. This confirmed that the effect of cooling on US-alone trials was significant [$F(1,5) = 5.122$, $p < 0.05$], but this was not the case for CS+US trials [$F(1,5) = 3.372$, $p = 0.076$]. Thus, the significant main effect of cooling in 2 x 3 design was due to the reduction in peak amplitude in US-alone condition.

Discussion

Cooling of the dentate-interpositus nucleus in well trained animals abolished the CR and reduced the amplitude of URs in US-alone trials, but failed to show significant effect on reflex facilitation latency and peak amplitude measurements of the UR within CS+US trials. Although the animal does not lose its capability of responding to the US on CS+US trials, the UR diminishes in the US-alone condition. A possible explanation could be that there is some US/UR-circuit modification whose maintenance is dependent on a functional DIPN. This modification would also correlate with the existence of a robust CR, because no change could be seen in the initial phase of the training, as was shown in the Experiment 1. Thus, cooling of the DIPN abolishes both the actual discrete conditioned skeletal muscle response and the CR-related reflex facilitation, but not CS-mediated reflex facilitation.

General Discussion

Reflex facilitation could be argued to be affected by at least three different factors. First, reflex facilitation is a nonassociative process. The NMR to the US increases in amplitude during US preexposure treatment (Saladin & Tait, 1986). The present data suggests the same. The NMR amplitude increased after 60 presentations

of the US-alone trials in the explicitly unpaired group. The treatment is, of course, not exactly the same as US preexposure, but Weisz & Waltz (1990) have shown that intertrial intervals longer than 4 seconds in trace conditioning paradigm are too long for the CR or the reflex facilitation to occur (ITI in our experiment varied between 20 and 40 seconds). It is our experience that few subjects show URs exceeding 0.5 mm to the airpuff in the very beginning of the experiment. However, usually less than ten presentations of the airpuff is enough to produce constantly criterion-exceeding responses thereafter. Thus, in some way, the animal must 'learn' the reflex in itself. It is not very far fetched to assume that the context the subject is placed in also plays a role in this process. This, of course, would mean that what is called nonassociative reflex facilitation, could actually be affected by context-related reflex facilitation, which would make it an associative process in addition to nonassociative process of autofacilitation of the US/UR circuit. Presumably, nonassociative reflex facilitation exists, but its presence is very difficult to prove in a complex surroundings as is the case in experimental settings like ours. It would be of interest to compare reflex modification in a changing context during the early phase of conditioning. Changing the context in an early phase of training as compared to the control group in a stable context should result in retarding the increased rate of nonassociative reflex facilitation if the context has acquired meaning.

Second, reflex facilitation is a conditioning-related process. Pairing the CS and the US results in more vigorous URs to the US used. This has been shown to be the case only after a robust level of conditioning has been reached (Schreurs, Oh, Hirashima, & Alkon, 1995). After one day of treatment we could not see any difference in the paired and unpaired groups in their responding to the US alone trials. The conditioning-related facilitation of the UR, shown to correlate with robust level of CRs, appears to be dependent of the DIPN, as the URs in US-alone trials reduced, but the URs in the CS+US trials did not, when UR amplitudes during cooling were compared to the amplitudes immediately before and after cooling. This is not in line with previous findings (e.g., Clark & al., 1992; Clark & al., 1984; Lavond & al., 1985; Yeo & al., 1985; Steinmetz & al., 1992; Ivkovich & al., 1993), which indicates the opposite; the URs in US-alone trials remain on the same level during DIPN inactivation while URs in paired trials decrease in amplitude or the lesioning does not have permanent effect on UR amplitude. Clark et al. (1992) explained that this result

illustrates the effect of removing the CR amplitude from the UR amplitude on paired trials. However, Ivkovich et al. (1993) reported numerical but not statistical decrease in UR amplitude just after the lesioning. It could be that in our study the interpositus inactivation had the same kind of temporary effect.

Third, reflex facilitation in itself is a result of conditioning, that is, reflex facilitation is an indication of a conditioned arousal state, and as such it is, the first sign of learning in the process of conditioning. Thompson and his associates (Richardson & Thompson, 1984; Thompson, Thompson, Kim, Krupa, & Shinkman, 1998) have argued that in addition to the sensory/response eliciting value, the US also is an aversive signal. It has been shown that stimulation of the dorsal accessory olive as a US could be associated with a CS but this association would involve no signs of aversiveness (Mauk, Steinmetz, & Thompson, 1986). Perhaps it is the aversiveness of the stimulus that gets associated to the CS separately from the behavioral response by the conditioned state of arousal. This would then be seen as CS-mediated reflex facilitation despite cooling of the DIPN. The storage of this association would not be dependent of the DIPN, since CS-mediated reflex facilitation was still present during cooling. Further, reflex facilitation has been shown to be relatively specific for the properties of the CS (Weisz & LoTurco, 1988; Weisz & McInerney, 1990). This further confirms the view that there is an association between the CS and the US that is maintained somewhere in the nervous system. Our data suggests that in addition to the CR, the conditioning-related enhancement of the UR also stops during cooling. This is analogous to the phase of conditioning where the CRs are not yet developed; there is no enhancement of the UR in US-alone situation either.

The data suggest that the DIPN does not have a role only in acquisition and maintenance of the CR-memory trace but also has a role in modification of the US/UR-circuit. Cooling of the DIPN significantly reduced the UR in test trials where only the airpuff, but not the CS-tone, was present. Therefore, the cooling did not have a general effect on animal's ability to perform the appropriate response. As the cooling did not have significant effect on the CS-mediated reflex facilitation, it could be argued that the memory trace for the conditioned facilitation is located elsewhere. The studies by Weisz et al. (1990) suggest that limbic system possibly has a role in this process. Further, electrical stimulation of the amygdala prior to the US presentation facilitates the UR (Whalen & Kapp, 1991). Another

possibility is that the CS modifies the US/UR circuit directly. It has been shown that electrical stimulation of auditory nuclei as a CS supports acquisition of the CR, but the stimulation of the cochlear nucleus (which is the first relay site in the auditory CS pathway) as a CS also produces reflex facilitation, whereas stimulation of the superior olive, inferior colliculus or medial geniculate nucleus as a CS do not as shown by Nowak, Kehoe, Macrae & Gormezano (1999). They further notice that in addition to projecting to the pontine nucleus, the cochlear nucleus also projects to the spinal trigeminal nucleus, which is the primary sensory relay for the US. Stimulus-evoked neural activity has been shown both to the CS and US in the trigeminal complex, as well as CR-related activity (Clark & Lavond, 1996). However, the learning-related neural unit activity in the trigeminal complex is shown to be abolished during interpositus inactivation (Clark & Lavond, 1996). Still, the trigeminal complex shows activity to the CS during interpositus inactivation and as the mesencephalic reticular formation and the trigeminal nucleus has been shown to receive fibers from the central nucleus of the amygdala (Hopkins & Holstege, 1978; Takeuchi, Satoda, Tashiro, Matsushima, & Uemura-Sumi, 1988) it is quite possible that the CS has an effect on the UR even when the CR is blocked by interpositus inactivation. Despite crucial involvement of the amygdala in the maintenance (but not initiation) of the reflex facilitation (Weisz et al., 1996), it is a poor candidate for a site where plasticity for the CS-mediated reflex facilitation occurs. Namely, Richardson and Thompson (1984) have shown that amygdaloid activity to the stimuli used in this type of conditioning is of substantially longer latency than would be sufficient to have an effect in the intratrial time course. This, however does not rule out the possibility that amygdaloid activity may be related to the state-related changes rather than information-related changes in eyeblink conditioning (Richardson & Thompson, 1984) and therefore to be essential to facilitation of conditioning.

Conclusions and Suggestions.

In localizing the possible memory engram of the CS-evoked emotional response, the present study suggests that it is essential to differentiate subtypes of reflex facilitation since all forms of it are probably not due to the altered arousal state, but autofacilitation of the UR itself. This most likely happens somewhere along the US/UR-circuit, whereas the associative, CS-mediated reflex facilitation probably reflects the modulative properties of the state

of the animal, whose control are located in the cerebral aspects of the nervous system.

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Figure captions:

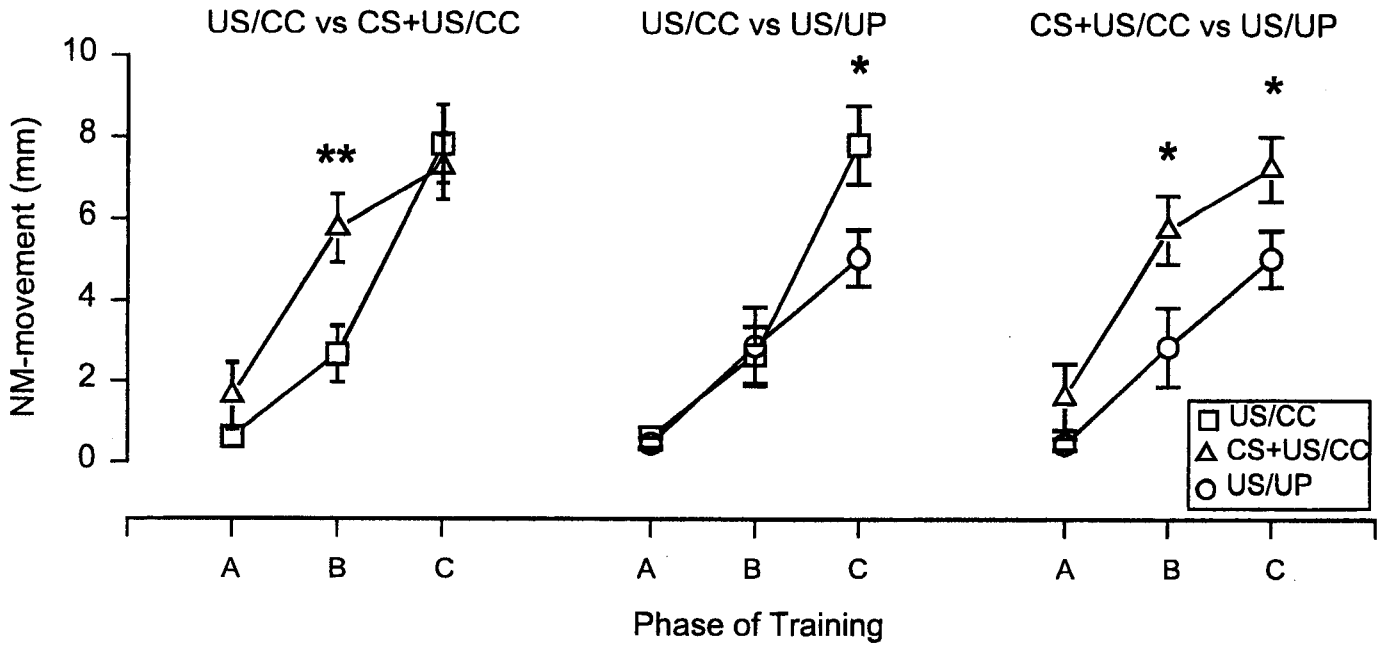
Figure 1. Mean peak amplitudes [± SEM] of the unconditioned responses on US-alone trials in CC group (triangles), CS+US trials in CC group (rectangles), and US trials in unpaired group (circles) are plotted as a function of the training phase in Experiment 1. (A, the first trial in the first session; B, the last trial in the first session; C, the last trial in the last session). The mean peak amplitudes are plotted pairwise based on experimental as they were analyzed. Stars indicate significant differences between the NM amplitudes on the trials (*, $p < 0.05$; **, $p < 0.01$).

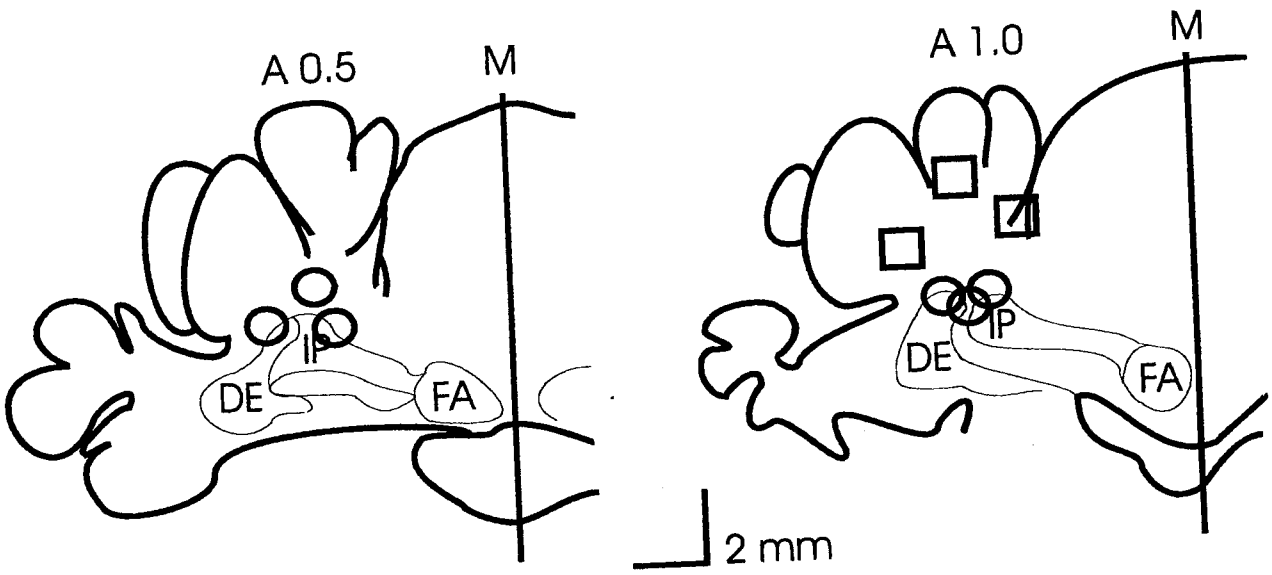
Figure 2. Locations of the cold probe tips in all nine experimental subjects in Experiment 2. Circles stand for the subjects in which the conditioned responding was abolished during probe activation. The cold probe tip locations for the animals which did not show statistically significant abolition of the CR during cooling are marked with rectangles, their data were excluded from further analysis. Key: DE, dentate nucleus; FA, fastigial nucleus; IP, interpositus nucleus; M, midline.

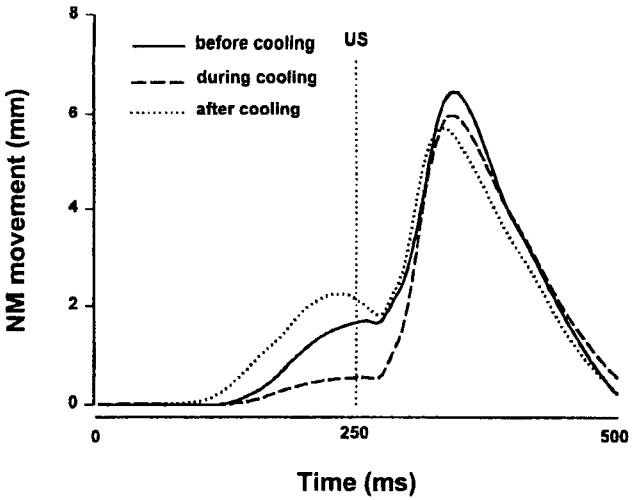
Figure 3. The averaged nictitating membrane movement in well trained animals with accurate cold probe implantation during the different blocks in the cooling session as measured on CS+US trials (22 trials/block) in Experiment 2. As seen, the responding on the CS period is of substantially smaller amplitude during cooling. Generally, the subjects lacked any movement during that period but the temperature fluctuates to some extent during cooling, which causes the release from blocking on some trials.

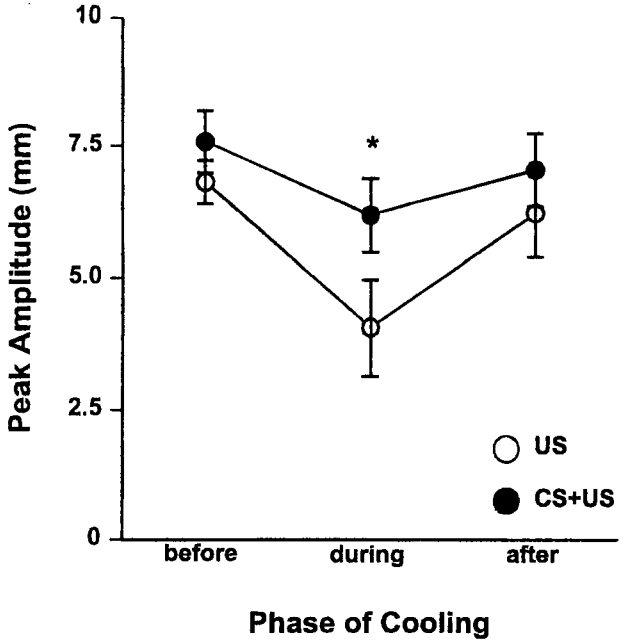
Figure 4. Mean peak amplitudes [± SEM] of the unconditioned responses on US-alone trials and CS+US trials as a function of the phase in the cooling session in Experiment 2. Star indicates a significant difference between the NM amplitudes on US-alone and CS+US trials.

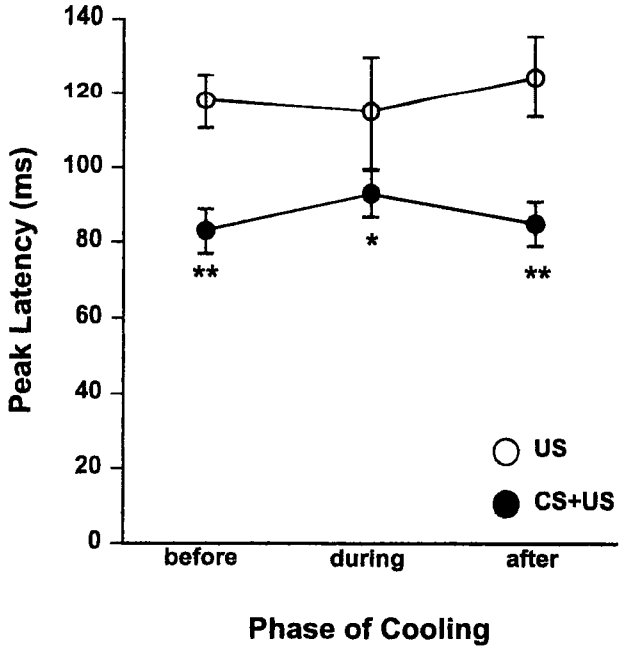
Figure 5. Mean peak latencies [± SEM] of the unconditioned responses on US-alone trials and CS+US trials as a function of the phase in the cooling session in Experiment 2. Stars indicate significant differences between the NM amplitudes on US-alone and CS+US trials.


US/CC vs CS+US/CC
US/CC vs US/UP
CS+US/CC vs US/UP
NM-movement (mm)
10
8
6
4
2
0
**
*
*
*
US/CC
CS+US/CC
US/UP
A
B
C
A
B
C
A
B
C
Phase of Training


A 0.5
M
IP
DE
FA
A 1.0
M
IP
DE
FA
2 mm


before cooling
during cooling
after cooling
US
NM movement (mm)
8
6
4
2
0
0
250
500
Time (ms)


10
7.5
5.0
2.5
0
Peak Amplitude (mm)
*
US
CS+US
before
during
after
Phase of Cooling


140
120
100
80
60
40
20
0
Peak Latency (ms)
**
*
**
US
CS+US
before
during
after
Phase of Cooling