BEHAVIORAL AND ELECTROPHYSIOLOGICAL STUDIES ON CELLULAR MECHANISMS OF CLASSICAL CONDITIONING MEDIATED BY THE RED NUCLEUS IN CAT.

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Abstract

We developed a behavioral and neural model for classical conditioning in the cortico-rubrospinal system and examined neuronal modification at the cortico-rubral synapses underlying the establishment of the classical conditioning.

1. The conditioned stimulus (CS) was applied to cerebral peduncle (CP) in cats with lesions of the corticofugal fibers caudal to the red nucleus (RN). The unconditioned stimulus (US) was an electric shock to the skin of forelimb which produced flexion of the limb. After pairing of the CS and the US at an interval of 100 msec for about a week, an initially ineffective stimulus to CP induced forelimb flexion. The shortest latency (8 msec) of electrical activity in biceps brachii muscle in response to the CS was consistent with the shortest time required for the transmission of impulses along the cortico-rubrospinal pathway to the muscle. The interposito-rubrospinal system could not have been the site of plastic change, because the threshold for elbow flexion induced by stimulation of nucleus interpositus (IP) of the cerebellum did not change before and after the conditioning. Since the cortico-rubrospinal and the interposito-rubrospinal system share the common pathway below RN, it was suggested that a modification of the cortico-rubral synapses was responsible for this behavioral change (Chapter 2).

2. By extracellular and intracellular recordings of the RN cell activity, we investigated signaling effectiveness at the cortico-rubral synapses before and after establishment of the classical

conditioning. The increased responsiveness of RN cells to the CS was correlated with acquisition of the conditioned forelimb flexion, i.e. RN cells responded to the CS with higher firing probability in the animals which received the paired conditioning than that in the animals which received the CS alone or pairing of the CS and the US at random intervals or that in the naive animals which did not receive any training. Monosynaptic excitation of RN cells in response to the single pulse to CP was most enhanced in the animals which received the paired conditioning. By contrast, response of RN cells, as well as the behavioral response, induced by stimulation of the cerebellar interpositus nucleus (IP) was not enhanced after the paired conditioning. The difference between the responses to the stimulation of CP and IP suggests that the primary site of neuronal change is the cortico-rubral synapses (Chapter 3).

3. In the animals that received the paired conditioning, the excitatory postsynaptic potentials (EPSPs) induced by stimulation of CP had fast-rising components superposed on the normal slow-rising EPSPs. On the other hand, most of the CP-EPSPs recorded in the naive animals showed a slow time course. The slow time course of the CP-EPSPs has been attributed to the peripheral localization of the cortico-rubral synapses on the dendrites of RN cells. The electrotonic length of RN cells in the animals which received the paired conditioning was not shorter than that in the naive animals. Therefore, it is suggested that the appearance of the fast-rising component in the CP-EPSPs is caused by formation of the new cortico-rubral synapses on proximal portion of the soma-dendritic membrane of RN cells. Since it has

been established that new synapses formed by collateral sprouting are retained for more than several months, the formation of new synaptic connections could underlie long-lasting behavioral modification (Chapter 4).

4. We tried to reduce the neuronal circuit of the US pathway. We considered that the locus coeruleus nucleus (LC), the main origin of central central noradrenergic projection, may be one of the important systems mediating effect of skin shock to the cortico-rubral system. Noradrenergic projection into RN of cat was demonstrated immunohistochemically by using an antiserum against the dopamine-B-hydroxylase. After pairing the LC stimulus, instead of the US, with the preceding CP stimulus (CS) for a week, the initially ineffective CP stimulus induced forelimb flexion. On the contrary, applying the LC or CP stimulus alone did not produce the conditioning of forelimb flexion. Acquired conditioned responses were retained for more than two weeks, and extinguished after applying the CP stimulus alone. Reacquisition occurred within a few days. Unitary activity of RN cells was well correlated with the behavioral modification, i.e. RN cells responded to the CP stimulus with higher probability than that in the LC-alone, CP-alone or naive animals. By contrast, either unitary response to the IP stimulus or spontaneous discharge of RN cells did not significantly differ among these four groups of animals. These common features between the paired CS-US and CP-LC conditionings suggest that stimulation of the LC following the CP stimulus may modify the efficacy of signal transmission at the cortico-rubral synapses in the same way as in the CS-US classical conditioning (Chapter 5).

Chapter 1.

General introduction

One of the most important properties of the brain is capacity of neuronal plasticity. In addition to adaptations acquired gradually by evolution during millions of years, the brain can also change its function in response to experience during an individual lifetime. The most interesting neuronal plasticity during a lifetime may be exhibited in learning and memory: learning refers to the process in which the animal acquire the new brain function, and memory refers to the process through which the animal retains the new function.

Although there is a large body of work dealing with the neuronal correlate of learning (cf. Thompson et al, 1984), neuronal mechanism of it has been unsolved for a long time. In these ten years, however, learning and memory can be approached with the methods of cell biology. Currently, the most productive research strategy for investigating the neuronal basis of learning and memory is the model system approach: selection of an identifiable nervous system that is essential for learning to occur. This approach has been successfully applied in adaptation of vestibulo-ocular reflex in rabbit (Ito, 1982), habituation, sensitization and classical conditioning of siphon-withdrawal reflex in Aplysia(Kandel, 1983), and classical conditioning of forelimb flexion mediated by the red nucleus in cat (Tsukahara, Oda Notsu, 1981).

We selected classical conditioning mediated by the red nucleus (RN) as a model system to analyse the neuronal substrates of learning and memory. Synaptic plasticity in the red nucleus (RN) offers an opportunity to study neuronal mechanisms underlying learning mainly for two reasons. Firstly, the afferent and efferent neuronal connections and the synaptic organization have been well characterized (Massion, 1967; Hongo et al., 1969; Allen and Tsukahara, 1974; Illert et al., 1976). Secondly, the cortico-rubral fibers sprouted and formed new functional synapses on RN cells not only after lesion of the other synaptic inputs (Tsukahara et al., 1975; Murakami et al., 1982), but also after cross-innervation of the forelimb flexor and extensor nerves. In parallel with adaptation of voluntary forelimb movements after the cross-innervation, new cortico-rubral synapses were formed on the RN cells innervating upper spinal segments (Tsukahara and Fujito, 1976; Tsukahara et al., 1982; Fujito et al., 1982; Murakami et al., 1984). These findings suggest that formation of new synapses may be a neuronal mechanism underlying behavioral plasticity. Especially for the long-lasting behavioral plasticity, the formation of the new connection at the cortico-rubral synapses may be noticeable because there is a wealth of evidence suggesting that protein synthesis is required for long-term memory (cf. Kandel and Schwartz, 1982) and because the newly formed cortico-rubral synapses were stable for more than a few months in the preparations mentioned above.

Chapter 2.

Classical conditioning mediated by the red nucleus in cat.

INTRODUCTION

We asked whether plasticity in the cortico-rubral system might also mediated a behavioral modification such as learning. There is already evidence that the RN is important for classical avoidance responses. Smith (1970) had previously found that unilateral lesion of the RN abolished conditioned flexion of the contralateral forelimb which was established by pairing a tone as the CS with forelimb shock as the US.

We attempted to simplify this paradigm by limiting both the afferent pathway from the CS to the RN and the pathway mediating the conditioned response. The CS was applied to the cortico-rubral fibers in form of electric pulses and the corticofugal outflow was restricted mainly to the cortico-rubrospinal by section of the corticofugal pathways just caudal to the RN.

First, we examined whether behavioral change occurs after pairing the CS with the US, and then compared effect of pairing two stimuli at fixed interval with that of pairing them at random intervals or applying the CS alone to examine the behavioral change is an associative or nonassociative effect. Secondly, we tried to indendify the primary site of neuronal change underlying the classical conditioning by comparing excitability of the cortico-rubrospinal pathway with that of the interposito-rubrospinal pathway.

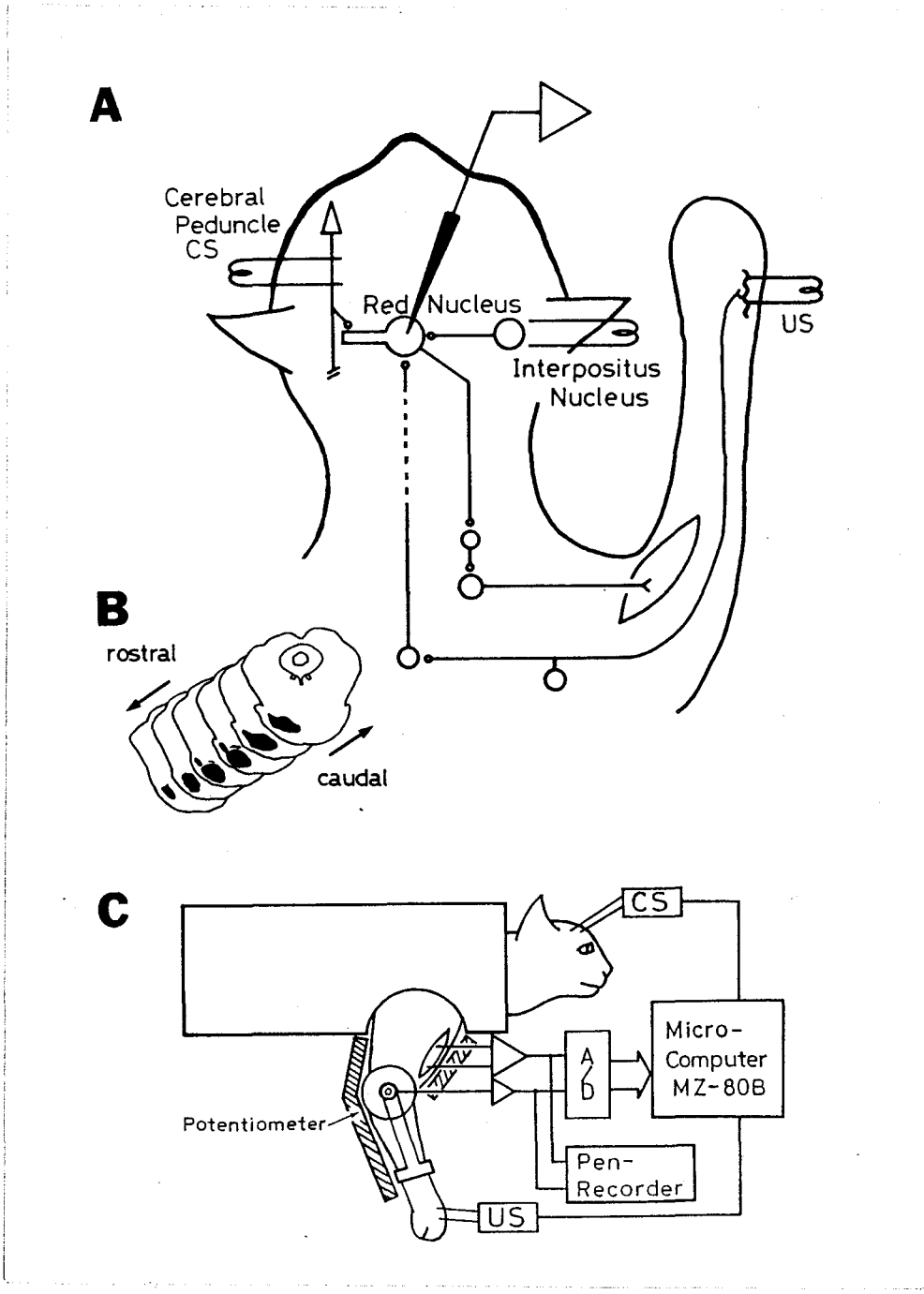

Fig. 1. Diagram to illustrate the experimental arrangement. A: Neuronal circuit for the classical conditioning mediated by the red nucleus. The CS was applied to the cerebral peduncle. The US was delivered to forearm skin just proximal to the wrist joint. B: Drawings of histological sections through the mid brain to illustrate the lesion in the cerebral peduncle caudal to the red nucleus. C: The setup used for applying the CS and the US and measuring movements at the elbow joint. Measures of the joint angle and electrical activity of the biceps muscle were stored and analysed by a microcomputer.

METHODS

Surgical procedures

Adult cats were used: 31 cats for extracellular unitary recording and 66 cats for intracellular recording. Before start of the training, the cats were operated under sodium pentobarbitone anesthesia. The cerebral peduncle was sectioned at the level caudal to RN to eliminate motor reactions mediated by the corticofugal fibers other than cortico-rubral fibers. The cerebral peduncle was lesioned by passing current (1 mA for 30 sec) at AP 0 and A 0.5 of the Horseley-Clarke coordinates (Fig. 1B). Bipolar stimulating electrodes were inserted stereotaxically into the left CP (A 8.5) and the right IP (Fig. 1A).

Training procedures

About ten days after the surgery, the cats received pretraining period to determine the current intensity for the CS. It usually took 4 to 6 days to determine the value. The CS to CP consisted of a train of five electric pulses with an interpulse interval of 2 msec. Each pulse, 100 usec in duration, was followed by a pulse with reversed polarity to prevent electrode polarization. The US, an electric shock with a duration of 10 msec, was delivered to the shaved skin of right forearm. Current intensity of the CS, which ranged from 0.1 to 1.0 mA, was determined so as to produce forelimb flexion at a probability of 0.2 during the pretraining period.

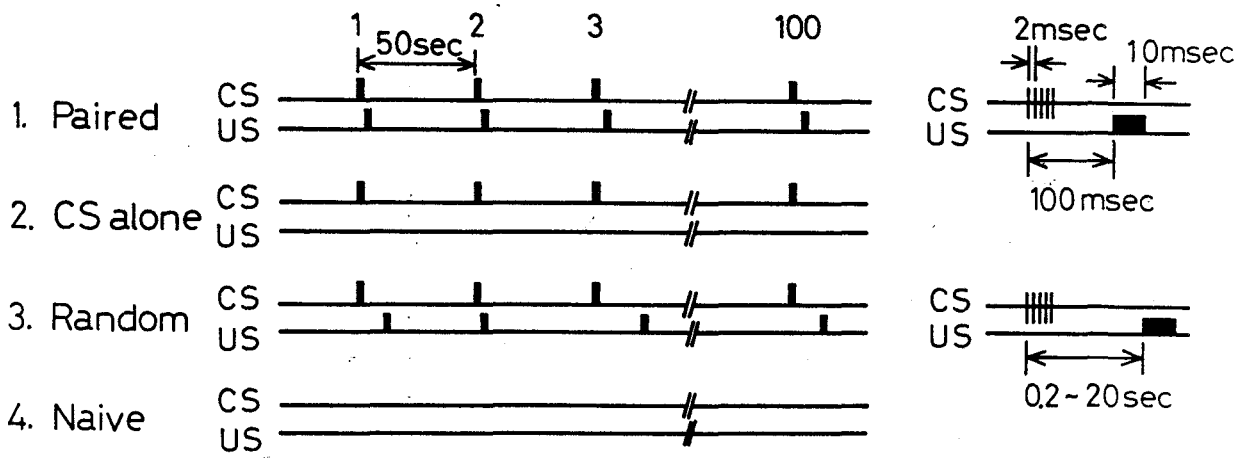

Fig. 2. Diagrammatic representation of the training and naive procedures. Paired: the CS was paired with the US at the fixed interval of 100 msec. CS alone: only the CS was applied. Random: the CS was paired with the US at random intervals ranging from 0.2 to 20 sec. In these three group, The CS with or without the US was applied once every 50 sec and a total of 100 stimuli in a day. Naive: animals received only the surgical and pretraining procedures.

Cats were mounted on a specially designed frame. Forelimb flexion was measured with a potentiometer attached to the elbow joint. The output voltage of the potentiometer was sampled at every 4 msec and stored in a microcomputer. The resolution of measurement of the joint angle was 0.02 degree. The elbow flexion exceeding 0.06 degree within 100 msec after the onset of the CS was judged as a positive response. The response was not judged if sway of the forelimb exceeded 0.02 degree during 80 msec before the onset of the CS. A microcomputer was used to control the strength and timing of the CS and US and to judge a positive response (Fig. 1C).

The training procedure started one day after the pretraining period. Animals for extracellular unit recordings were divided into four groups: paired (N=14), random (N=7), CS-alone (N=5) and naive (N=5) (Fig. 2). Animals for intracellular recordings were divided into paired (N = 38) and naive group (N = 28). The animals in the paired group received the CS paired with the US at the fixed interval of 100 msec. Those in the random group received the CS and the US, but the time interval between them was randomly varied from 0.2 sec to 20 sec. The animals in the CS-alone group received the CS alone. In these three groups, the CS with or without the US was applied once every 50 sec and a total of 100 paired or unpaired stimuli were provided in a day. Training period consisted of one to two weeks' sessions. The effect of training was estimated by the percentage of positive responses out of total trials, which will be referred to as "score of performance". To compare the change in the efficacy of

signal transmission along the cortico-rubrospinal and the interposito-rubrospinal pathway, the minimum current intensity of the stimulus to CP or IP to produce 100 % score of performance was determined after the end of the each day's training session. These currents will be referred to as "CP-100 % performance current" and "IP-100 % performance current", respectively. The animals in the naive group underwent the surgical procedures and the pretraining procedure which were the same as those for the other groups of animals.

Histology

After the end of the experiments, the animals were anesthetized with sodium pentobarbitone and DC current (500 uA, 10 sec) were passed through the stimulating electrodes. Then the animals were perfused with 10 % formalin solution. The degree of lesion of CP, the location of the stimulating electrodes and also tracks of recording electrodes in the experiment for unitary or intracellular recordings of RN cells as mentioned in Chapters 3, 4 and 5 were confirmed by serial sections stained with methylene blue.

Statistical analysis

Statistical comparisons were made using a one-tailed t-test for correlated means. If the population variances were the same a Student's t-test was used, and in other cases a Welch's t-test

was used. All the data are expressed as the mean ± standard error of mean (S.E.M.).

The histological procedures and statistical analysis as mentioned here were also employed in the unitary or intracellular recording of RN cells as shown in Chapter 3 to 5.

RESULTS

Modification of behavior

The initially ineffective CS induced forelimb flexion after pairing the CS with the US at the fixed intervals. Figures 3A, B and C show examples of mechanogram of elbow flexion in an animal of the paired group. On the 2nd day of training, the CS alone scarcely produced positive responses (Fig. 3A), whereas the US produced large elbow flexion at every trial (Fig. 3B). After the CS-US pairing for 6 days, the CS produced forelimb flexion which often exceeded the threshold elbow joint angle of 0.06 degree (Fig. 3C).

An example of the electrical activity recorded from the biceps brachii muscle during one on the training session is illustrated in Fig. 3D. The latencies of the first spike observed in the eletromyographic records from the onset of the CS were measured and the frequency distribution of these latencies is shown in Fig. 3E, which includes records from three cats on the 8th, 9th and 13th day of the paired training. The latencies ranged from 8 to 34 msec.

Figure 4A shows an example of the time course in the change of score of performance. The score of performance increased gradually until it reached a plateau on about the 7th day of the paired conditioning (acquisition in Fig. 4A). From the 11th day of training, the cat received pairing the US with the CS in reversed order. the score of performance returned to the

pretraining level within 5 days (extinction in Fig. 4A). In parallel with the increase in the score of performance, the 100 % performance current progressively decreased (Fig. 4B). With extinction, it returned to the pretraining level.

Figures 5A and B summarize the time course in the change of the responses to the CS in three groups of animals. The animals in the paired group showed gradual increase in the score of performance during the training period (Fig. 5A). The score on the 8th day (78.2 $\pm$ 5.1 %, n=14) was significantly higher than that of the last day of the pretraining period (19.4 $\pm$ 1.5 %; $p < 0.001$). There was no significant increase in the score of performance in the other two groups (Fig. 5B). The score on the 8th day in the paired group was significantly higher than those in the random group (28.2 $\pm$ 7.1 %, $p<0.001$) or in the CS-alone group (17.9 $\pm$ 10.9 %, $p<0.01$). The acquired conditioned response could be extinguished by providing the CS alone or reversing the order of the CS and the US (as shown in Fig. 4). These results showed that this behavioral learning can be categorized into the associative classical conditioning (Kandel, 1976; Rescorla and Holland, 1976).

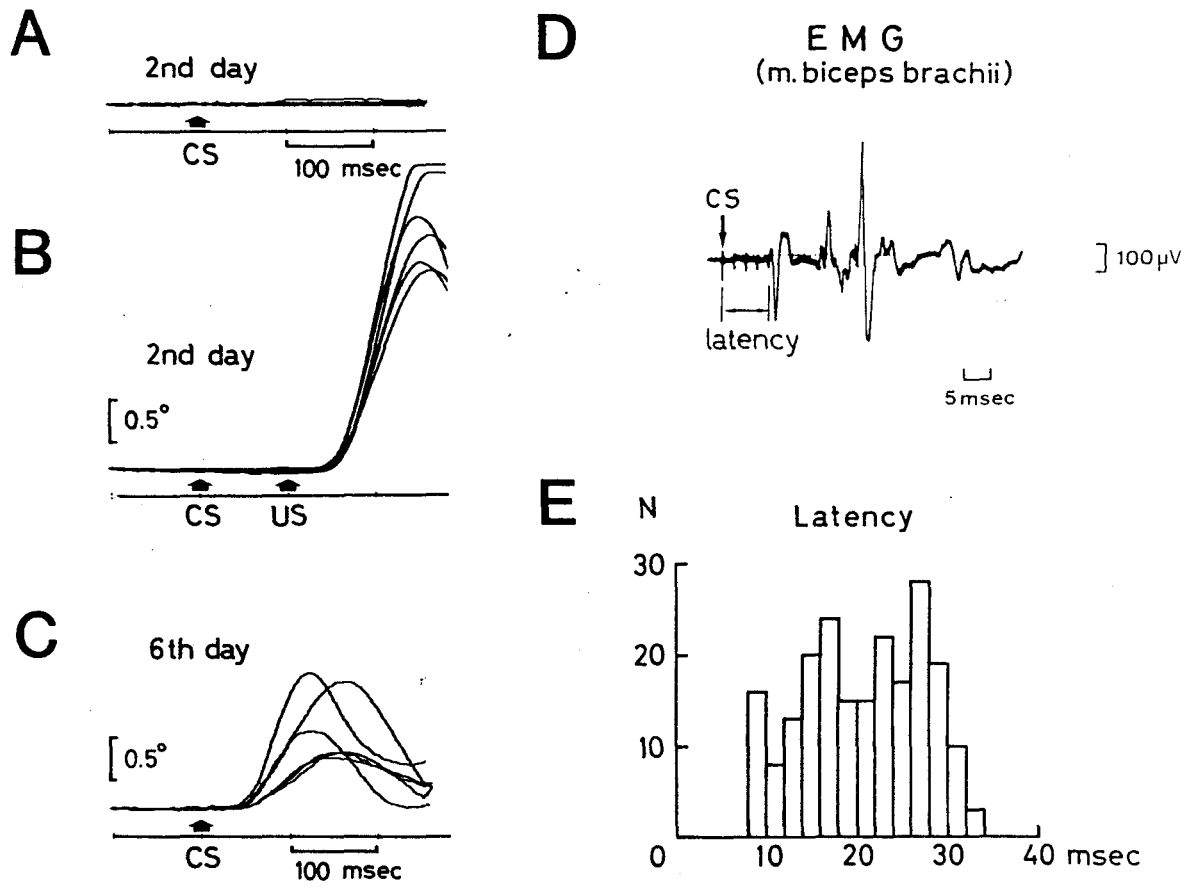

Fig. 3. Acquisition of the conditioned forelimb flexion. A to C: Sample records of mechanogram of forelimb flexion during the paired conditioning. A and B: Flexion of the elbow joint on the 2nd day of training. The CS produced no response (A), but the US produced large elbow flexion (B). Arrows indicate the onset of the CS and US. C: Flexion of elbow joint in the same animal on the 6th day of training. The CS alone produced positive elbow flexion. The upper traces are the superposed records of six examples of the mechanogram. Time and angle calibration are common for A to C. D: Electromyogram of biceps brachii muscle. Down arrow: onset of the first pulse of the CS. E: Frequency distribution of the latencies of the first pulse in the electromyographic record from the onset of the CS. Ordinate: number of spikes. Abscissa: latencies in msec.

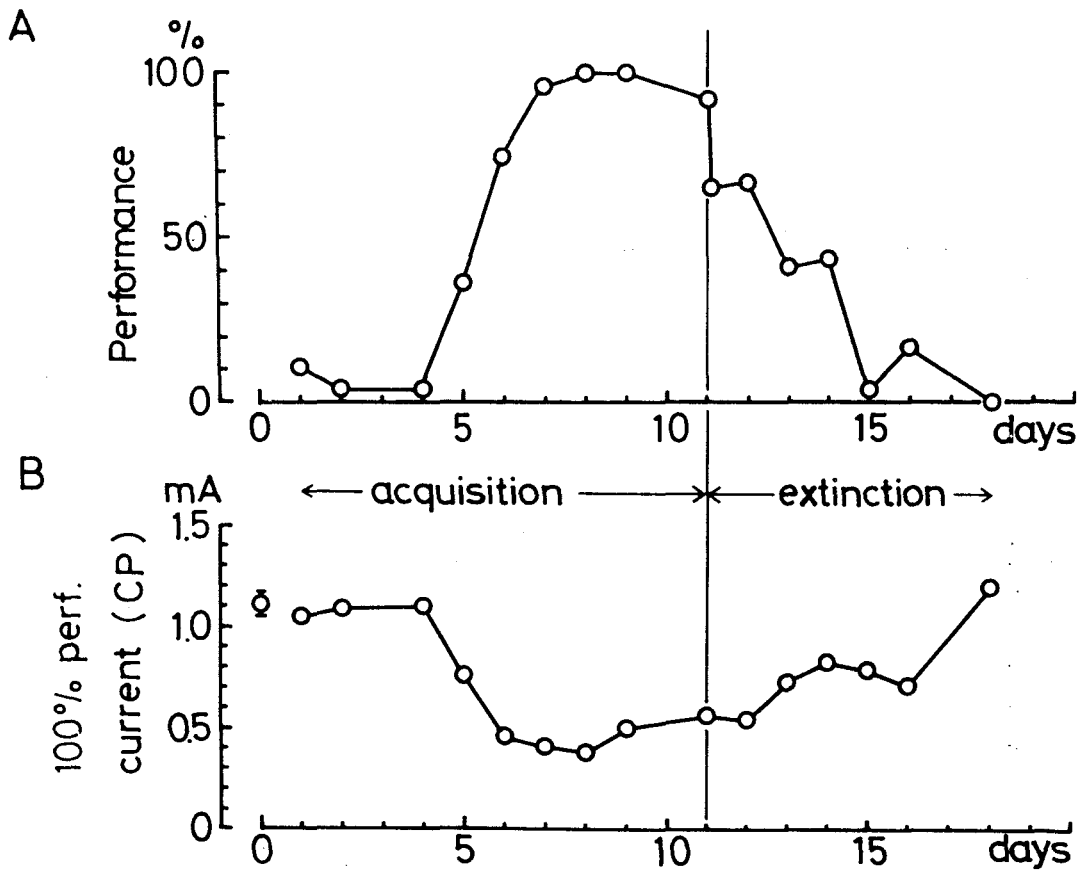

Fig. 4. The time course of classical conditioning. A: The score of performance during forward and backward conditioning in a cat. Ordinate: score of performance. Abscissa: day after onset of training. From the first to 11th day, the CS was paired with the US at an interval of 100 msec. After the 11th day, the stimulus sequence was reversed (i.e., US-CS) with an interval of 900 msec. B: Minimum current of the CP stimulus for eliciting 100 % performance (CP-100 % performance current) during the training in the same cat.

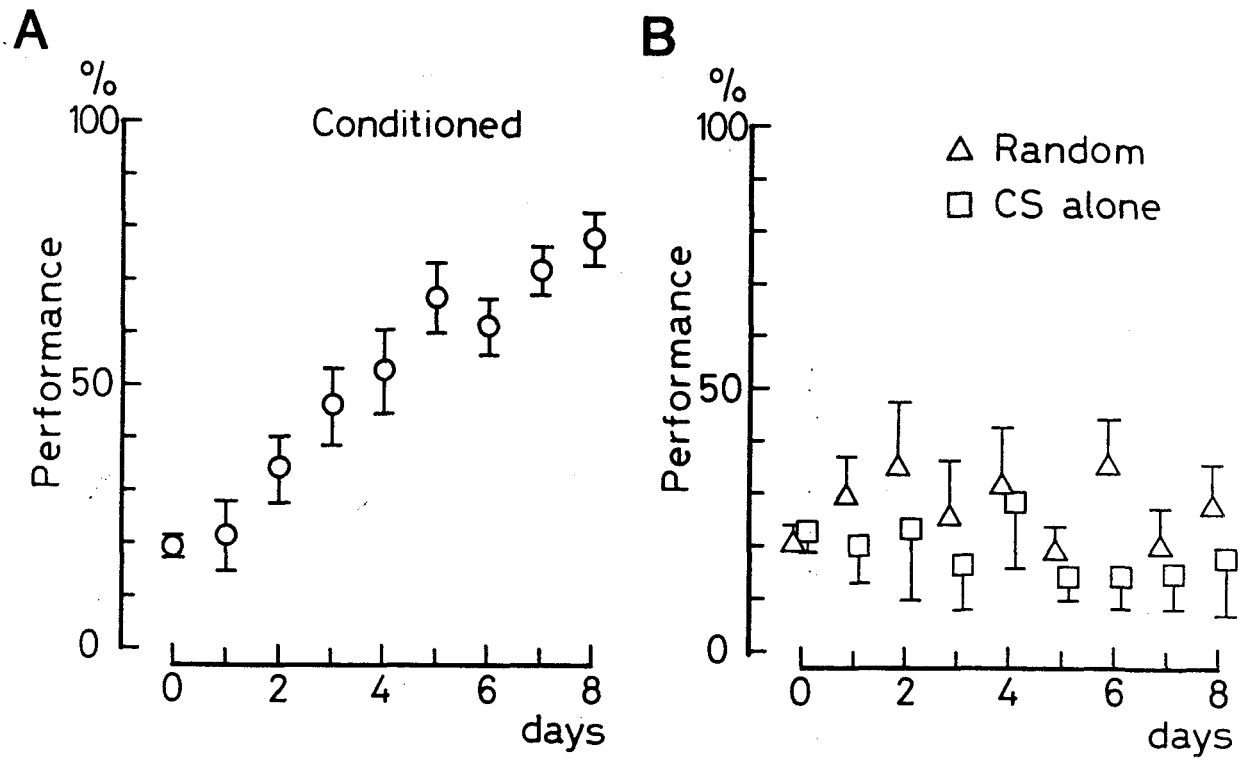

Fig. 5. Mean time course of change in the score of performance. Summarized data of the animals in the paired group (A), the CS-alone group or the random group (B) which were used for the analysis of unitary activity of RN cells. The score of the 0th day represents percentage of positive responses to the CS on the last day of the pretraining period. Ordinate: Mean and S.E.M. of the score of performance. Abscissa: day after onset of training.

Primary site of neuronal change

To decide which part of the neuronal pathway was modified after the paired conditioning, we compared the minimum current of CP or IP stimulus to produce 100 % score of performance in the animals of the paired group (n=14). In parallel with the increase in the score of performance, the CP-100 % performance current progressively decreased during the training period (Fig 6A). The CP-100 % performance current on the 8th day (59 $\pm$ 5.1 % of that during the pretraining period) was significantly smaller than that during the pretraining ($p < 0.001$). By contrast, the IP-100 % performance current did not change significantly (Fig. 6B). The IP-100 % performance current on the 8th day was 116 $\pm$ 10.4 % of that during the pretraining period. These results indicate that neuronal transmission along the cortico-rubrospinal pathway increased after the paired conditioning while that along the interposito-rubrospinal pathway did not.

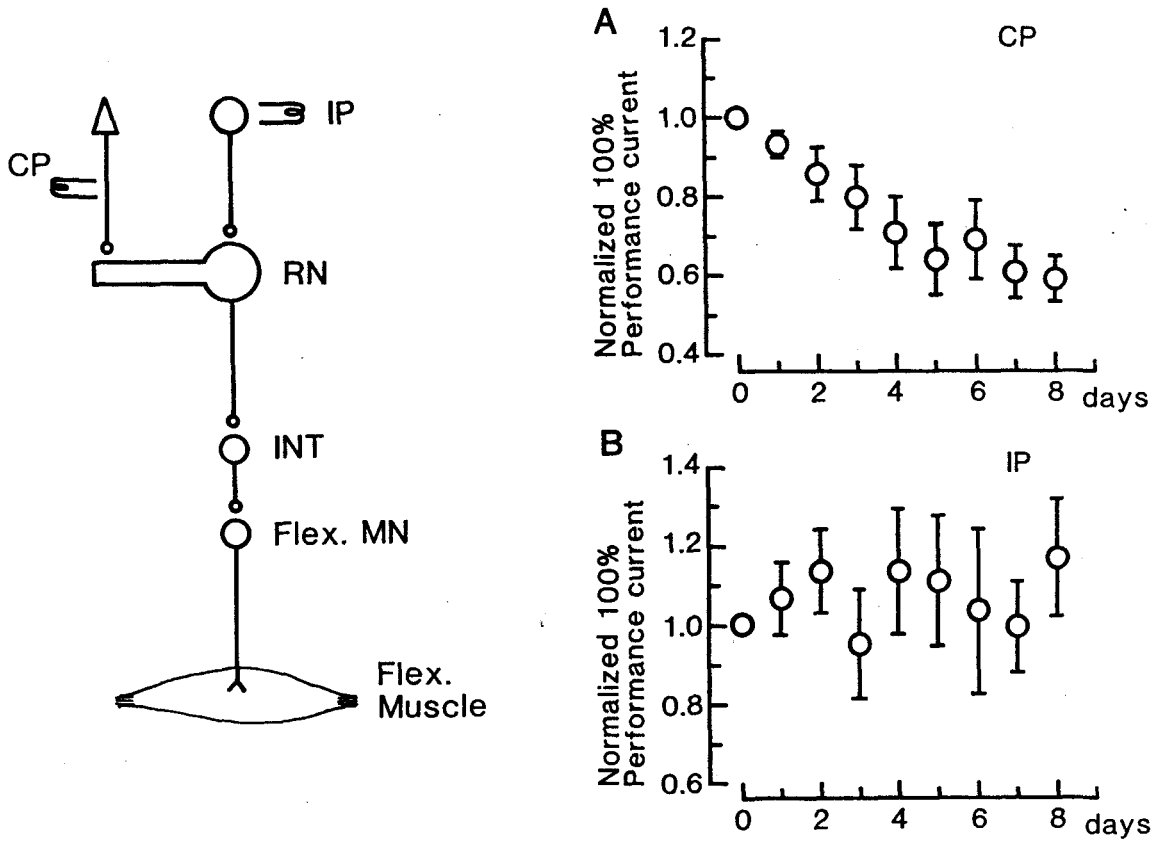

Fig. 6. Primary site of neuronal change after the paired conditioning. A: Minimum current intensity of CP simulation for producing 100 % performance (CP-100 % performance current). B: Minimum current intensity of IP stimulation for producing 100 % performance (IP-100 % performance current), which was tested in the same cats as in A. These current intensities were normalized by those of pretraining level. CP: cerebral peduncle, IP: interpositus nucleus of the cerebellum, RN: red nucleus neurons, INT: interneurons interpolated in the rubrospinal system, Flex. MN: flexor motoneurons of forelimb. Flex. Muscle: flexor muscle of forelimb.

DISCUSSIONS

The present study show that pairing the CS to the cerebral peduncle and the US to the forelimb skin increased effectiveness of transmission along the cortico-rubrospinal pathway to the forelimb flexor motoneurons in response to the CS. The results of the experiments where the CS was paired with the US at random intervals or the CS was presented alone exclude the possibility that the enhanced efficacy of transmission is due to some phenomena such as lesion-induced sprouting of the cortico-rubral fibers after the surgical lesions of the cerebral peduncle. These experiments suggest that the behavioral modification is categorized as the associative classical conditioning.

The pathway mediating the conditioned responses in this preparation is considered to be relatively simple, since the corticofugal fibers were surgically eliminated at the level caudal to the RN and the CS electrodes were implanted in the cerebral peduncle. The lesion at the cerebral peduncle excludes the possible contribution of the cortico-spinal, cortico-pontine or cortico-reticular pathway. Therefore, we considered that the main pathway mediating the conditioned responses is the cortico-rubrospinal pathway. The latency of electrical activity evoked in the biceps brachii muscle in response to the CS supports this view.

The shortest latency of the electrical activity of the biceps brachii muscle was 8 msec, which accords well with the shortest time required for the signal transmission along the

cortico-rubrospinal pathway from the CP to the biceps muscle. This is calculated as follows: (1) 1 msec, for conduction from the CP to RN and the onset of cortico-rubral EPSPs (Tsukahara and Kosaka, 1968); (2) 2 msec, from the onset of the cortico-rubral EPSPs to the spike initiation of RN neurons, assuming that RN neurons are excited during the rising phase of the cortico-rubral EPSPs which have a mean time to peak of 3.6 msec (Tsukahara et al., 1975); (3) 2 msec, from the onset of spike initiation in the RN neurons to the onset of EPSPs in forelimb motoneurons (Illert et al., 1976); (4) 3 msec, from the onset of EPSPs in the motoneurons to the initiation of electrical activity in the biceps brachii muscle. The total of 8 msec is considered to be the shortest conducting time of impulse.

The neuronal change that correlates to the acquisition of the conditioned response may not appear in the neuronal system below the RN, since the efficacy of signal transmission along the interposito-rubrospinal pathway did not change, whereas that along the cortico-rubrospinal pathway remarkably increased. Since these two pathways are common caudal to the RN, it is suggested that the site of modification underlying the conditioning is the cortico-rubral synapses, and some substrates underlying the establishment of the classical conditioning can be found in either the terminals of the cortico-rubral fibers or transmission from the terminals to the somata of the RN neurons.

Chapter 3.

RN cell activity associated with the classical conditioning

INTRODUCTION

As mentioned in the previous section, the behavioral studies suggest the increased efficacy of signal transmission from the cortico-rubral fibers to the RN neurons as a result of the classical conditioning procedure. As the second step, it is necessary to test whether the synaptic transmission at cortico-rubral synapses was actually enhanced after the establishment of conditioning. We examined unit activity of RN cells in response to the stimulation of CP. Generally it is difficult to determine the causal relationship between change in neuronal acitivity and behavioral modification of learning. The classical conditionig mediated by the RN, however, has certain advantages also for unitary studies, since the neuronal pathway for the conditioned responses was restricted to an identified neuronal circuit and the CS was applied to the monosynaptic input to the RN inform of electric pulses. Analysing the unitary activity within short latency after the CS makes it possible to detect change in synaptic efficacy at the cortico-rubral synapses by extracellular recording of RN cell activity. We recorded single-unit responses from the RN in the four groups of animals after the behavioral studies, i.e. paired, random, CS-alone and naive.

METHODS

Rubral unit recording

After the training period, extracellular single unit recordings were performed from RN cells. Recording from the naive animals were performed 7-10 days after the end of the pretraining period. Animals were restrained in bags and their heads were fixed on a stereotaxic apparatus by means of screws implanted in the skull. Under local anesthesia with Xylocaine (Fujisawa Pharmacy.), a small opening was made through the dura, and a glass-insulated tungsten microelectrode was penetrated into the left RN stereotaxically. The uninsulated tips of the electrodes ranged from 6 to 10 um in length and 5 to 8 um in diameter at the end of the glass insulation. The cells which responded with latencies less than 2 msec to stimulation of the contralateral IP were identified as RN cells. Impulse discharges of RN cells were fed to a minicomputer through a comparator with the time resolution of 50 usec. The peri-stimulus time histograms (PSTHs) were computed from 100 to 200 traces of responses. Spontaneous discharge rate was caculated from the unit discharge during the pre-stimulus period in the PSTHs.

RESULTS

RN cell activity is modified after conditioning

(1) RN cell responses to a train of pulses to CP

Figure 7 shows examples of PSTHs of RN cells recorded in three different groups of animals. The RN cell in a cat of the paired group responded with a high probability to the CS applied to CP (Fig. 7A). As exemplified here, strong excitation was often observed within 11 msec after onset of the CS. In contrast with the cells in the paired group, the RN cell in a cat of the random group exhibited a response with lower frequency. (Fig. 7B), and that in the CS-alone group showed no appreciable response (Fig. 7C).

To evaluate the change in excitability of RN cells, we measured the number of spikes which were observed within 11 msec after the onset of the CS. The sampling period, 11 msec, was based on the latency of the fifth pulse of the CS, 8 msec, plus that of the monosynaptic activation from the CP (see below). Figure 3D shows the mean number of spikes within 11 msec after the onset of a CS from the cells recorded in the animals of the paired, random, CS-alone or naive group. The number of spikes of each cell was calculated by dividing total number of spikes during the initial 11 msec by total number of trials. The number of spikes of RN cells recorded in the paired group was 0.87 $\pm$

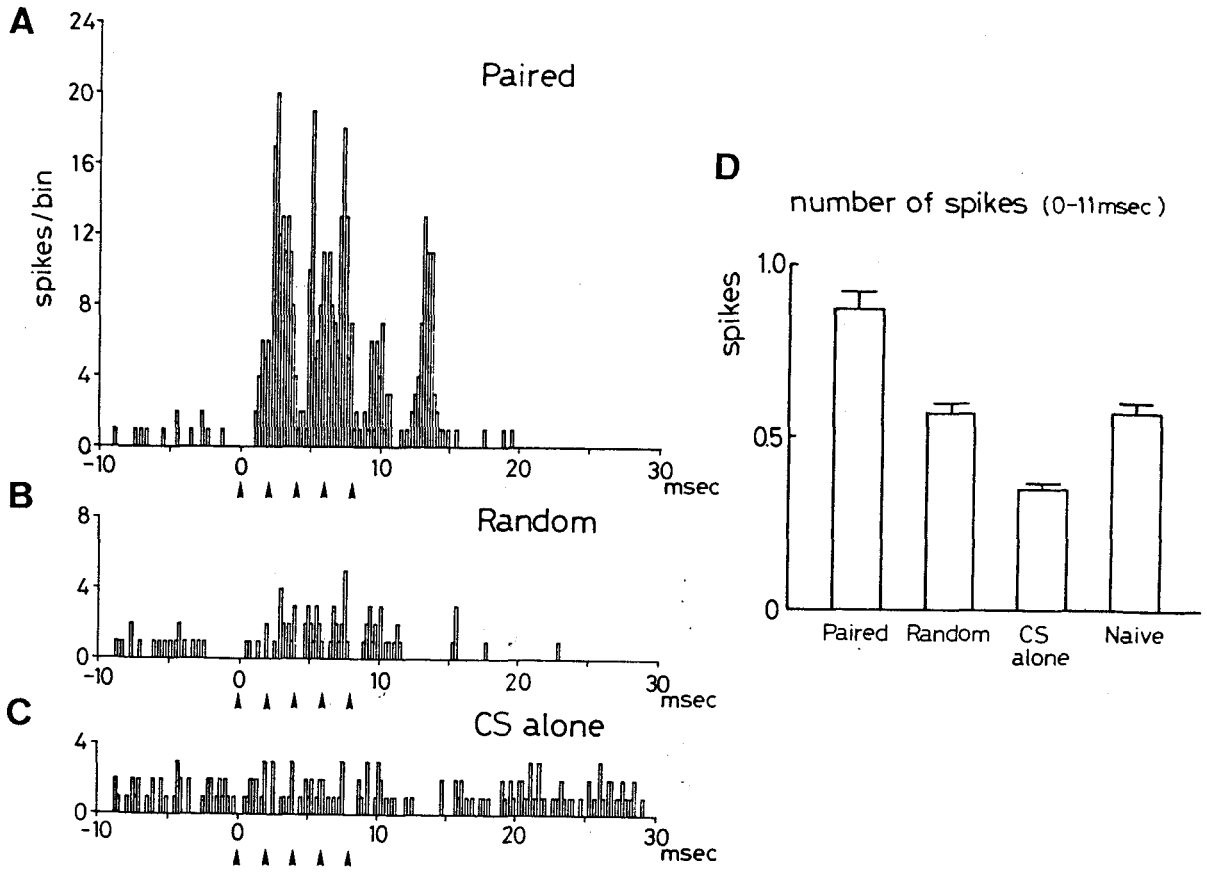

Fig. 7. Responses of RN cells to the CS. A to C: PSTHs of the responses of RN cells in three groups of animals. These PSTHs were sum of 100, 150, and 200 traces. The current intensity of the CS was 0.6, 0.6 and 0.3 mA in A, B and C, respectively. Abscissa: time after the onset of the first pulse of the CS. All PSTHs have 0.2 msec/bin. Arrowheads denote the onset of five pulses of the CS. Ordinate: number of spikes/bin. D: Number of spikes within 11 msec after the onset of CS. The number of spikes for each cell was calculated by dividing the total number of spikes during the initial 11 msec by total number of trials. Each bar represents the mean (± S.E.M.) number of spikes of the RN cells in four groups of animals.

0.05 spikes (123 cells). Those of the random, CS-alone and naive groups were 0.57 ± 0.03 spikes (86 cells), 0.35 ± 0.02 spikes (122 cells) and 0.57 ± 0.03 spikes (93 cells), respectively. A statistical analysis demonstrated that RN cells responded to the CS with higher probability in the paired group than in the other groups of animals ($p < 0.01$). The number of spikes of the RN cells in the CS-alone group appears to be lower than those of the other groups($p < 0.01$). The spontaneous firing rate of the RN cells were 26.1 ± 1.6 spikes/sec in the paired group, 21.7 ± 1.6 spikes/sec in the random group, 19.2 ± 1.2 spikes/sec in the CS-alone group and 23.3 ± 1.3 spikes/sec in the naive group.

(2) RN cell response to single-pulse stimulus to CP

The difference in the responsiveness of the RN cells to a train of pulses among four different groups of animals could be ascribed to the difference in the polysynaptic activity. To examine whether monosynaptic responses of RN cells to the CS is different among four different groups of animals, we investigated the RN cell response to a single-pulse stimulus to CP. The responses observed within 3 msec after the onset of the CP stimulus were regarded as monosynaptic and analysed further, because the earliest polysynaptic responses, the disynaptic inhibitory postsynaptic potentials, are observed 3.9 msec after the CP stimulus. (Tsukahara et al., 1968; Allen and Tsukahara, 1974; Tsukahara et al., 1975; Larsen and Yumiya, 1980). The CP

was stimulated with the same intensity and duration as was used for the CS in the training. Figures 8A, B and C show examples of the responses to the single-pulse stimulus. These RN cells are the same as shown in Fig. 7A, B and C, respectively. The RN cell in the paired group exhibited a prominent response with a unimodal peak. By contrast, the cells recorded in the random group or the CS-alone group did not show such a large response in the PSTHs.

Figure 8D summarizes the mean number of spikes of RN cells recorded from four groups of animals. In the animals of the paired group, the number of spikes within 3 msec after the CP stimulus was 0.17 $\pm$ 0.02 (85 cells). This value was significantly higher than those in the CS-alone group (0.07 $\pm$ 0.01; 102 cells; $p<0.005$) and the naive group (0.12 $\pm$ 0.01; 84 cells; $p<0.005$), and slightly higher than those in the random group (0.13 $\pm$ 0.01; 69 cells; $p<0.05$). These results indicate that the enhancement of monosynaptic cortico-rubral transmission occurred specifically after the paired conditioning.

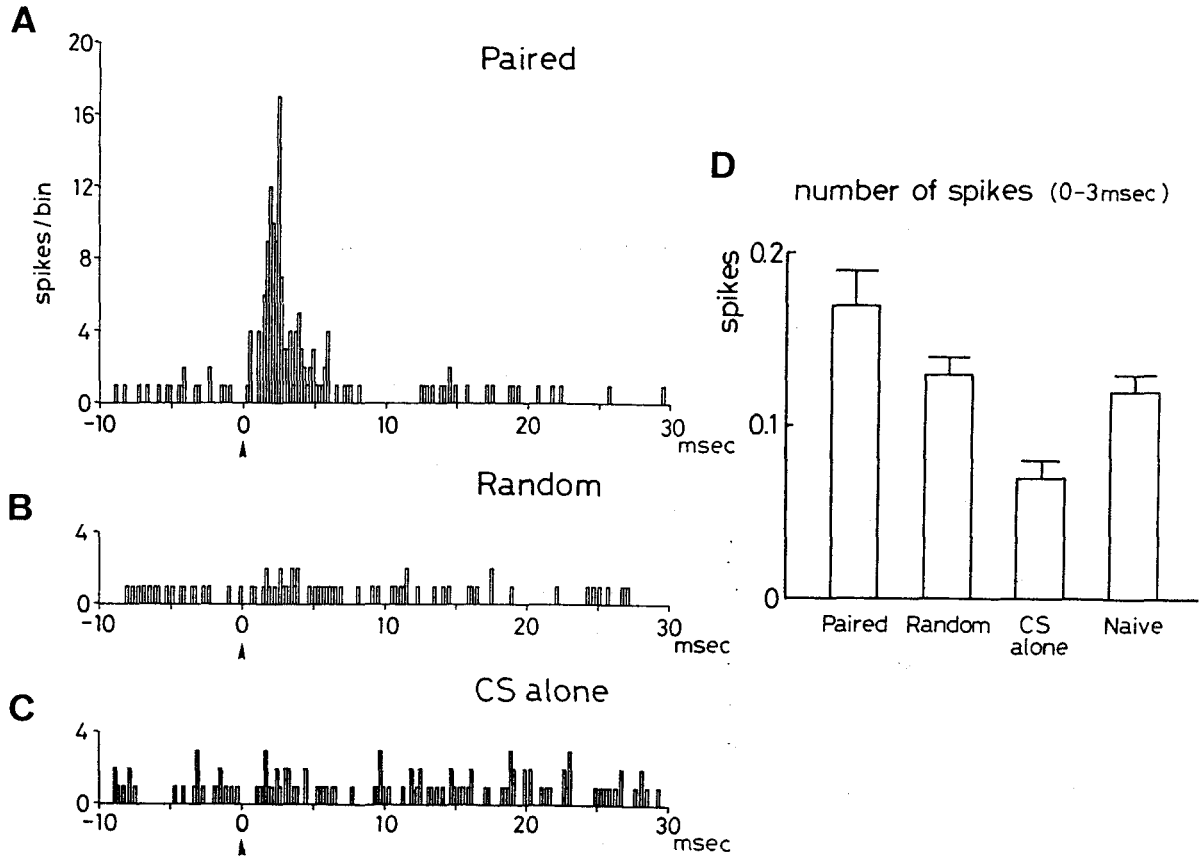

Fig. 8. Responses of RN cells to single-pulse stimulation applied to CP. A to C: PSTHs of the responses of the RN cells were acquired from the same RN cells, responses of which to the CS were shown in Fig. 3 A, B and C, respectively. These PSTHs were sum of 200 (A), 200 (B) and 200 (C) responses to single-pulse stimuli to CP with the same intensity as that of the CS. Arrowheads show the onset of the stimulus. D: Number of spikes within 3 msec after the onset of the stimulation of CP. Each bar represents the mean ($\pm$ S.E.M.) number of spikes of RN cells in four groups of animals.

RN cell response to IP stimulation does not change after conditioning

The increased responsiveness to the stimulation of the CP could be explained by lowered threshold of RN cells. To test this possibility, the firing response of RN cells to the stimulation of IP was compared among three groups of animals (Fig. 9). Current intensity was the same as the IP-100 % performance current of the pre-training period. The response within 2 msec after the stimulation of IP was analysed, because the latencies of the monosynaptic activation of RN cells to IP stimulation ranged from 0.9 to 2.2 msec (Eccles et al., 1975). The mean number of spikes within 2 msec after the single-pulse stimulation of IP in the paired group (0.52 $\pm$ 0.042, 66 cells) was not higher than that in the CS-alone group (0.59 $\pm$ 0.027, 92 cells) or that in the naive group (0.71 $\pm$ 0.034, 58 cells). Thus, it is unlikely that the increased responsiveness of RN cells to stimulation of CP is due to the change in threshold of RN cells.

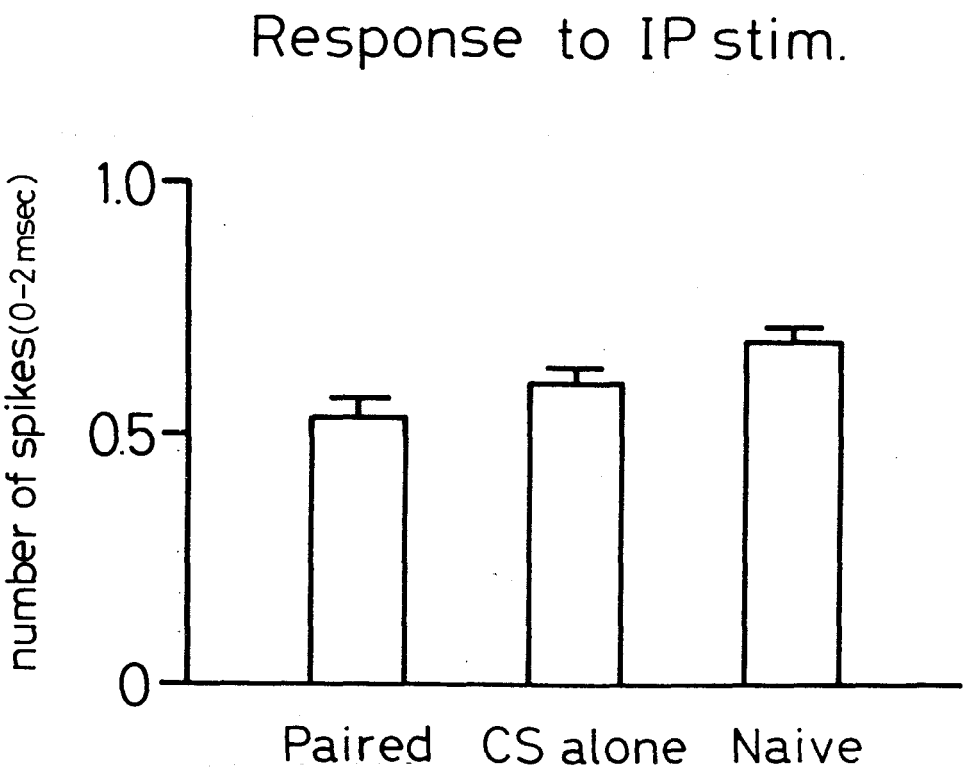

Fig. 9. Responses of RN cells to the simulation of the IP. Mean (± S.E.M.) number of spikes within 2 msec after the single-pulse stimulation of the IP. Current intensity of the stimulus was the same as that of the IP-100 % performance current of the pre-training period.

Discussion

Analysis of the unit activity showed that the increased responsiveness of RN cells to the stimulation of CP was correlated with acquisition of the conditioned forelimb flexion.

Modification of RN cell activity

The firing responses of RN cells to the CS showed a good correlation between the RN cell activity and the behavioral results: the responsiveness of the RN cells in the animals of the paired group was greater than that in animals of the random, the CS-alone or the naive group. The responsiveness of the RN cells in the CS-alone group was lower than that of naive animals. It may be correlated with the previous observation that extinction of the conditioned responses occurred by presenting the CS alone for a week (see Chapter 2; Tsukahara, Oda and Notsu, 1981).

Since the CS activated the monosynaptic input to RN, we could detect the synaptic efficacy at the cortico-rubral synapses by recording the unit activity of RN cells. In fact, we focused our interpretation on the unit activity within 3 msec after the single shock to CP, which were considered as the monosynaptic excitations from CP (Fig. 8). The responses of RN cells within 2 msec after the stimulation of IP were also considered monosynaptic excitation from IP (Fig. 9). Therefore, the difference in the responses of RN cells to the stimulation of CP and IP among different groups of animals suggests possible

mechanisms underlying the conditioning as follows: (1) formation of the new cortico-rubral synapses on the proximal dendrites or the somata of the RN cells, (2) an increase in the transmitter release or the sensitivity of receptors at the cortico-rubral synapses, (3) shortening of the electrotonic length of the RN cell dendrites, (4) lowering in the threshold for spike initiation of the RN cells by changing the active and/or passive characteristics of the membrane of the RN cell soma or increased tonic synaptic input to RN cells, (5) decreased effect of inhibitory synaptic transmission (cf. Tsukahara et al., 1968), or (6) enhanced frequency potentiation of the cortico-rubral EPSPs (cf. Murakami et al., 1977).

The fourth possibility could be excluded by the observation that either the responses of RN cells to the stimulation of IP or the threshold current of the IP stimulus to produce the forelimb flexion did not change after the paired conditioning. It may not be necessary to assume the fifth and sixth possibilities, because the increase in the monosynaptic excitation from CP offers a satisfactory explanation for the increase in the responsiveness of RN cells to the CS. Therefore, first to third possibilities are suggested by the analysis of unit activity of RN cells.

To examine the remaining possibilities, we performed intracellular recording of the RN cells and analysed the cortico-rubral EPSPs and electrical properties of the RN cells. The results of them will be shown in the next section.

Chapter 4.

Appearance of fast-rising component after the classical conditioning

INTRODUCTION

One of the possible mechanisms to explain the elevated synaptic transmission would be collateral sprouting of the cortico-rubral fibers, which was demonstrated after lesions of the inputs from the IP (Tsukahara et al., 1975; Murakami et al., 1982) or after cross-innervation of peripheral flexor and extensor nerves (Tsukahara et al., 1982; Fujito et al., 1982; Murakami et al., 1984). Since previously demonstrated collateral sprouting of cortico-rubral fibers was associated with the reduction in time to peak of the cortico-rubral EPSPs, we performed intracellular recordings from RN cells to examine whether similar change in the time course of the cortico-rubral EPSPs occurred.

In the RN cells in the paired group, a fast-rising component appeared in cortico-rubral EPSPs and the electrotonic length of them did not differ from that of the naive animals. These results suggest the occurrence of the cortico-rubral sprouting at the proximal portion of the RN cell.

METHODS

Intracellular recording of the RN cells

The cats were anesthetized with sodium pentobarbitone (35 mg/Kg) and supplemental doses were administered as required. The animals were immobilized by gallamine triethiodide and artificially respirated. Glass microelectrode filled with 2 M K-citrate or 2 M K-methylsulphate were used and tips of the electrodes were beveled to make the resistance 8-12 MO. The cells activated antidromically from C_1 spinal segment and orthodromically from the contralateral nucleus interpositus were identified as RN cells. RN cells with stable membrane potentials and spike amplitude of more than 50 mV were analysed in the present experiment.

To investigate electrical properties of the RN cells, the voltage responses to step current injected through the recording electrode were analysed. The voltage resposes were fed to a microcomputer (micro NOVA) with sampling intervals of 50 usec and afterward analysed by a minicomputer (Eclipse S/140).

RESULTS

Change in the time course of cortico-rubral EPSPs after the paired conditioning

A total of 119 cells were recorded from 38 cats in the paired group, the score of performance of which attained 56.5 ± 5.18 % during the training. Ninety-eight cells were recorded from 28 cats in the naive group. The mean latencies of the CP-EPSPs were 0.90 ± 0.018 msec for RN cells from the paired group and 0.94 ± 0.018 msec for those from the naive group.

Figures 10A and B show examples of the cortico-rubral EPSPs recorded in a cat of the paired group and that of the naive group, respectively. The cortico-rubral EPSPs in the cats of the paired group had a fast-rising component, which was sometimes superposed on the slowly-rising one. The time course of the CP-EPSPs in the naive group was similar to that of normal cats.

The frequency distribution of the time-to-peak of the CP-EPSPs is illustrated in Fig. 10C and D. For the dual peaked EPSPs the initial peak was used to determine the time-to-peak of the EPSPs. The time-to-peak of the CP-EPSPs in the cats of the paired group (2.2 ± 0.09 msec, n = 119) was significantly shorter than that of the naive cats. (3.4 ± 0.16 msec, n = 98; $p<0.01$). Time-to-peak of the CP-EPSPs recorded in the naive group was not significantly different from that of the normal

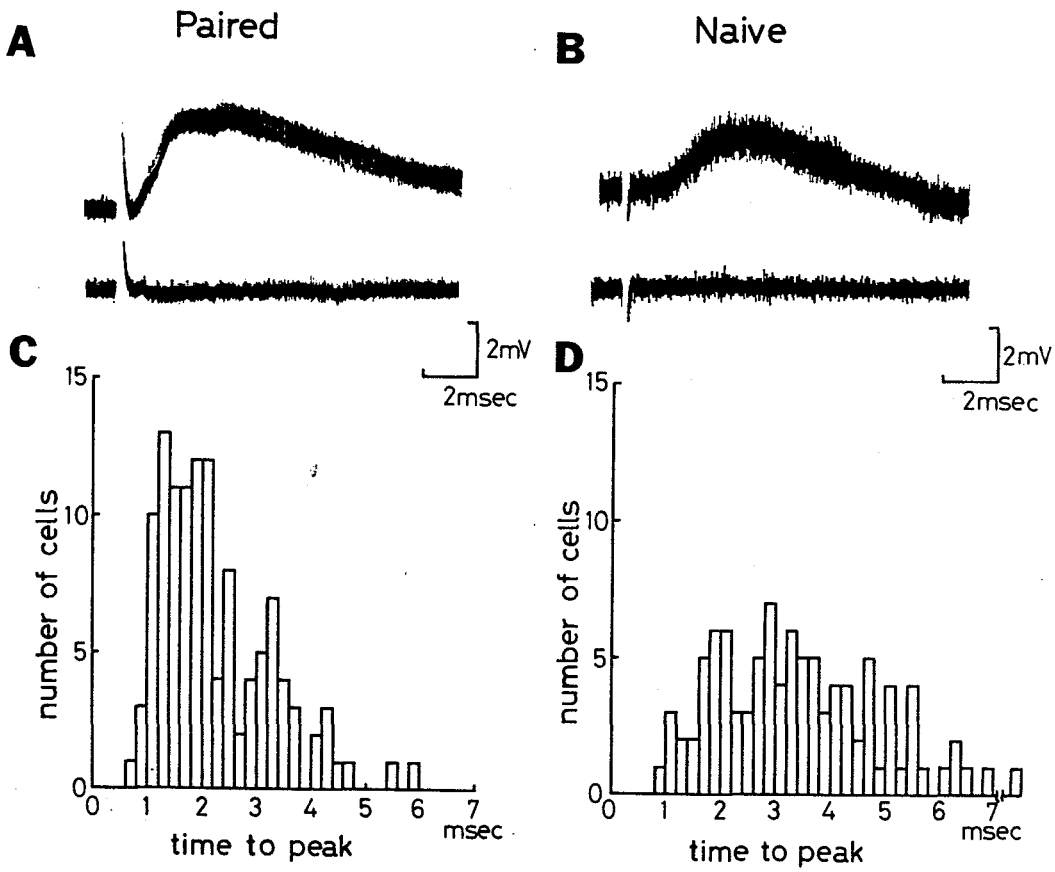

Fig. 10 Change in the time course of the cortico-rubral EPSPs after the paired conditioning. A and B: CP-EPSPs recorded in a cat of the paired group (A) and that recorded in a cat of the naive group (B). C and D: frequency distribution of the time to peak of the CP-EPSPs in the cats of the paired group (C) and that of the naive group (D). Abscissa: time to peak of the CP-EPSPs. Ordinate: number of cells.

cats (3.6 ± 0.14, n=100, cf. Tsukahara et al., 1975; p>0.1). These results indicate that a fast-rising component appeared after the paired conditioning.

Cable properties of RN cells before and after the paired conditioning

The most straightforward interpretation of the appearance of the fast-rising component in the cortico-rubral EPSPs is to assume that cortico-rubral terminals sprouted to form new synaptic contacts at a proximal portion of the RN cell membrane (Fig. 11-1). However, an alternative explanation is that the paired conditioning causes a change in the electrical cable properties of RN cells (Fig.11-2).

To test the latter possibility we estimated the electrotonic length of RN cells. The estimation was performed by measuring the membrane voltage transient following the application of the rectangular pulse to the soma of the RN cell. We analysed 10 RN cells from the animals of the paired group and 8 RN cells from the naive animals. Spike amplitudes of which ranged from 60 to 86 mV. Figure 12A illustrates an example of a membrane transient response of an RN cell in a naive cat. The voltage response was approximated by the sum of exponential functions of different time constants. The mean value of the largest time constant, T_0, was 6.7 ± 0.72 msec in the paired group and 6.4 ± 1.09 msec in the naive group. The mean value of the shorter time constant,

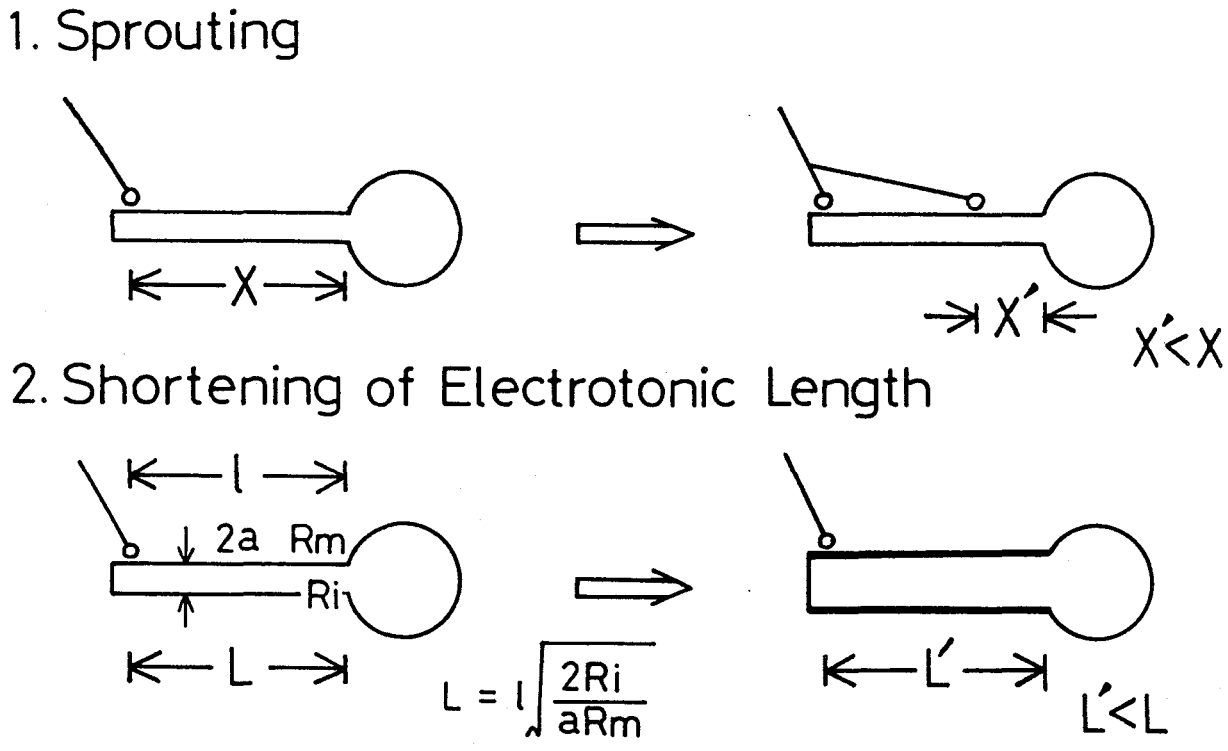

Fig. 11. Possible mechanisms for appearance of the fast-rising component in cortico-rubral EPSPs.
1: Sprouting of the cortico-rubral fibers and formation of new synapses on the proximal portion of the RN cell. Distance from the cortico-rubral synapse to the soma of RN cell is shortened (from X to X') after the conditioning.
2: Shortening of the electrotonic length of the dendrite of RN cell (from L to L'). The electrotonic length is determined by length (l), radius (2), specific membrane resistance (R_m) and intracellular resistivity (R_i) of the dendrite.

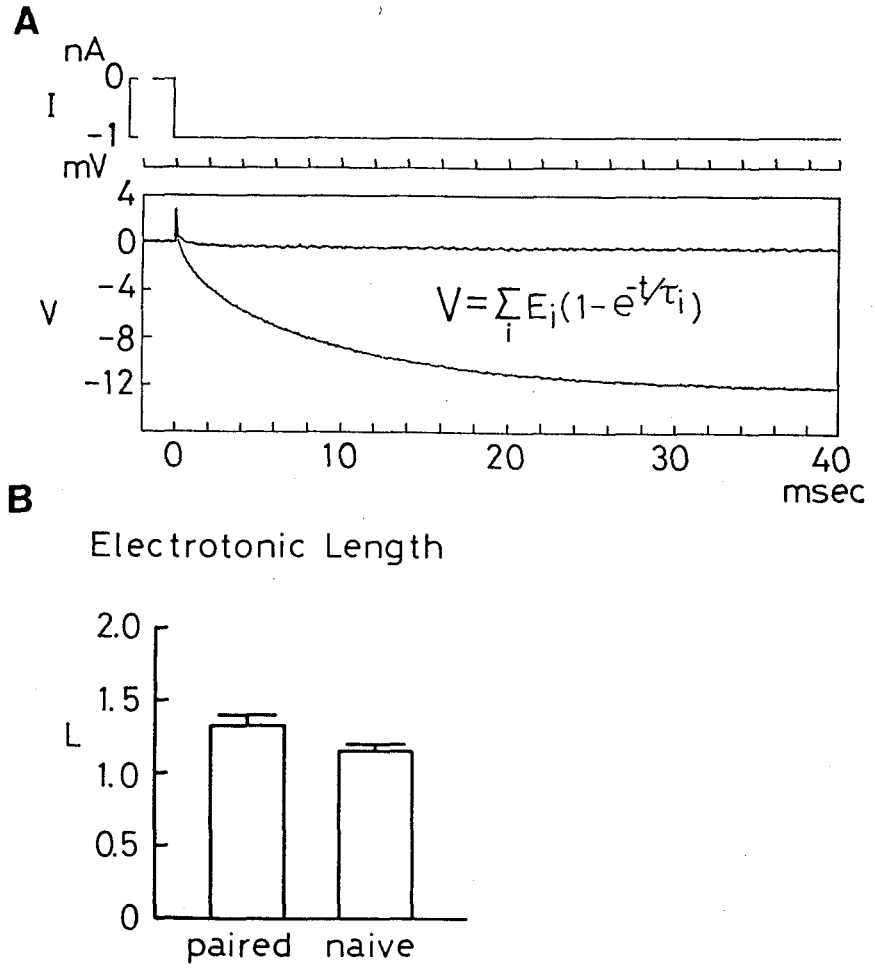

Fig. 12. Cable properties of RN cells before and after the paired conditioning. A: the membrane transient response (V) of an RN cell induced by applying the a step current (I) through microelectrode. The upper trace of V is the corresponding extracellular recording. By using the Newton's method, the membrane response was approximated by the sum of exponential functions with different time constants. The largest time constant, T_0, was 8.9 msec and the second time constant, T_1, was 1.2 msec. B: The electrotonic length (L) of RN cells of the paired group and those of the naive group. L was estimated from the Rall's equation (see in the text).

T_1, was 1.0 $\pm$ 0.14 msec in the paired group and 0.74 $\pm$ 0.134 msec in the naive group. From the ratio of the two time constants, it is possible to estimate the electrotonic length, L, of RN cell by using the following equation (Rall, 1969):

$$L = \frac{\P}{\sqrt{T_0/T_1 - 1}}$$

The mean L value in the RN cells of the paired group, 1.32 $\pm$ 0.076, was not significantly different from that of the naive group, 1.14 $\pm$ 0.051 ($p>0.05$) (Fig. 11B). Therefore, it seems unlikely that the reduction of time to peak of the cortico-rubral EPSPs was caused by reduction of electrotonic length of RN cells. These results suggest that the appearance of the fast-rising component in the cortico-rubral EPSPs after the paired conditioning was caused by formation of the new cortico-rubral synapses on the proximal portion of RN cells.

DISCUSSION

Cellular mechanism for modification of RN cell response

The cellular mechanisms possibly responsible for elevated responsibility of RN cells to the CS are: (1) formation of the new cortico-rubral synapses on the proximal dendrites or the somata of the RN cells, (2) an increase in the transmitter release or the sensitivity of receptors at the cortico-rubral synapses, (3) shortening of the electrotonic length of the RN cell dendrites, (4) lowering in the threshold for spike initiation of the RN cells by changing the active and/or passive characteristics of the membrane of the RN cell soma or increased tonic synaptic input to RN cells, (5) decreased effect of inhibitory synaptic transmission (cf. Tsukahara et al., 1968), or (6) enhanced frequency potentiation of the cortico-rubral EPSPs (cf. Murakami et al., 1977). The fourth, fifth and sixth possibilities could be excluded by the analysis of unit activity of RN cells as mentioned in Chapter 3.

The intracellular recording of RN cells demonstrated that a fast-rising component newly appeared in the cortico-rubral EPSPs after the paired conditioning. This result suggested the first and third possibilities. However, we did not find significant difference in the electrotonic length between RN cells in the paired group and those in the naive group. Furthermore, cortico-rubral EPSPs recorded in the animals of the paired group sometimes had dual peaks (Fig. 10A). The time to the

second peak was similar to the time to peak of the cortico-rubral EPSPs in normal cats (Tsukahara et al., 1975). This observation suggests that the fast-rising component was superposed on the normal EPSPs. The fast-rising component could be a regenerative potential, such as a dendritic spike (Spencer and Kandel, 1961; Llinas and Sugimori, 1980). This is unlikely because the amplitude of the fast-rising component was increased in a graded manner when the stimulus intensity was increased. Furthermore, the fast rising cortico-rubral EPSPs exhibited frequency potentiation (cf. Murakami et al., 1977).

Therefore, it seems reasonable to assume that the formation of new synapses occurred on the proximal portion of the RN cell dendrites or somata. Recently, an electronmicroscopic examination of the location of the cortico-rubral synapses showed that the cortico-rubral synapses were formed on large dendrites or somata of the RN cells after the paired conditioning, whereas the cortico-rubral synapses in the naive animals existed mainly on the small dendrites as those of the normal animals (Murakami et al. 1987). This observation suggested that the proportion of cortico-rubral synapses contacting with the proximal dendrites and somata increased after the paired conditioning.

Concerning the second factor, it is not possible to decide whether it occurs or not from the present study. It is necessary to analyse the unitary EPSPs mediated by the cortico-rubral synapses.

Change in the synaptic site may contribute to RN cell responsibility

It remains to be examined whether the shortening of the time to peak of the cortico-rubral EPSPs correlates with the firing activity of RN cells or not. Fetz and Gustafsson (1983) demonstrated that shape of the PSTH of spike response produced by the simple EPSPs in cat motoneuron resembled shape of temporal derivative of the EPSPs. If this is the case for the RN cells in cat, formation of the cortico-rubral synapses on the proximal dendrites or somata may contribute to the increased firing probability of RN cells in response to the CP stimulus, because synaptic action at the proximal portion of RN cells causes not only shortening of the time to peak of the EPSPs but also steepening of the rising slope of the EPSPs. We estimated this relationship by calculating the theoretical EPSPs from the Rall's compartmental model. The model consists of ten compartments, with equal electrotonic lengths of 0.11. This value represents the actual RN cell well, because the electrotonic length of the RN cell was 1.1 (Tsukahara et al., 1975). An alpha-function (1/a = 350 usec) is utilized to simulate the excitatory conductance change. The wave form of this conductance was obtained from a new iterative single-electrode voltage clamp method applied to RN cells in cat (Murakami et al., 1987). Figure 13 shows the relationship between the time to peak and the maximum slope of the theoretical EPSPs. The maximum slope of the EPSPs becomes much steeper when the time to peak of the EPSPs was decreased to less than about 2 msec. Therefore, if the probability of spike

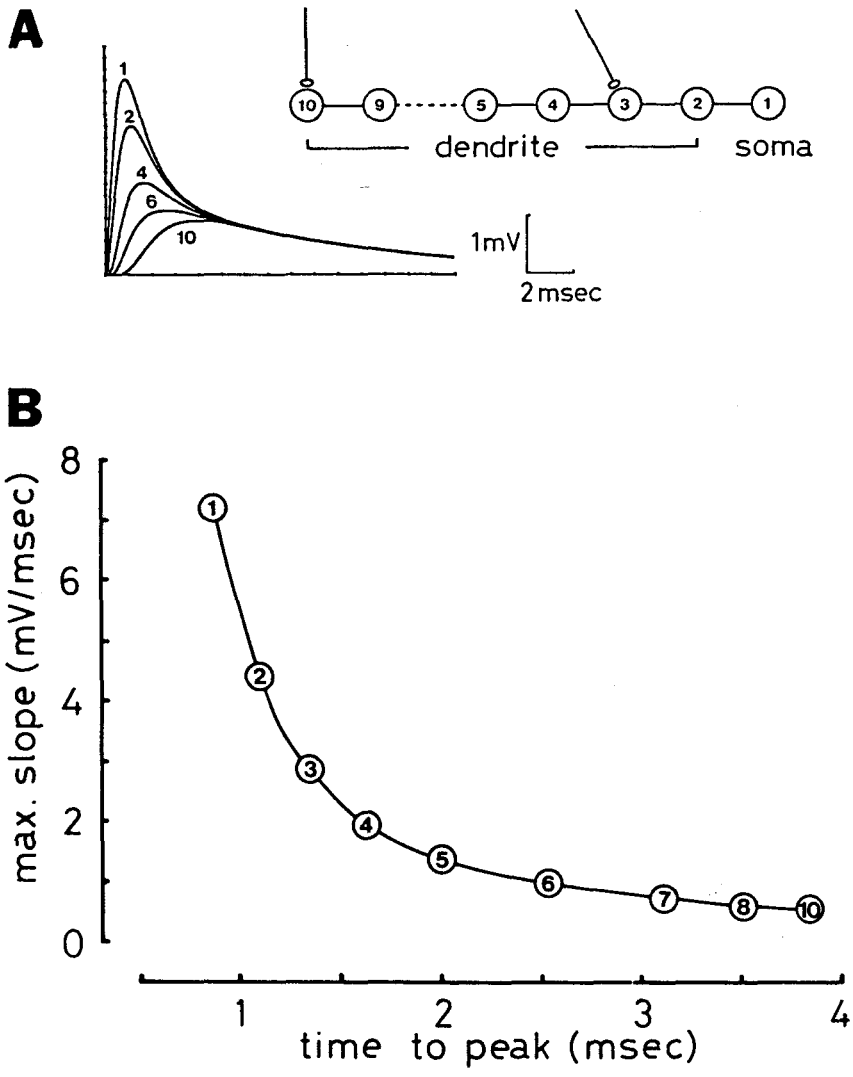

Fig. 13. Change in the shape of the cortico-rubral EPSPs after formation of new synapses. A: Theoretical EPSPs calculated from the Rall's compartmental model. The model consists of ten compartments having equal electrotonic length, 0.11 (right). The EPSPs (left) were obtained by applying the excitatory conductance change with the time course of an alpha-function (1/a=350usec). The number on each curve represents the compartment where the conductance change occurred. B: the relationship between the time to peak and the maximum slope of the computed EPSPs. Numbers 1-10 in each circle indicate corresponding EPSPs.

discharge is proportional to the slope of the rising phase of the cortico-rubral EPSPs, change in the synaptic site may contribute actually to the neuronal activity of RN cells in response to the stimulation of CP.

It has been established in the sprouting of the cortico-rubral fibers after IP lesion (Tsukahara et al., 1975) or cross-innervation of peripheral nerves (Tsukahara et al., 1982) that the newly formed synapses are retained for more than several months. Therefore, the newly formed cortico-rubral synapses could be maintained during the retention period of the classical conditioning, more than a few weeks (Tsukahara, Oda and Notsu, 1981). It was reported that increases in number, size and vesicle components of active zones as well as an increase in the number of synaptic varicosities were found as a possible neuronal mechanism underlying long-lasting sensitization in a marine mollusk, Aplysia, (Bailey and Chen, 1983, 1987; Glantzman et al., 1987). These observations suggest that morphological change at the synapses may play a role in the maintenance of long-term memory in mammals and invertebrates.

Chapter5

Associative conditioning and modification of rubral cell activity produced by paired stimulation of the cortico-rubral fibers and locus coeruleus nucleus.

INTRODUCTION

As mentioned above, associative classical conditioning mediated by the red nucleus in cat was established by stimulating the cortico-rubral fibers as the CS and the forelimb skin as the US (Tsukahara et al, 1979, 1981; Oda et al, in press). The advantages of this preparation are that the primary site of neuronal change was identified as the cortico-rubral synapses and that plastic change of the synapses was analysed qualitatively and quantitatively by electrophysiological and morphological methods. The next and important question is how the synaptic plasticity of RN neurons is induced by pairing the CS with the US. For answering this question, it is necessary to identify the essential system for the learning.

However, the pathway from the US site to the primary site of neuronal change remained to be identified. We considered that the locus coeruleus nucleus (LC) may be one of the systems mediating the effect of skin shock to the cortico-rubral synapses, since the LC neurons are activated typically by the noxious stimulus such as skin shock (Nakamura, 1977), and the LC cells are one of origins of the central catecholaminergic or

indolaminergic neuron systems which project diffusely in the brain including the RN (Swanson and Hartman, 1975; Moore and Bloom, 1979; Foote, Bloom and Aston-Jones, 1983) and may work essentially for brain plasticity.

In the present study, we first examined whether the associative learning is induced by pairing the CS to the cortico-rubral fibers with the stimulus applied to the LC instead of the US to the forelimb skin. Next, we examined change in the efficacy of signal transmission at the cortico-rubral synapses by comparing unitary activity of RN cells in response to the CP stimulus among cats receiving paired CP-LC conditioning, those receiving the CP or the LC stimulus alone, and the naive animals. RN cell activity in response to the CP stimulus among the cats after receiving paired stimuli or unpaired stimuli or naive animals.

MATERIALS AND METHODS

Surgical Procedure

Adult cats (2.5 - 5.0 Kg) were used. To eliminate motor reactions through the corticofugal fibers other than cortico-rubral fibers, the left cerebral peduncle (CP) was lesioned by passing DC current (1 mA for 30 sec) at ten different points at AP 0 and A 0.5 of Horseley-Clarke coordinates. Bipolar stimulating electrodes were inserted stereotaxically in the left CP (at A 8.5) and the right interpositus nucleus (IP) of the cerebellum. Concentric electrode was inserted to the left LC and the tip of the electrode was positioned at P3.0, L2.0, H-2.0 of Horseley-Clark coordinate(Fig. 14A). Positions of stimulating and recording electrode tips were marked electrolytically and verified histologically. Screws were implanted in the skull to fix the cat's head on an apparatus during later unit recording from the RN.

Training Procedures and Behavioral Analysis

Parameters of the CP stimulus used in training procedure were the same as the CS used in the previous preparation (see Chapter 2). Strength of the CP stimulus was determined during pretraining period about 10 days after the surgery. The CP stimulus consisted of a train of five bipolar electric pulses with a duration of 100 usec for each polarity and an interpulse interval of 2 msec. Current intensity of the CP stimulus was selected as that produced positive forelimb flexion at rate of 20

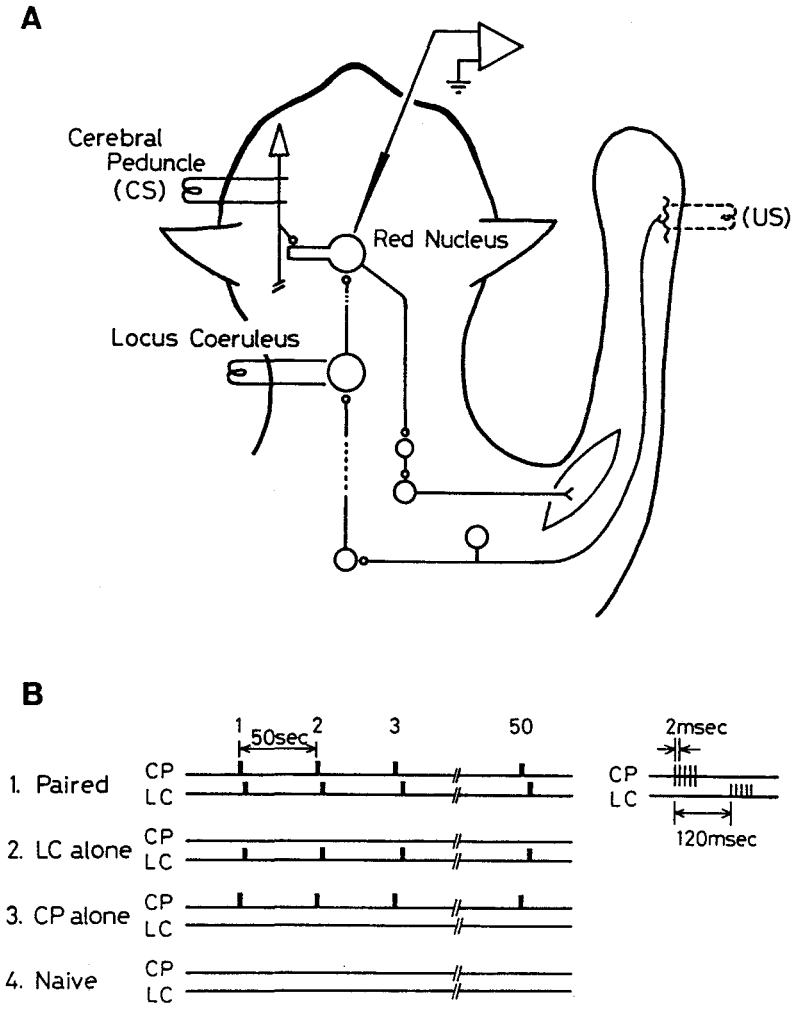

Fig. 14. Diagram to illustrate the experimental arrangement where the LC was stimulated instead of the US applied to forelimb skin in the previous preparation. A: Neuronal circuit for the associative conditioning. The stimuli to the CP and the LC were a train of five electric pulses. B: Diagrammatic representation of the training and naive procedures. Paired: the CP stimulus was paired with the LC stimulus at the fixed interval of 120 msec. LC alone: the LC stimulus was presented alone. CP alone: the CP stimulus was presented alone. Fifty times of paired or unpaired stimuli were presented everyday.

%. The positive forelimb flexions were determined as elbow flexion of more than 0.06 degree induced within 100 msec after onset of the CP stimulus. Current intensities of the CP stimulus ranged from 0.4 to 1.2 mA.

Stimulus applied to the LC was a train of five cathodal pulses with a duration of 100 usec and an interpulse interval of 2 msec. The intensities of it ranged from 100 to 300 uA.

A microcomputer was used to control strength and timing of these stimuli and judge the forelimb responses. Details were described previously (see Chapter 2; Tsukahara, Oda and Notsu, 1981; Oda et al, in press).

In the training procedures, animals were divided into four groups: paired (N=10), LC-alone (N=7), CP-alone (N=6), and naive (N=5) (Fig. 14B). In the paired group, the CP stimulus was paired with the following LC stimuli by a fixed interval of 120 msec. The paired stimuli were applied once every 50 sec and totally 50 times in a day. Training period was usually 8 days. In the LC-alone or the CP-alone group, the unpaired stimulation of LC or CP alone was applied, respectively. Parameters of these stimuli, number of total trials and period of training were the same as those used in the paired group. In the naive group, cats received only the surgical procedure and the pretraining procedure to determine the strength of the CP stimulus.

Effect of the training was investigated by counting the number of positive forelimb flexion responses induced by the CP stimulus. Percentage of the positive responses in a total trials

of each day was referred to as the score of performance on that day.

Rubral Unit Recordings

After the training procedures, extracellular single unit recordings were obtained from the RN cells in the four groups of animals. Recordings from the naive animals were performed 7-10 days after end of the pretraining period.

Animals were restrained in bags and their heads were fixed on a stereotaxic apparatus by means of screws in the skull. A glass-insulated tungsten microelectrode was inserted into the left RN stereotaxically through a small opening in the dura on the parietal cortex. Single units recorded in RN region were identified as the RN cells by their monosynaptic excitatory responses to stimulation of the contralateral IP of the cerebellum. These responses had latencies less than 2 msec. Peristimulus time histograms (PSTHs) were computed from 100 to 500 (usually 200) traces of responses. Tracks and tips of the recording electrodes were ascertained by histological sections after the unitary recording.

Histology

Noradrenergic projection into the RN was examined immunohistochemically by using antisira to two catecholaminergic-sythesizing enzymes, tyrosine hydroxylase (TH) and dopamine-B-hydroxylase (DBH). These antisera were antigenically specific

to bovine TH and DBH. Adult cats (3.0 -4.0 Kg) were anesthetized with sodium pentobarbitone and perfused transcardially with 2 l of phosphate-buffered-saline (PBS, pH 7.4, 50 mM phosphate buffer, 0.9 % NaCl), followed first by 3 l of fixative containing 4 % formaldehyde and 0.1 % glutal aldehyde in 120 mM phosphate buffer (pH 7.4) and then by 1 l of second solution consisting of 4 % formaldehyde in 120 mM phosphate buffer (pH 7.4). The brains were dissected out and sectioned at a thickness of 50 um on a microslicer, using chilled PBS (pH 7.6, 50 mM phosphate buffer, 0.9 % NaCl) as bath fluid.

The sections were incubated in 10 % normal goat serum for 1 hour and then rinsed in PBS (pH 7.6) with 0.1 % Triton X-100 (D-PBS). The sections were then incubated in rabbit-anti DBH serum at dilution of 1:4000 with 0.1 % NaN_3 for 40 hours at room temperature and processed for enzyme immumohistochemistry with the ABC method (Vector). As a control, non-immunized rabbit serum was applied instead of the rabbit anti-DBH serum. After being washed with D-PBS, the sections were further treated with biotinated anti-rabbit IgG serum (1:200 dilution, Vector) for 30 min, rinsed in D-PBS, and then treated with ABC (1:100 dilution, Vector) for 1 hour. All of the processes were carried out at room temperature. All the serum used here were diluted with 1 % normal goat serum in D-PBS. The specificity of the anti-DBH serum used in the present study has been reported elsewhere (Nagatsu et al., 1977; Nagatsu, 1983).

The sections were then reacted in PBS containing 0.05 % diaminobenzidine tetrahydrochloride and 0.01 H_2O_2 for 10 min at room temperature. After being washed, the sections were mounted

onto gelatine coated slides, and air dried.

The location of the LC electrode tips were also examined in the TH or DBH immunohistochemically stained sections.

RESULTS

Conditioned Forelimb Flexion

Figure 15A shows the examples of mechanogram of elbow flexion in a cat on the first and 7th day of the paired conditioning. On the first day, the elbow flexions were rarely induced by either the CP or LC stimuli. On the 7th day, the CP stimulus produced the positive flexion responses which were evoked before the onset of the LC stimuli. We recorded electrical activity of the biceps brachii muscle on that day. Latencies of the first spike in the electromyographic records from the onset of the first pulse of CS ranged from 8.2 to 25 msec (n=40). The shortest latency accords with the shortest time for impulse transmission along the cortico-rubrospinal pathway from the CP to the biceps brachii muscle as mentioned previously (see Chapter 2; Tsukahara, Oda and Notsu, 1981).

Figure 15B shows change in the score of performance in a cat. During the first 8 days, the cat received the paired CP and LC stimuli 50 times everyday. The score of performance increased gradually in this period.

After the paired conditioning, we examined how long the acquired responses were maintained. The score of performance for ten CP stimuli was counted once every 4 days after the paired conditioning period. Higher score of performance was maintained for more than two weeks after the end of paired conditioning (Fig. 15B).

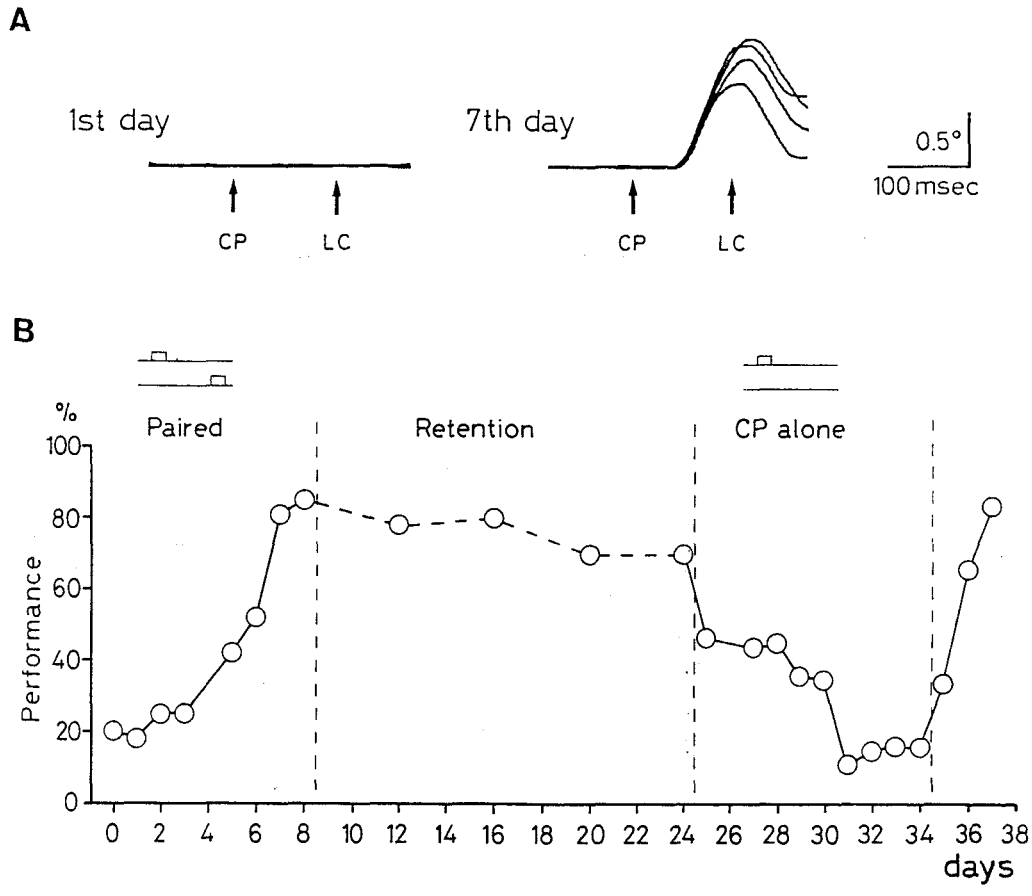

Fig. 15. Sample record of associative conditioning. A: Forelimb flexion in response to paired stimulation of the CP and LC. On the first day of paired conditioning, neither the CP nor LC stimulus produced the forelimb flexion. On the 7th day of training, the positive forelimb flexion induced by the CP stimulus. These traces are superposed of four examples of the mechanogram. B: Time course of the acquisition, retention, extinction and reacquisition of the conditioned forelimb flexion in a cat. Score of the 0th day represents percentage of positive responses to the CP stimulus on the last day of pre-training period. Ordinate: score of performance; Abscissa: day after onset of training.

Acquisition of the conditioned forelimb flexion in three groups of animals was summarized in Fig. 16. Mean score of performance from the twelve cats in the paired group increased gradually during 8 days' training period (Fig. 16A). The score on the 8th day (mean $\pm$ S.E.M.: 73.1 $\pm$ 4.2 %, N=12) was significantly higher than that during the pre-training period (18.1 $\pm$ 1.8 % ; $p<0.001$, t-test). On the contrary, the score of the animals which received the LC stimuli alone during the training session showed only a slight, but not significant, increase (16.7 $\pm$ 5.1 % during the pre-training period, 33.3 $\pm$ 0.0 % on the 8th day, N=7; $p>0.1$) (Fig. 16B). The animals which received the CP stimuli alone did not show any sign of increase in the score (22.8 $\pm$ 4.2 % during the pre-training period, 17.9 $\pm$ 8.5 % on the 8th day, N=5; $p>0.2$) (Fig. 16B).

These results suggest that this behavioral change can be categorized into the associative conditioning which needs pairing of two stimuli to produce the conditioned responses. This suggestion is supported by the result of extinction experiment as shown in Fig. 15B. After the paired conditioning procedure, the cat received 50 times presentation of the CP stimuli alone everyday. The score of performance gradually decreased and reached its pre-training level within 5 days' extinction procedure.

It should be noticed that since the LC stimuli did not produce the forelimb flexion, the LC stimuli is not the unconditioned stimulus such as the forelimb shock in the previous preparation (see Chapter 2; Tsukahara, Oda and Notsu, 1981;

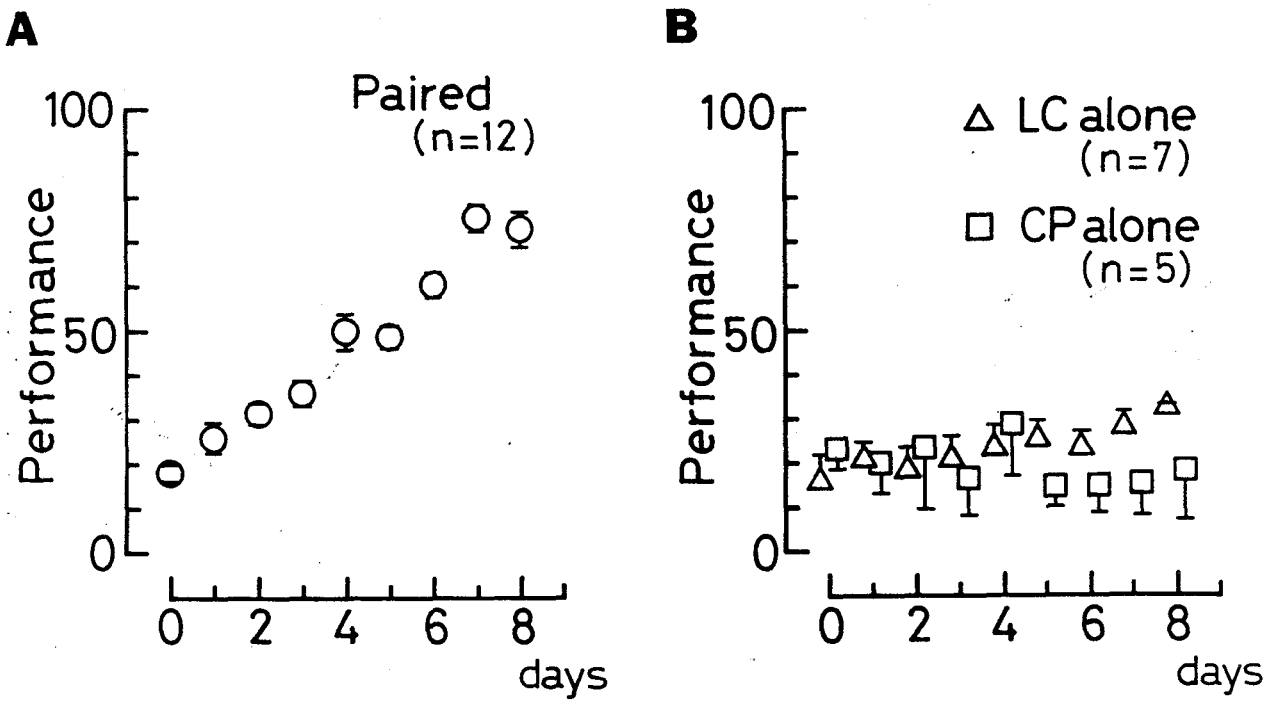

Fig. 16. Mean score of performance during the paired, CP alone and LC alone training. Ordinate: mean and S.E.M. of the score of performance. Abscissa: day after the onset of training. The score of performance of the paired group increased significantly during 8 days' training, but that of the CP alone or the LC alone group did not increase. Score of the 0th day represents that of the last day of pre-training period.

Oda et al. in press). The stimulation of the LC could increase the efficacy of neuronal transmission in the cortico-rubrospinal system when it was paired with the preceding stimulus applied to the cortico-rubral fibers.

The LC nucleus is the effective site of stimulation to produce the paired conditioning

We investigated the relation between sites of stimulation near the LC nucleus and acquired score of performance on the last day of paired conditioning. After the last day of training, the position of LC electrode's tip was marked electrolytically and observed in the slices which were labeled immunohistochemically by using an anti-TH or an anti-DBH serum. Figure 17 summarizes positions of the stimulus sites. The clusters of medium-size (30-50 um in diameter, Leger and Hernandez-Nicaise, 1980) and TH- or DBH-containing neurons were identified as the dorsal LC (LCD) and LCa.

In the animals in which the LCD or the LCa was stimulated, the score of performance reached above 50 % on the 8th day of training (Fig. 17A). On the contrary, the cats in which ventral sites to the LCa, the superior cerebellar peduncle or the periventricular gray was stimulated did not acquired the conditioned forelimb flexion at the rate above 50 % (Fig. 17B).

These results demonstrate that the effective sites for the stimulus which is paired with the CP stimulus are the LCD or LCa. Since these nuclei include a large proportion of catechol-

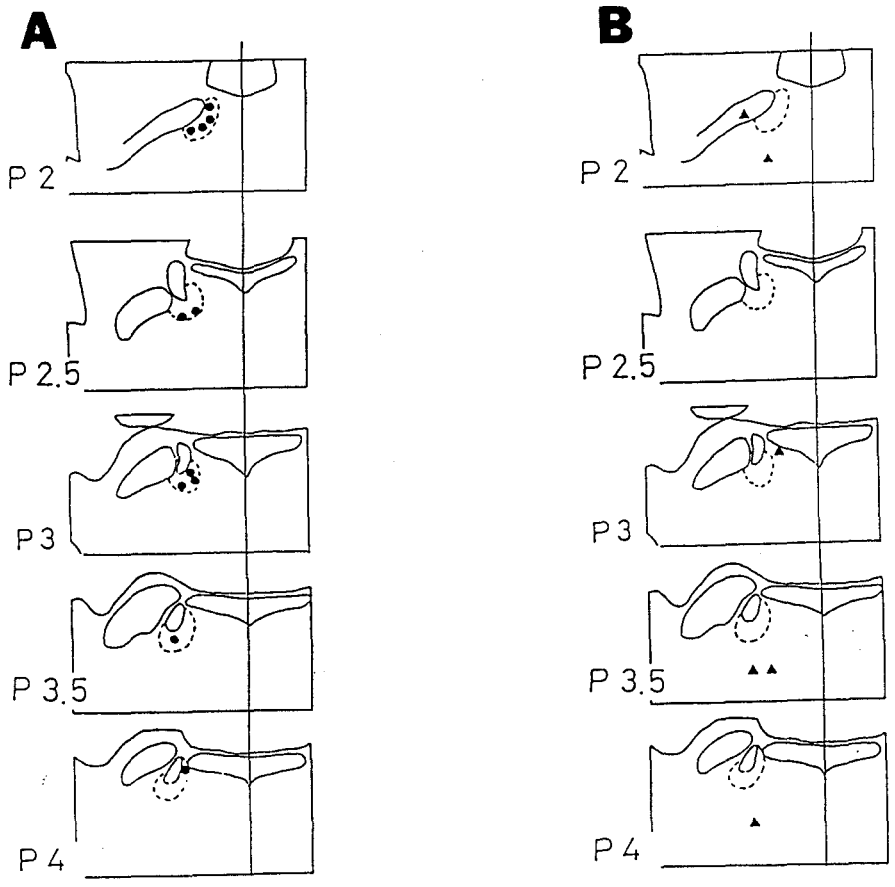

Fig. 17. Relation between the sites of stimulation around the LC nucleus and acquired score of performance. Areas enclosed with dotted line represent the dorsal LC and LCa, where medium-size and TH- or DBH-containing neurons were clustered. The position of the LC electrodes' tips of the cats whose score of performance reached above (filled circles in A) or below 50 % (filled triangles in B) were identified in the frontal sections immunohistochemically labeled by using an anti-TH or anti-DBH serum.

aminergic neurons and these catecholaminergic neurons project to almost every part of the central nervous system, it is suggested that pairing cortico-rubral activation with chatechol aminergic activation of the central nervous system may increase the efficacy of signal transmission along the cortico-rubrospinal pathway.

Modification of RN cell activity after conditioning

The next question is whether the behavioral change reflected the efficacy of neuronal transmission at the cortico-rubral synapses. To examine this, we recorded the RN cell activity extracellularly from four groups of animals: paired (N=3), LC-alone (N=2), CP-alone (N=4) and naive (N=5). In the unitary recording procedure, the animals were delivered CP or IP stimuli without LC stimuli. The cells identified as the RN cells responded to stimulation of the contralateral IP with stable latencies ranging from 1.0 to 1.8 msec.

Figure 18 shows examples of the PSTHs of spike responses to the CP stimulus recorded from three RN cells in cats in the paired, LC-alone or CP-alone group. The responses to trains of five pulses were superposed for 200 trials for each cells. The RN cell in the paired group responded with higher firing probability, and there were several peaks of responses in the PSTH (Fig. 18A). In many cases, main responses occurred within 11 msec after the onset of CS. The RN cell in the LC alone group showed a small responses with lower frequency (Fig. 18B). The

RN cell in the CP alone group showed no appreciable response (Fig. 18C).

To evaluate the excitability of RN cells, we compared number of spikes occurring within 11 msec after the CP stimulus, since the latest monosynaptic activation may occur within 3 msec after the last pulse of the CP stimulus (see Chapter 3 and Ref. Oda et al, in press). The number of spikes of each cell was calculated by dividing the total number of spikes within the initial 11 msec by the total number of trials. Figure 18D shows the mean number of spikes sampled from RN cells recorded in the paired, LC-alone, CP-alone and naive groups.

The number of spikes of RN cells recorded in the paired group was 0.99 $\pm$ 0.05 spikes (107 cells). Those of the LC alone, CP-alone and naive groups were 0.69 $\pm$ 0.04 spikes (80 cells), 0.35 $\pm$ 0.02 spikes (122 cells) and 0.57 $\pm$ 0.03 spikes, respectively. A statistical analysis demonstrated that RN cells responded to the CP stimulus with higher probability in the paired group than in the other groups of animals ($p < 0.001$). As mentioned in Chapter 3, number of spikes of RN cells in the CP alone group was lower than those of the other groups ($p < 0.01$). The number of spikes of RN cells in the LC-alone group appears slightly higher than that of the naive group, but there is no significant difference between them ($p>0.1$). These results show that change in activity of RN cells in response to the CP stimulus correlated with the behavioral modification after training.

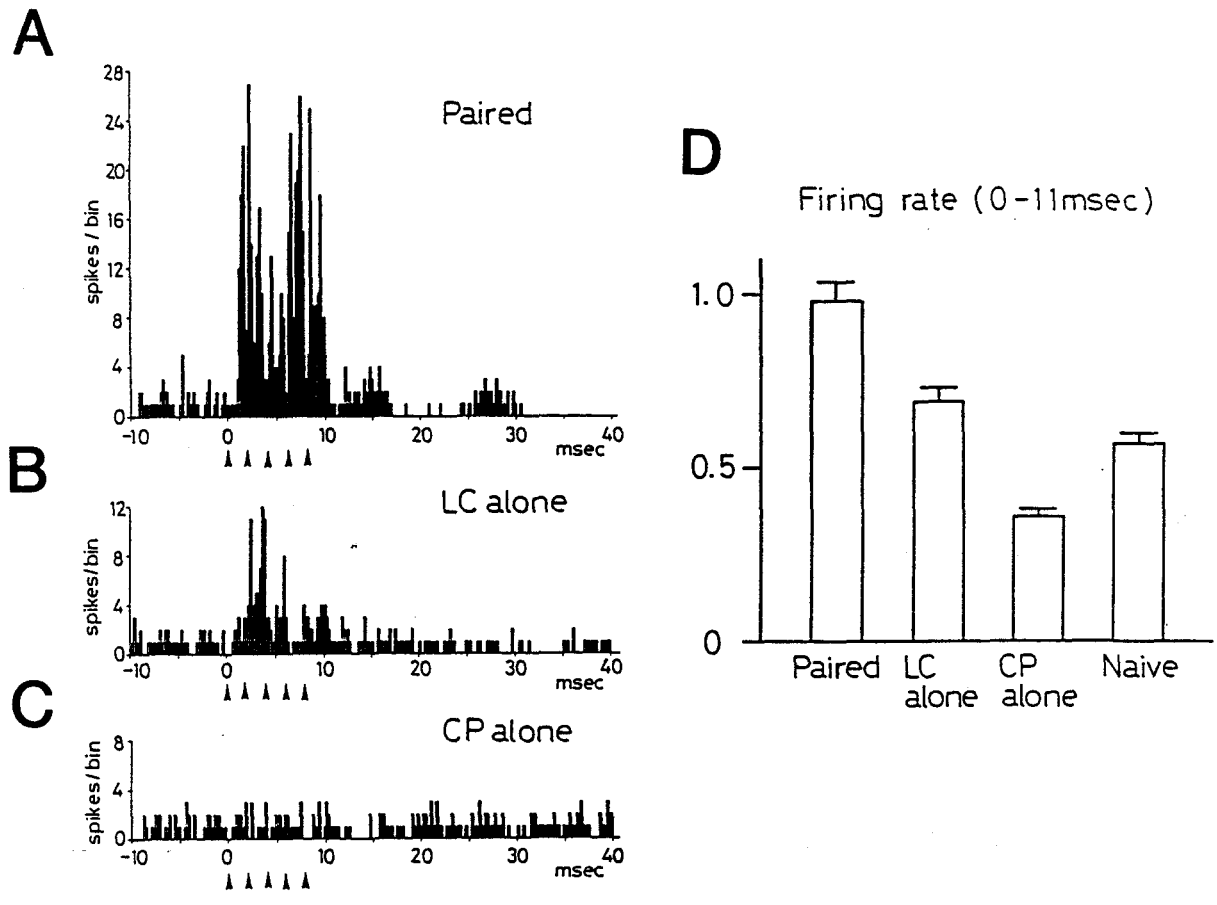

Fig. 18. Responses of RN cells to the CP stimulus. PSTHs of responses of RN cells in paired (A), LC-alone (B) and CP-alone group(C). The CP stimulus consists of five electric pulses (arrowheads) with intensity of 0.75, 1.2, 0.3 mA, respectively. Abscissa: time after the onset of the first pulse of the CP stimulus. All PSTHs have 0.2 msec/bin. Ordinate: number of spikes/bin. D: mean (± S.E.M.) number of spikes within 11msec after the onset of the CP stimulus, summarized in four groups of animals.

Modification of monosynaptic response of RN cells to the CP stimulus

There was a significant difference in the RN cell response to the CS between the paired group and the other groups. Activity of the RN cells correlated closely with the behavioral results. To investigate the mechanisms underlying the change in the RN cell responses, it is necessary to identify the primary loci responsible for the activity change. Therefore, we focused our attention on monosynaptic excitation more precisely.

We applied single-pulse stimuli to CP with the same intensity used for the CP stimulus and a duration of 100 usec. Figures 19A, B and C shows example of responses to the single-pulse CP stimulus. These PSTHs were acquired from responses of the same cells as shown in Fig. 18. The RN cell in the paired group exhibited a prominent response with a unimodal peak. The response rose fast after onset of the stimuli, reached its peak at 1.6 msec and ceased at about 5 msec (Fig. 19A). By contrast, the cell in the LC-alone group showed a slight response with peak at 3.2 msec (Fig. 19B). The cell in the CP-alone group did not have positive response exceeding the pre-stimulus spontaneous firing level (Fig. 19C).

We compared the spike activities evoked by the single-pulse stimulation of the CP by counting number of spikes observed initial 3 msec after the stimulus for 200 successive trials and dividing it by total number of trials. These response with short latency do not include the polysynaptic activation mediated by

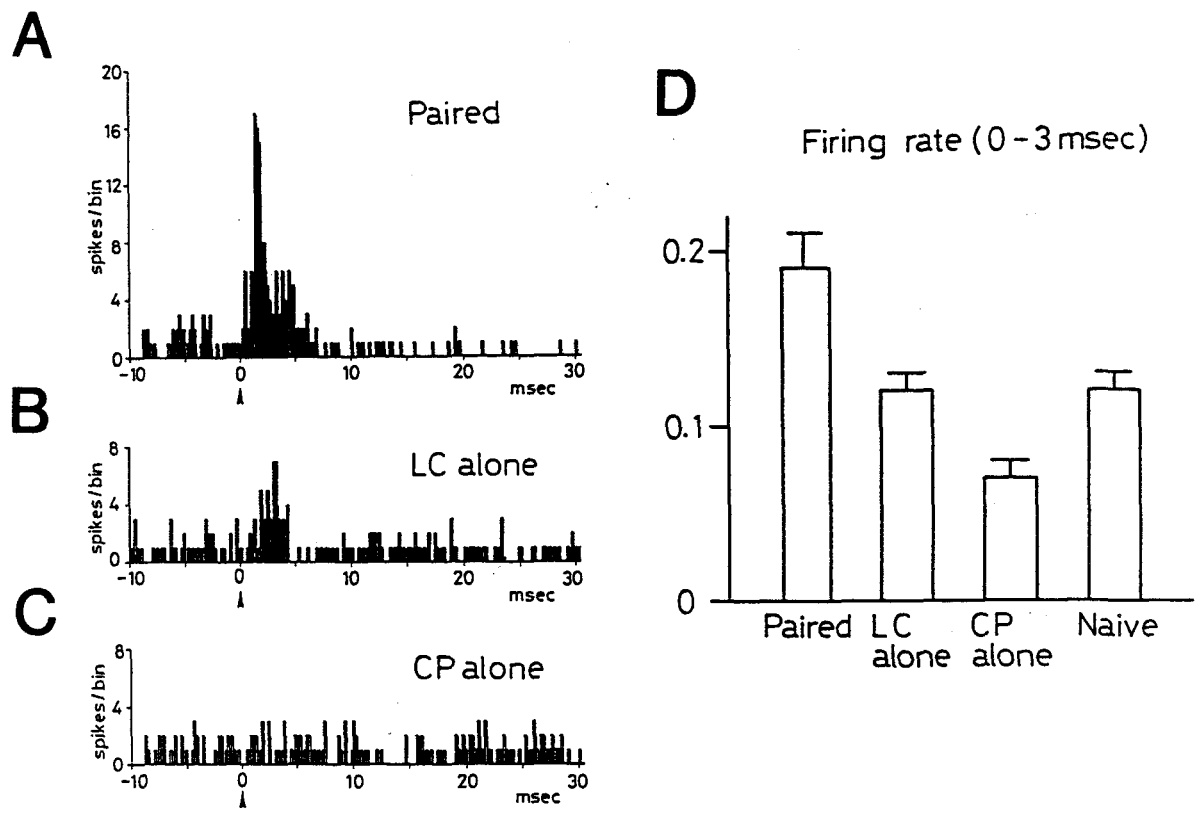

Fig. 19. Response of RN cells to single-pulse stimulation applied to CP. A to C: PSTHs of the responses of the RN cells were acquired from the same RN cells as shown in Fig. 18A, B and C, respectively. These responses were sum of 200 responses to single-pulse stimuli applied to the CP with the same intensity as that of the CP stimulus. Arrowheads show the onset of stimulus. D: Number of spikes within 3 msec after the single-pulse stimulus to CP. Each bar represents the mean (±S.E.M.) number of spikes of RN cells in four groups of animals.

the inhibitory interneurons in the RN cells, the rubro-olivo-interpositus pathway, the cerebral cortex, and etc. (Toyama, Tsukahara and Udo, 1968; Tsukahara, Fuller and Brooks, 1968; Appelberg and Jeneskog, 1973; Allen and Tsukahara, 1974; Eccles, Scheid and Taborikova, 1975).

Figure 19D shows histograms of mean number of spikes in response to the single-pulse CP stimuli in four groups of animals. Mean and S.E.M. of number of spikes induced during initial 3 msec after the CP stimulus was 0.19 $\pm$ 0.02 spikes/3 msec (90 cells) in the paired group, 0.12 $\pm$ 0.01 spikes/3 msec (71 cells) in the LC-alone group, 0.07 $\pm$ 0.01 spikes/3 msec (102 cells) in the CP-alone group and 0.12 $\pm$ 0.01 spikes/3 msec (82 cells) in the naive group). Statistical analysis demonstrated that the activity of RN cells in the paired group significantly higher than in the LC-alone, CS-alone or naive group ($p<0.02$). The cell activity of the CP-alone group was lower than that of the naive group ($p<0.05$). These results indicates that monosynaptic activation through the cortico-rubral synapses was enhanced specifically after the paired conditioning in parallel with the acquisition of the conditioned forelimb flexion.

However, these results could be explained also by the lowered threshold of RN cells that was caused by increased background input to RN cells or change in the membrane properties of RN cells. We examined this possibility by investigating the monosynaptic activation of RN cells from the interpositus nucleus of the cerebellum and spontaneous firing rate of RN cells.

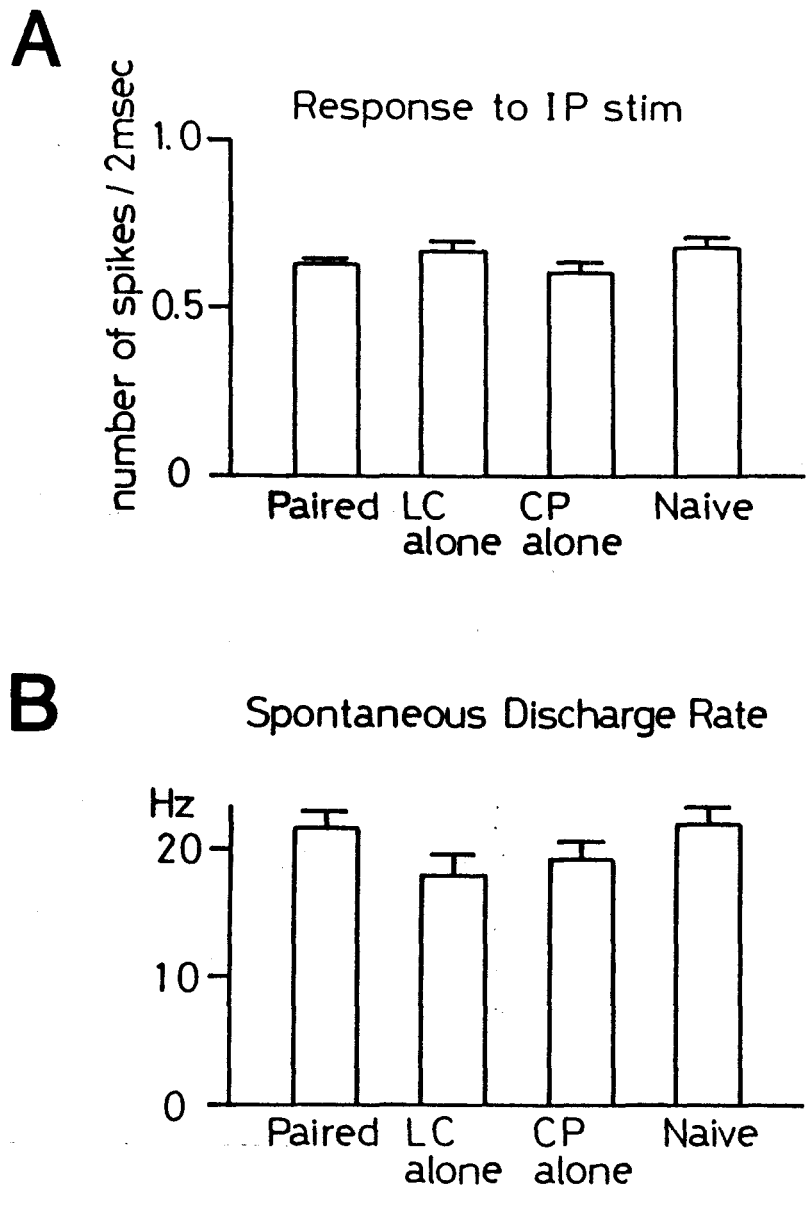

Fig. 20. Comparison of responses to the stimulation of IP and spontaneous discharge of RN cells among four groups of animals. A: Response of RN cells to the stimulation of IP. Mean (± S.E.M.) number of spikes within 2 msec after the single-pulse stimulation of IP. Current intensity of the stimulus was the same as that of the IP-100 % performance current during the pretraining period. B: Mean (± S.E.M.) spontaneous firing rate of RN cells, which was calculated from the spike discharge during the prestimulus period of the PSTHs for the response to the CP stimulus.

We compared the spike activity evoked by single-pulse stimulation of the IP by counting the number of spikes observed during initial 2 msec after the stimulus for 100 successive trials and dividing it by total number of trials. As mentioned in Chapter 3, the responses within 2 msec after the IP stimulus are estimated as monosynaptic responses. Figure 20A summarizes the mean number of spikes of RN cells in four groups of animals. There was no significant difference among them. There is no significant difference in spontaneous firing rate of RN cells among four groups (paired: 21.7 $\pm$ 1.4 spikes/sec, 106 cells; LC-alone: 18.1 $\pm$ 1.7 spikes/sec, 73 cells; CP-alone: 19.2 $\pm$ 1.2 spikes/sec, 111 cells; naive: 23.3 $\pm$ 1.3 spikes/sec, 89 cells; Fig. 20B).

Therefore, these results suggest that the increased activity of RN cells in response to the CP stimulus after the paired conditioning is caused by increased effectiveness of signal transmission at the cortico-rubral synapses rather than by the lowered threshold of RN cells.

Noradrenergic Projection into the RN

In the present study, it has been shown that pairing the stimuli to the cortico-rubral fibers and that to the LC is essential to produce the conditioned forelimb flexion and to increase the efficacy of transmission at the cortico-rubral synapses. It remained to be examined whether the modification at the cortico-rubral synapses might be caused by the direct projection from the LC to the RN or the projection from the LC to

the other system which finally affect the transmission at the cortico-rubral synapses. As the first step to examine this, we tried to demonstrate the adrenergic projection in the RN by detecting the DBH containing nerve terminals in the RN immunohistochemically. DBH is the enzyme to convert dopamine to noradrenaline and useful as a anatomical marker for noradrenergic and adrenergic neurons. Figure 21 shows an example of DBH-containing fibers in the RN. These fibers have varicosities, which have been suggested to be catecholaminergic terminals. These DBH-positive fibers with varicosities were sometimes observed on or near the somata of the RN cells. Therefore, these data indicate noradrenergic innervation of the RN in cat and suggest a possibility that some of the noradrenergic fibers terminate on the RN cells.

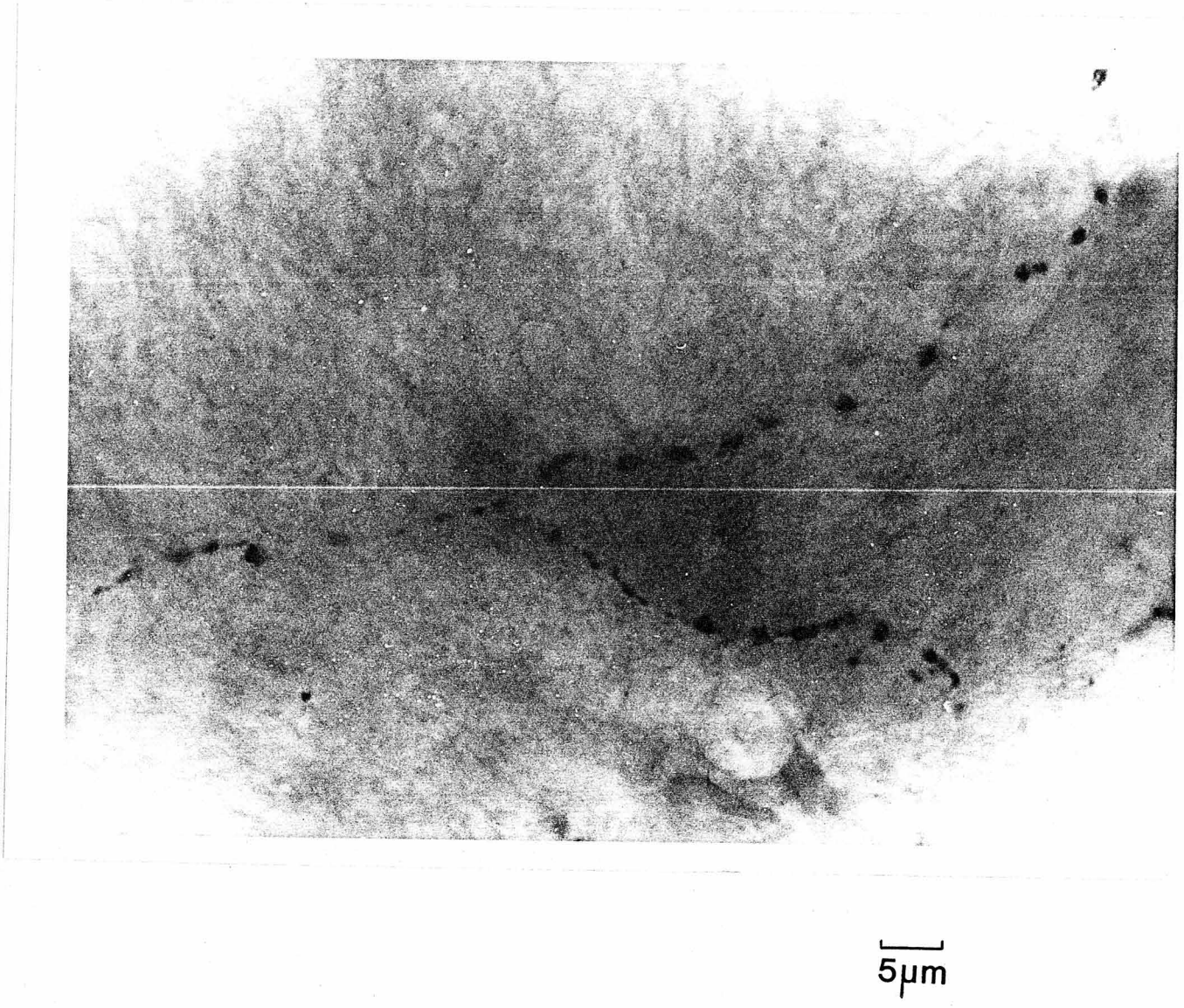

Fig. 21. Noradrenergic projection into the red nucleus in cat. Dopamine-B-hydroxylase (DBH) containing fibers with varicosities near an RN neuron. The section was reacted with a rabbit anti-DBH serum and labeled with the ABC method.

DISCUSSION

In this study, we tried to reduce neuronal circuit of the US pathway by applying stimulus to the LC instead of the US to forelimb skin, since the LC neurons fire typically when animals receive noxious stimulus such as skin shock (Nakamura, 1977) and may play some important role for neuronal plasticity(see Ref. Saper, 1987). Pairing the CP stimulus with the LC stimulus at a fixed interval induced forelimb flexion as the conditioned response.

Stimulation of the LC is effective to produce the associative conditioning

It is not easy to insert electrode exactly into the LC and to stimulate exclusively the LC. We modified slightly the stereotaxic coordinates of the LC in accord with the weight of cat. We applied the stimulus pulses through concentric electrode with the current intensity less than 300 uA to minimize current spread to the trigeminus nucleus or the cerebellar peduncle near the LC.

In six animals out of 17 cats, however, the stimulus electrodes were inserted out of the LC as shown Fig. 17B. They were inserted in superior cerebellar peduncle, edge of the periventricular gray and pontine reticular formation ventral to the LC. In either of these cases, the score of performance did not reach above the level of 50 %. And, in all the cases when the electrode tips were positioned in the LC (LCD or LCa, Fig.

17A), the score of performance exceeded 50 %. These results strongly suggest that stimulation of the LC itself is effective to produce the associative conditioning.

Associative conditioning produced by stimulation of CP and LC is similar to the classical conditioning mediated by the RN

Associative conditioning produced by the paired CP-LC stimuli has the several features identical with those of the classical conditioning produced by pairing the CP stimulus (CS) with the skin shock (US): (1) The conditioned forelimb flexion was acquired by pairing two stimuli at a fixed interval. Applying either alone or pairing two stimuli at random intervals (a recent observation not shown in the text) did not increase the score of performance. (2) The score of performance reached its peak after about one-week's training. (3) The conditioned forelimb flexion retained for more than a few weeks. (4) The latency of electrical activity evoked in the biceps brachii muscle by the CP stimulus accorded with the time required for the impulse transmission along the cortico-rubrospinal pathway from the CP to the muscle. (5) Extinction was produced by reversing the sequence of two stimuli, i.e. LC-CP. (6) Reacquisition of the conditioned forelimb flexion occurred faster than the original acquisition. (7) RN cell activity evoked by the CP stimulus is well correlated with the behavioral responses. Firing probability of RN cells in response to the CP stimulus increased especialy after the paired training. (8) Monosynaptic

response of RN cells to the CP stimulus was enhanced after the paired training. (9) Efficacy of neuronal transmission at the cortico-rubral synapses was enhanced by the paired training, whereas neither the response to the stimulation of the interpositus nucleus nor spontaneous discharge of RN cells changed. These results suggest that the primary site of neuronal change is the cortico-rubral synapses.

It should be noticed, however, that the LC stimulus did not evoke forelimb flexion as the unconditioned response. Therefore, the behavioral change induced by the CP-LC stimulation is not categorized as a classical conditioning in which the unconditioned response similar to the conditioned response is evoked by the US even before training.

From the common features between two preparations as mentioned above, we consider that neuronal modification underlying the associative learning established by the paired CP-LC training is similar to what occurs in the classical conditioning produced by pairing the CP stimulus with the skin shock, though the LC stimulus does not evoke forelimb flexion. It is possible to speculate that the LC stimulation can modify the efficacy of signal transmission at the cortico-rubral synapses in the same way as the US when it is paired with the preceding CP stimulus, though it remains to examine whether the LC stimulus affects the cortico-rubral synapses directly or indirectly.

GENERAL DISCUSSION

Formation of new synapses as a neuronal basis of learning and memory

Results of the present study (see Chapter 4; Tsukahara and Oda, 1981; Oda et al., in press) provide the first physiological evidence for the the hypothesis that sprouting and formation of new synapses producing the change in time course of EPSPs is the neuonal basis of classical conditioning in the mammalian central nervous system, as has long been assumed by previous authors (Cajal, 1911; Konorski, 1948; Hebb, 1949).

This result is supported by a morphological analysis of change in distribution of the cortico-rubral synapses after the conditioning in the same preparation as mentioned here (Murakami et al.,1987). An electron microscopic qualntitative study shows that the propotion of cortico-rubral synapses contactiong with somata or proximal dendirtes increased after the classical condtioning.

Recently morphological change at synapses was proposed as the neuronal basis of learning even in the invertebrate. Bailey and Chen (1983; 1987; 1988) reported that number, size and vesicle components of active zone, as well as extent of each sensory neuron's arborization increased in long-term sensitized Aplysia. In parallel with this study, Montarolo et al. (1986) showed that repetitive application of a presumed modulatory trasnmitter, serotonin, and protein synthesis is necessary for establishment of long-term sensitization by using in vitro co-

culture preparation of sensory and motor neurons. They also showed that the short-term sensitization was not affected by inhibit of the protein synthesis.

There are also evidences for involvement of morphological change in neurons in long-term memory. In rats given extensive training on multiple maze pattern, dendritic fields of the visual cortex pyramidal neuron were larger than in control (Greenough, Juraska and Volkmar, 1979; Chang and Greenough, 1982). In addition to earlier studies demonstrating structural change in nervous system after injury of brain, these studies showed that enviormental change involving learning produced structural change in the adult central nervous system.

More recently, it has become clear that the arrangement of synaptic connections in the mature nervous system can undergo striking changes even during normal functioning (Purves et al., 1986). On the other hand, it has been established that newly formed synapses can be retained, maybe dynamically, for more than months or years. All of these results suggest that morphological change at synapses such as number or pattern of synaptic connections can be a neuronal substrate for memory strage during behavioral modification.

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