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| Title        | Electrophysiological Studies on Cellular Mechanisms Underlying an Associative Learning Mediated by the Red Nucleus in the Cat |
| Author(s)    | 伊藤, 南                                                                                                                         |
| Citation     | 大阪大学, 1994, 博士論文                                                                                                              |
| Version Type | VoR                                                                                                                           |
| URL          | <a href="https://doi.org/10.11501/3075299">https://doi.org/10.11501/3075299</a>                                               |
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**Electrophysiological Studies on Cellular Mechanisms  
Underlying an Associative Learning Mediated by the Red  
Nucleus in the Cat.**

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This thesis is based on the following articles.

1. **Appearance of new component in the cortico-rubral EPSPs after the establishment of classical conditioning in the cat.**  
Oda Y., Miyasaka S., Ito M., Tsukahara N.  
J. physiol. Soc. Japan 45, 1983.
2. Classical conditioning acquired by paired stimulation of inputs to the red nucleus from the cerebrum and the locus coeruleus.  
Oda Y., Ito M., Kishida H., Yoshimi K., Nagatsu I., Tsukahara N.  
Neurosci. Res. Suppl. 3:s56, 1986.
3. Associative conditioning acquired by paired stimulation of inputs to red nucleus from cerebrum and locus coeruleus: modification of rubral cell activity.  
Oda Y., Kishida H., Ito M., Nagatsu I.  
Neurosci. Res. Suppl. 5:s112, 1987.
4. Modification of rubral cell activity underlies associative classical conditioning induced by paired stimulation of corticorubral fibers and locus coeruleus.  
Oda Y., Kishida H., Ito M., Nagatsu I.  
Soc. Neurosci. Abstr. 13:839, 1987.
5. **Formation of new cortico-rubral synapses as a possible mechanisms for classical conditioning mediated by the red nucleus in cat.**  
Oda Y., Ito M., Kishida H., Tsukahara N.  
J. Physiology (Paris) 83:207-216, 1988.

6. Formation of new corticorubral synapses associated with establishment of classical conditioning in cat.

Ito M., Oda Y.

Neurosci. Res. Suppl. 9:s85, 1989.

7. Formation of new corticorubral synapses as a possible mechanism for classical conditioning in cat.

Oda Y., Ito M.

Proc. IUPS XVII. S4051, 1989.

8. **Electrophysiological evidence for formation of new corticorubral synapses associated with classical conditioning in the cat.**

Ito M., Oda Y.

Exp. Brain Res. (in press)

## Summary

The present study was performed to clarify whether or not structural plasticity of synaptic connections underlies classical conditioning mediated by the red nucleus (RN) in the cat. Conditioned forelimb flexion is established by pairing electrical conditioned stimuli (CS), applied to corticorubral fibers at the cerebral peduncle (CP), with a forelimb skin shock (the unconditioned stimulus; US), but not by applying the CS alone nor by pairing the CS and US at random intervals. In the previous studies showed that the firing probability of rubrospinal neurons (RN neurons) in response to the CS was well correlated with acquisition of the conditioned forelimb flexion and that the primary site of neuronal change underlying establishment of the conditioned forelimb flexion was suggested to be at corticorubral synapses. In the present study, I investigated corticorubral excitatory postsynaptic potentials evoked by the CP stimulation (CP-EPSPs) in RN neurons in order to identify the neuronal mechanism underlying establishment of classical conditioning.

In normal cats, the CP-EPSPs had a typical slow-rising phase, which has been attributed to the distal location of corticorubral synapses on the dendrites of RN neurons. In contrast, in animals which received paired conditioning, subsequent CP stimulation evoked potentials with a fast-rising time course. In control groups of cats which received the CS alone, the CS randomly paired with the US or only the same surgical operations as the conditioned animals, most of the CP-EPSPs displayed slow-rising EPSPs which were similar to those observed in normal cats. The mean time from onset to peak of the potentials in the conditioned animals was significantly shorter than those seen in other groups. Therefore, the appearance of the fast-rising potential correlates well with the acquisition of the conditioned forelimb flexion.

The amplitude of the fast-rising potential was gradually changed with stimulus

intensity. It had a short onset latency following CP stimulation (0.9ms) which was similar to that of the slow-rising EPSP in normal cats. It followed high-frequency stimulation up to 100Hz. These results suggest that the newly appearing fast-rising potential was a monosynaptically evoked EPSP. Fast-rising EPSPs were also induced by stimulation of the sensorimotor cortex (SM). Since the SM-EPSP was occluded by the CP-EPSP, the SM cortex is, at least in part, a likely source of the fast-rising EPSPs. Fast-rising SM-EPSPs were also observed at the unitary level. The SM-EPSPs in the conditioned animals exhibited somatotopical representation in their cortical origin as has been described in normal cats. The electrotonic length was calculated from the voltage transient responses to current steps injected into the RN neurons. There was no concomitant change in the electrotonic length following the classical conditioning. Furthermore, the fast-rising EPSPs were often observed as if they were superposed on the slow-rising EPSPs which were observed in normal animals. These observations suggest that the appearance of the fast-rising EPSPs is due to the formation of new corticorubral synapses on the somata or the proximal dendrites of the RN neurons, and not as a result of a reduction in the electrotonic length of the RN neurons.

The present study provides further evidence that this type of structural plasticity of synaptic connections underlies establishment of the classically conditioned forelimb flexion.



## CHAPTER 1.

### GENERAL INTRODUCTION

Ability of learning provides us flexibility which is necessary for us to adapt ourselves to always changing environment and circumstances. After several number of trails, we can perform appropriate motor control with no apparent effort. Furthermore, we can perform the obtained motor control even after long intervals. Both adaptive systems and learning systems are able to adjust their parameters to achieve optimal motor control in accordance with repeated exploration of the optimal conditions and adequate motor control. Learning system should have also mechanisms for maintaining the results of preceding experiences in form which is ready for utilizing in any time. Ability of learning, acquisition and persistence, may be one of essential neural functions and has been a fundamental target of the neurophysiology.

Synaptic connections in the mammalian central nervous systems are flexible even in adults. Formation of new synapses has been found in various preparations following partial denervation (for reviews, see Cotman et al. 1981; Tsukahara 1981). Furthermore, partial denervation is not the sole cause of synapse formation. For example, Tsukahara and his colleagues demonstrated formation of new corticorubral synapses following the cross-union of forelimb flexor and extensor nerves (Tsukahara et al. 1982; Fujito et al. 1982; Murakami et al. 1984). New synapses were formed on the proximal dendrite or somata of the red nucleus neurons (RN neurons) innervating upper spinal segments in parallel with adaptation of the voluntary forelimb movements following the cross-union. This study indicated that the formation of new synapses can underlie behavioral modification. It has long been hypothesized that learning may base on physiological and morphological plasticity of synapses and synaptic connections.

Reinforcement of pre-existing connections or formation of new connections may participate in the long-lasting processes of learning (Tanzi 1893; Cajal 1911; Konorski 1947; Hebb 1949). Although enormous efforts have been given to investigate neuronal correlates of learning (Thompson et al. 1984), the contribution of synapse formation to learning is not yet clear. We are still far from understanding what are the neuronal mechanisms underlying learning, especially, with long time course of weeks, months, years or life time. This is mainly because complex neural circuits of central nervous systems in intact animals prevent us from identifying primary site where neuronal correlates occur. Identification of the primary site is necessary for connecting electrophysiological, anatomical or biological experimental data and behavioral modifications resulted from learning procedures. Recently, several laboratories are breaking this difficulty by selecting appropriate learning paradigm in which the identifiable nervous system is essential to induce behavioral modifications; adaptation of vestibulo-ocular reflex in rabbit (Ito 1982), habituation, sensitization and classical conditioning of siphon-withdrawal reflex in *Aplysia* (Kandel 1976), and classical conditioning of forelimb flexion mediated by the red nucleus in cat (Tsukahara et al. 1981).

In order to get insights of neuronal correlates of learning with long time course, we used a simplified model of classical conditioning of forelimb flexion mediated by the RN in the cat (Tsukahara et al. 1981). There are several merits in using classical conditioning. 1. Since classical conditioning is associative process, it is potentially relevant to more complex forms of learning. 2. Classical conditioning involves stimuli which can be defined and manipulated by the experimenter. 3. Classical conditioning involves neuronal events which occurs in a known temporal sequence and which can be analyzed in correlation to external events observable to experimenters. Demerit is that both visual (auditory) conditioned stimuli and aversive unconditioned stimuli affect many neural systems

during the classical conditioning. Therefore, it should be noted that any conditioning experiment represents a complex process including many conditioned responses and neural sites which are not limited to those of particular interest of experimenter.

To deal with this complexity, numerous studies have been attempted to identify the minimal essential neuronal circuit required to maintain one particular conditioned response. Within this essential circuit, one can expect to find the essential neuronal modifications underlying long-term behavioral changes. However, vertebrate central nervous systems are too complicated to specify a simple circuit corresponding to a particular response. Thus, we used "reduced preparation" in which one or two elements of classical conditioning is replaced by a neurophysiological event within nervous system, that is to say, electrical stimulation to activate a particular neural site. The model was based on a classical conditioning of forelimb flexion, acquired by pairing a tone stimulus as a conditioned stimulus (CS) and forelimb electrical shock as an unconditioned stimulus (US). Since unilateral lesion of the RN or rubrospinal tract after the training abolished conditioned forelimb flexion, the conditioned response is thought to be mediated by the rubrospinal tract (Smith 1970; Voneida 1990). Furthermore, the afferent and efferent neural connections of the RN neurons are well characterized (Massion, 1967; Hongo et al. 1969; Allen and Tsukahara, 1974; Illert et al. 1976; Ito 1984). In order to simplify the neural circuitry which is involved in the conditioning, the pathways related to the conditioned response were restricted to the corticorubrospinal pathway. 1. Only weak electrical stimulation was applied to the cerebral peduncle (CP) as the CS. 2. Corticofugal fibers were sectioned at a level caudal to the RN. After pairing the CS with the US repeatedly at a fixed interval of 100ms for a week, the initially ineffective CS induced pronounced forelimb flexion (Tsukahara et al. 1981).

An important advantage of this preparation was that the primary site for the

conditioning was suggested to be at the corticorubral synapses. 1. The shortest latency (8ms) of electrical activity obtained in the biceps brachii muscle in response to the CS was consistent with the shortest time required for impulse transmission along the corticorubrospinal pathway to the muscle (Tsukahara et al. 1981). 2. The threshold of the CP stimulation which evoked forelimb flexion decreased after the paired conditioning, while threshold of another monosynaptic input from the nucleus interpositus of the cerebellum did not change and these two pathways are common caudal to the RN (Tsukahara et al. 1981; Oda et al. 1988). 3. The lesion of the corticofugal fibers excluded possible contributions of the corticospinal, corticopontine or corticoreticular pathways.

Therefore, electrophysiological studies on the synaptic transmission at the corticorubral synapses and the electrophysiological properties of the RN cells may give us clues to the neuronal correlates underlying acquisition and retention of the classical conditioning.

## CHAPTER 2.

### Appearance of the fast-rising corticorubral EPSPs following the classical conditioning

## INTRODUCTION

As mentioned in the previous chapter, the primary neural site of the classical conditioning mediated by the RN was thought to be at the corticorubral synapse. Oda and his colleagues conducted single unit recordings from the RN neurons (Oda et al. 1981, 1988). They demonstrated that the averaged firing probability of the RN neurons in response to the CS was significantly higher in animals which received the paired conditioning (*paired* group) compared to that in animals which underwent surgical operations and pretraining procedures alone (*naive* group). In animals which received randomly paired CS and US (*random* group), the averaged firing probability was similar to that of the *naive* group. In animals which received the CS alone (*CS-alone* group), the averaged firing probability was rather lower than those of the other groups. On the other hand, neither the averaged firing probability of the RN neurons in response to stimulation applied to the nucleus interpositus of the cerebellum nor the spontaneous firing probability of the RN neurons differed among the four groups of animals. These results suggested that the acquisition of the classical conditioning is based on enhancement of the synaptic transmission efficacy at the corticorubral synapses.

As the next step, I conducted intracellular recordings from the RN neurons to examine a detail of changes in the synaptic transmission efficacy. In this chapter, I asked whether the classical conditioning induce changes in time course of the corticorubral excitatory postsynaptic potentials (EPSPs). I asked the relationship between behavioral modification and waveform of the corticorubral excitatory postsynaptic potentials (EPSPs). Intracellular recordings were performed in five

groups of animals; the *paired*, *CS-alone*, *random*, *naive* and *normal* groups. By comparing those between the paired and other control groups, we examined whether the difference in the firing probabilities in response to the CS correlate to the time course of the corticorubral EPSPs. Control groups including the *random* and *CS-alone* groups were served to test non-associative effects of the CS and/or the US. Normal cats were also examined in order to assess effect of the surgical procedures.

In the RN neurons in the paired group, fast-rising potentials appeared in the corticorubral EPSPs, while not in other groups. These results suggest that appearance of the fast-rising potential is causally related to the establishment of the classical conditioning.

## MATERIALS AND METHODS

### Surgical and training procedures

Experiments were performed on 116 adult cats (2.1-4.9kg). Seventy-eight of them were surgically operated under sodium pentobarbital anesthesia (initial dose, 35 mg/kg) prior to training as described previously (Tsukahara et al. 1981). Left corticofugal fibers were sectioned electrolytically at a level caudal to the RN (at AP0 and A0.5; Horsley-Clarke coordinates, 1mA for 30sec). In order to eliminate motor reactions mediated by the corticofugal fibers other than corticorubrospinal pathway, bipolar stimulating electrodes were inserted stereotaxically into the left cerebral peduncle (CP, at A8.5) for the CS (Fig.1).

About ten days after the surgery, the animals underwent a *pretraining period* of 4 to 6 days when the intensity of the required CS was determined. The CS consisted of a train of 5 biphasic pulses with a duration of 0.2msec and with an interpulse interval of 2msec (500Hz). Each pulse with duration of 100 $\mu$  was followed by a pulse with reversed polarity to prevent electrode polarization. Cats were mounted on a specially designed frame in which only right forelimb can move freely. Flexion of the right forelimb was monitored by a potentiometer attached to the elbow joint. The output voltage of the potentiometer was sampled at every 4msec and stored in a microcomputer. The resolution of measurement of the joint angle was 0.02°. A flexion was taken to be significant when it exceeded 0.06° within 100msec of the onset of the CS. Intensity of the CS which ranged from 0.2 to 1.4mA was selected such that significant forelimb flexion was induced in 20% of total trials. A microcomputer was used to control the stimulation and to judge positive responses.

The *training period* started on the first day after the end of the pretraining period and lasted for 7 to 10 days. The daily session consisted of an application of 50 paired stimuli. The animals were divided into five groups: (1) *paired* (n=35), (2)

*CS-alone* (n=7), (3) *random* (n=4), *naive* (n=34) and (4) *normal* (n=38). As shown in Fig.2, cats in the *paired* group received the CS paired with the US at a fixed interval of 100msec. The US was a weak electrical shock of 10msec duration applied to right forelimb skin and induced forelimb twitch as the unconditioned response. Animals in the *CS-alone* group received only the CS during the training period. Those in the *random* group received the CS and the US at intervals randomly varied from 200msec to 20sec during the daily session. In these three groups, the CS with or without the US was applied once every 50sec. Those in the *naive* group received only the same surgical operation and the same pretraining procedure and spent 7 to 14 days without receiving any stimulation. Those in the *normal* group received neither the operation nor the training procedure.

#### Intracellular recordings

Animals were anesthetized with sodium pentobarbital (initial dose, 35mg/Kg, supplemented as required), immobilized with gallamine triethiodide, and artificially respired. Intracellular recordings were obtained from the magnocellular portion of the left RN with glass micropipette filled with 2M K-methylsulphate or 2M K-citrate (resistance 5-12M $\Omega$ ). The electrode was inserted from exposed surface of the left hippocampus with a lateral angle of 15°. The RN neurons were identified by antidromic activation from the C<sub>1</sub> and/or L<sub>1</sub> spinal segment and orthodromic activation from the right interpositus nucleus as described previously (Tsukahara et al. 1967). Corticorubral fibers were stimulated at the left CP and sensorimotor cortex. Potentials were photographed and/or were digitized at 50 $\mu$ sec sample frequency and stored by a microcomputer for further analysis.

#### Histology

At the end of the experiments, DC current (500 $\mu$ A, 10sec) was passed through the stimulation electrodes and the animals were perfused with 10% formalin



solution under deep anesthesia with sodium pentobarbital. The extent of the lesion of corticofugal fibers, location of the stimulating electrodes and tracks of recording electrodes were examined later in serial sections stained with methylene blue.

### Statistical analysis

For statistical evaluation between two sets of values, the two-tailed Student's t-test was used. When the population variance was significantly different (F-test,  $p < 0.05$ ), the Welch's t-test was used. For the behavioral performance of the training, the paired t-test was used. For statistical evaluation of the difference among the five animal groups, one-way ANOVA test was used. Data are expressed as the mean  $\pm$  standard error of the mean (S.E.M.).

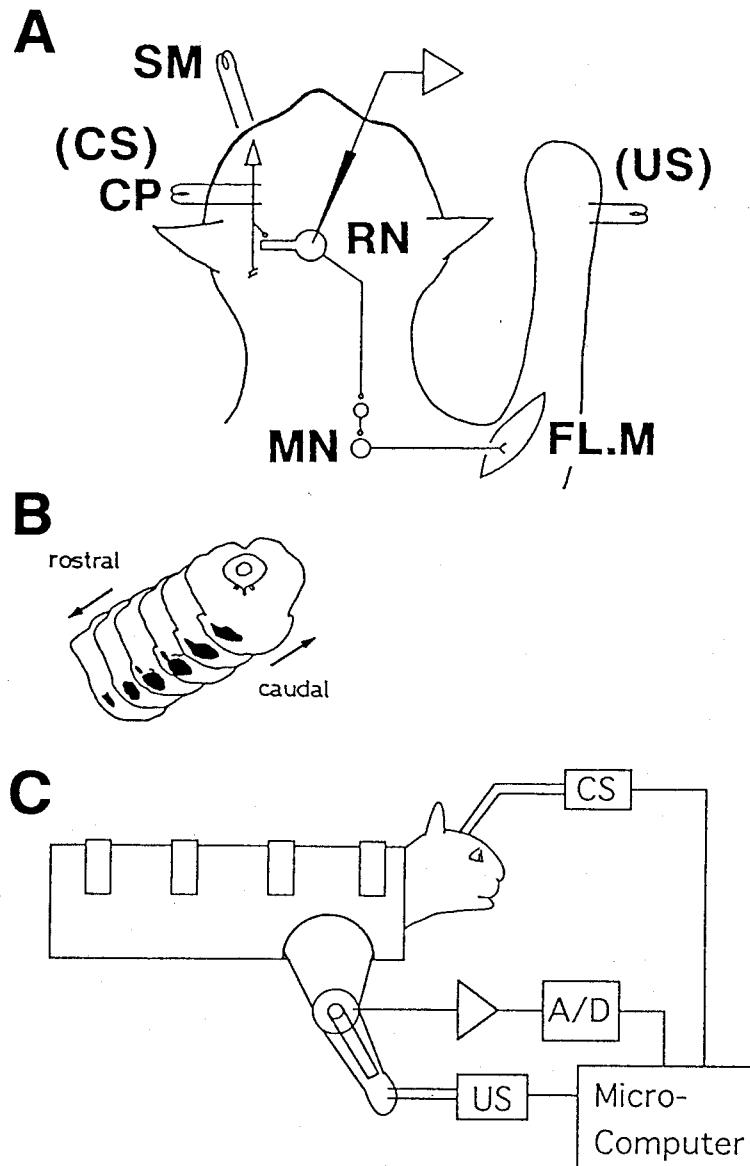


Fig. 1. A: A schematic diagram illustrating the neural circuit for classical conditioning mediated by the red nucleus (RN) in cats. The conditioned stimulus (CS) was applied to the left cerebral peduncle (CP) and the unconditioned stimulus (US) was delivered to the right forelimb skin. The corticofugal fibers below RN were eliminated to restrict the pathways for the conditioned response mainly to the corticorubrospinal pathway. Intracellular recordings were performed in the RN neurons. The corticorubral EPSPs were evoked by stimulation to the CP or the sensorimotor cortex (SM). MN: motoneuron. FL.M.: flexor muscle. B: Drawings of histological sections through the mid brain show lesioned area in the cerebral peduncle caudal to the RN. C: The set up used for applying the CS and the US and measuring movements at the elbow joint. Measured joint angle was stored and analyzed by a microcomputer.

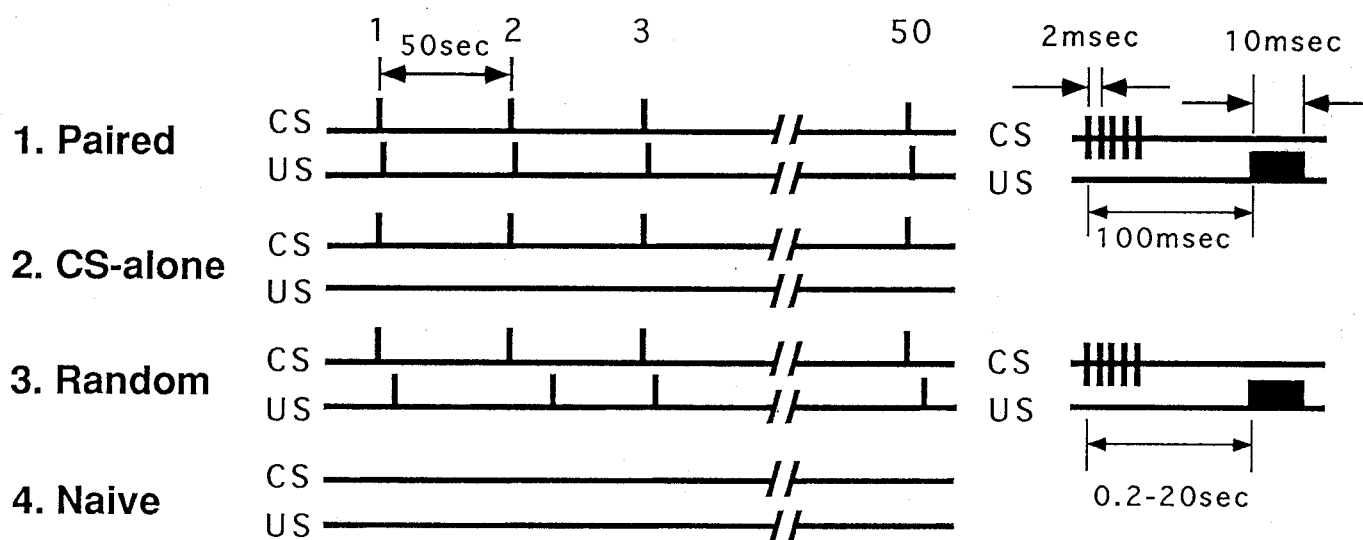


Fig. 2. Diagrammatic representation of the training and the naive procedures. *Paired* : the CS was paired with the US at a fixed interval of 100msec. The CS consisted of a train of 5 biphasic pulses with a duration of 0.2msec and with an interpulse interval of 2msec. *CS-alone* : only the CS was applied. *Random*: the CS was paired with the US at intervals randomly varied from 0.2 to 20sec. In these three groups, the CS was repeated every 50sec for a total of 50 times a day. *Naive* : animals underwent the surgical procedure alone.

## Results

### Behavioral modifications after the paired conditioning

The conditioned forelimb flexion was acquired after the *paired* conditioning as reported previously (Tsukahara et al. 1981; Oda et al. 1988). The behavioral response was described as a "score of performance", i.e., the percentage of positive forelimb flexion out of total trials in a one-day session (cf. Tsukahara et al. 1981). Figure 3 shows examples of the mechanogram of forelimb flexion in a cat which received the paired conditioning. On the first day of the training, the CS induced no flexion, while the US induced large flexion as unconditioned responses. After the conditioning, the CS also induced positive forelimb flexion. As shown in Fig.3D, in the *paired* group, the mean score of performance on the last day of the training period ( $45.9 \pm 5.8\%$ ,  $n=35$ ) was significantly higher than that on the first day ( $15.9 \pm 1.5\%$ ,  $n=35$ , paired t-test,  $t=5.97$ ,  $p<0.001$ ). In the *CS-alone* group, the score on the last day ( $16.6 \pm 5.3\%$ ,  $n=7$ ) was virtually the same as that on the first day ( $17.7 \pm 3.9\%$ ,  $n=7$ , paired t-test,  $t=0.27$ ,  $p>0.20$ ). In the *random* group, the score on the last day ( $26.5 \pm 5.7\%$ ,  $n=4$ ) was virtually the same as that on the first day ( $20.5 \pm 0.5\%$ ,  $n=4$ , paired t-test,  $t=1.06$ ,  $p>0.20$ ).

Figure 4 shows change in the score of performance in a cat. During first 13 days, the cat received the paired CS and US, 50 times in a daily session. After the paired conditioning, we tested how long the acquired conditioned forelimb flexion was maintained. The score of performance out of totally ten trials of CP stimulation was counted once every 4 days. High score of performance was maintained for more than four weeks after the end of the paired conditioning.

### Appearance of the fast-rising potentials after the paired conditioning.

One or two days after the end of the *training period*, we made intracellular recordings. Three hundred and seventy-eight rubrospinal neurons (RN neurons) were selected for analysis on the basis of stable membrane potential and spike amplitude larger than 55mV. The mean spike amplitudes of the five groups of animals were as follows;  $66.8 \pm 0.83\text{mV}$  (*paired*,  $n=111$ ),  $66.2 \pm 1.57\text{mV}$  (*CS-alone*,  $n=29$ ),  $58.7 \pm 1.79\text{mV}$  (*random*,  $n=23$ ),  $65.6 \pm 0.74\text{mV}$  (*naive*,  $n=104$ ) and  $67.9 \pm 0.85\text{mV}$  (*normal*,  $n=109$ ).

Figure 5A exemplifies corticorubral EPSPs evoked by the CP stimulation (CP-EPSPs) in a normal cat. They represented single-peaked potentials with slow-rising slope and simple decay as reported previously (Tsukahara and Kosaka 1968). In animals of the *naive*, *random* and *CS-alone* groups, CP stimulation evoked similar slow-rising EPSPs (Fig.5B,C,D). In contrast, in animals of the *paired* group, CP stimulation typically produced fast-rising potentials in the RN neurons, as shown in Fig.5E,F. Often a late component was also seen; a second peak or bulge following the early summit instead of simple decay (Fig.5F). Although varying intensity of the CP stimulation resulted in a grading of the amplitude of the fast-rising potentials, their time courses remained constant (Fig.5G). Hence, the fast-rising potential is likely an EPSP but not a regenerative potential such as a dendritic spike (Spencer and Kandel 1961; Purpura 1967; Llinás and Nicholson 1971).

To compare the time course of the CP-EPSPs among the five groups in a quantitative manner, the time interval from onset to peak of the EPSP (time to peak) was measured. For dual-peaked EPSPs as exemplified in Fig.5E, time to the initial peak was measured. Although the time to peak was distributed in a relatively wide range in each group and overlapped between groups, Fig.6 clearly shows (1) that fast-rising EPSPs with times to peak less than 2msec were frequently observed in the *paired* group, but rarely in other groups, and (2) that the distribution of the times to peak in the *paired* group was asymmetrical, indicating that short times to

peak were more frequent. Analysis of variance indicated significant difference of the mean time to peak across the five groups (ANOVA test,  $F=16.78$ ,  $p<0.001$ );  $2.3\pm0.10$ msec (*paired*,  $n=111$ ),  $3.3\pm0.14$ msec (*naive*,  $n=102$ ),  $3.3\pm0.27$ msec (*CS-alone*,  $n=29$ ),  $3.3\pm0.22$ msec (*random*,  $n=25$ ) and  $3.7\pm0.13$ msec (*normal*,  $n=109$ ). Post-hoc analysis indicated that the *paired* group was significantly different from the four control groups (*naive*, *CS-alone*, *random* and *normal* groups) (ANOVA test,  $F=63.23$ ,  $p<0.001$ ). There was no significant difference among the four control groups (ANOVA test,  $F=1.98$ ,  $p>0.10$ ).

Since each animal was a unit of the training procedure, we also evaluated intergroup difference of the time to peak on a per animal basis as shown in Fig.7. We calculated the mean time to peak of the CP-EPSPs in each animal. The averaged values per animal were  $2.1\pm0.14$ msec (*paired*, 35 cats),  $3.4\pm0.26$ msec (*naive*, 34 cats),  $3.2\pm0.28$ msec (*CS-alone*, 7 cats),  $3.7\pm0.41$ msec (*random*, 4 cats) and  $3.6\pm0.20$ msec (*normal*, 12 cats), respectively, and significantly different (ANOVA test,  $F=8.79$ ,  $p<0.001$ ). Post-hoc analysis indicated that significant difference between the paired group and the four control groups (*naive*, *CS-alone*, *random* and *normal* groups) (ANOVA test,  $F=33.51$ ,  $p<0.001$ ) and no difference among the control groups (ANOVA test,  $F=0.55$ ,  $p>0.25$ ). Individual deviation thus did not influence the measurements shown in Fig.6. Therefore, the fast-rising corticorubral EPSPs appeared in associative manner as well as the acquisition of the conditioned forelimb flexion.

The RN neurons in the *paired* group were divided into those innervating upper spinal segments (C-cells) and those innervating lower spinal segments (L-cells) by their antidromic activation only from the C<sub>1</sub> spinal segment or from both C<sub>1</sub> and L<sub>1</sub> spinal segments (Tsukahara and Kosaka 1968). As shown in Fig.8, the fast-rising EPSPs, however, appeared in both C-cells and L-cells; the mean times to peak were  $2.3\pm0.18$ msec (C-cells,  $n=36$ ) and  $2.4\pm0.13$ msec (L-cells,  $n=62$ ) (t-test,  $t=0.46$ ,  $p>0.20$ ).

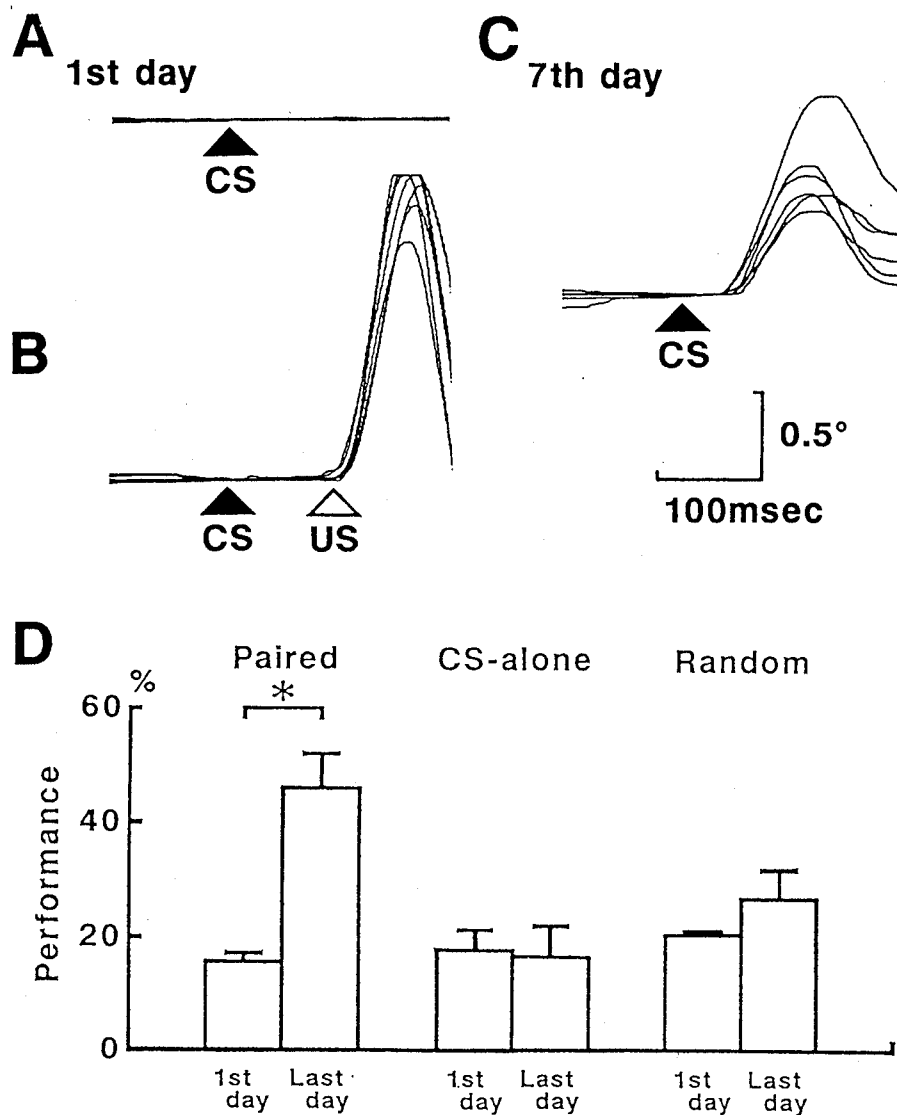


Fig . 3. Acquisition of the conditioned forelimb flexion. A-C: Sample records of mechanogram of forelimb flexion during the paired conditioning. Superposed records of six trials are shown. A,B: Flexion of the elbow joint on the first day of the training. The CS produced no responses, but the US produced large elbow flexion. Arrows indicate the onset of the CS and the US. C: Flexion of elbow joint in the same animal on the 7th day of the training. The CS alone produced positive elbow flexion. Calibration is common for A-C. D: The mean( $\pm$ S.E.M.) score of performance on the first and last day of the training in the *paired* (n=35), *CS-alone* (n=7) and *random* (n=4) groups. \*  $p < 0.001$  (paired t-test).

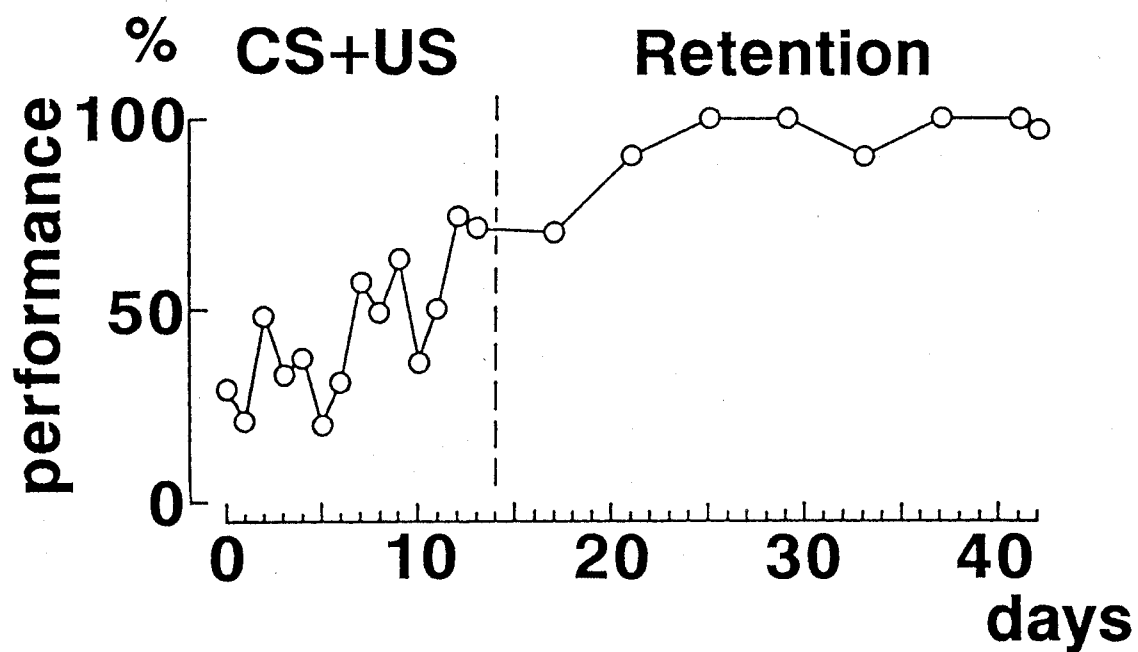


Fig. 4. The time course of the score of performance during acquisition and retention of the paired conditioning in a cat. Mean score during the *pretraining period* is shown at 0th day. From the first to 13th day, the CS was paired with the US at an interval of 100msec. After the 13th day, the CS alone was applied 10 times in a session in once every 4 days. After the 42th day, we examined extinction of the conditioned response (not shown).



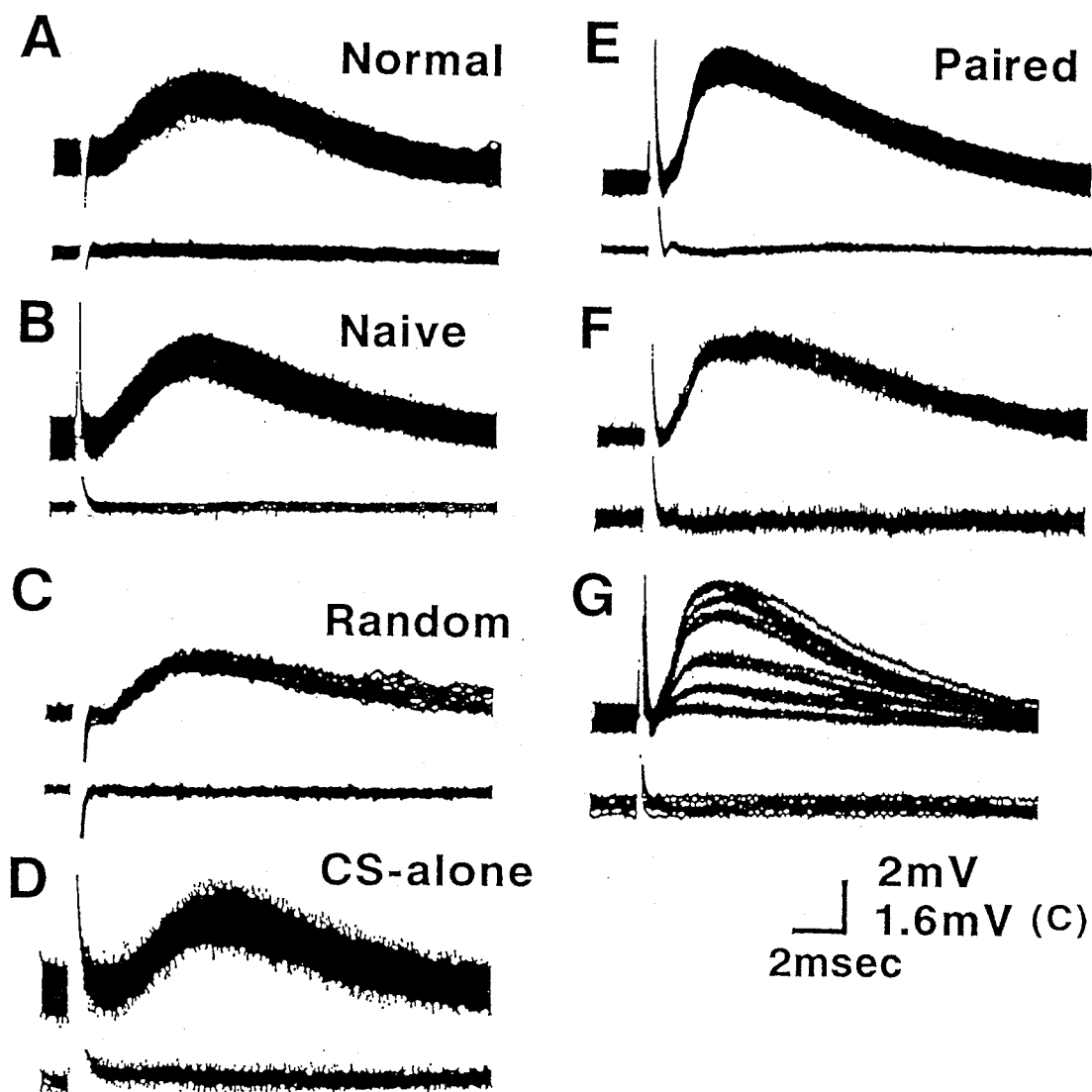
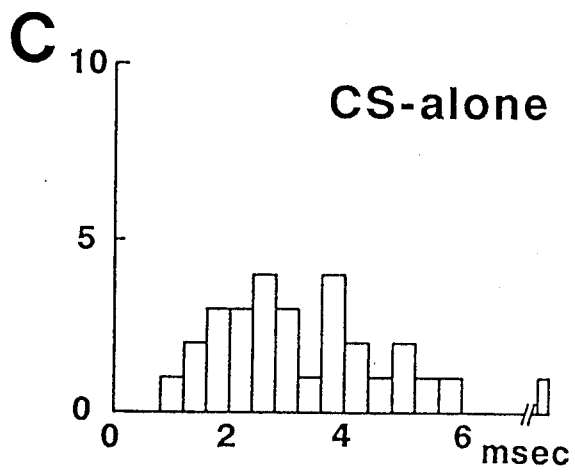
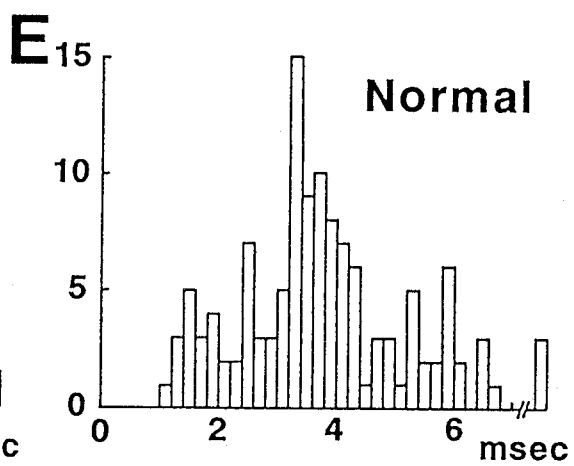
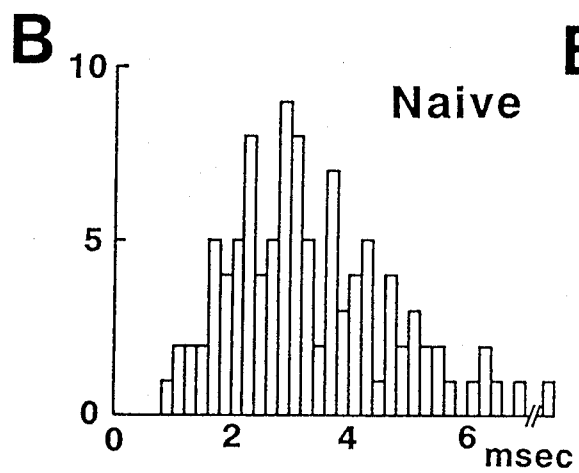
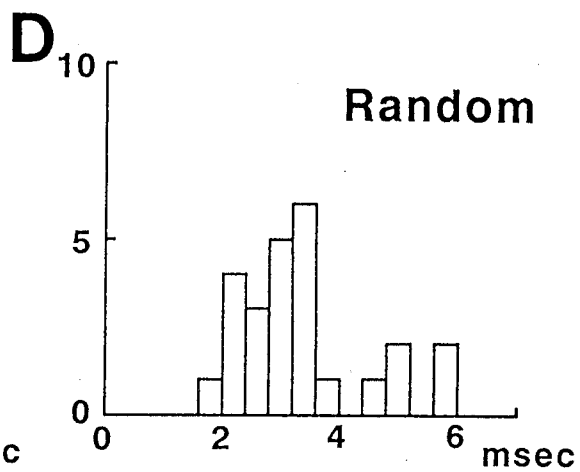
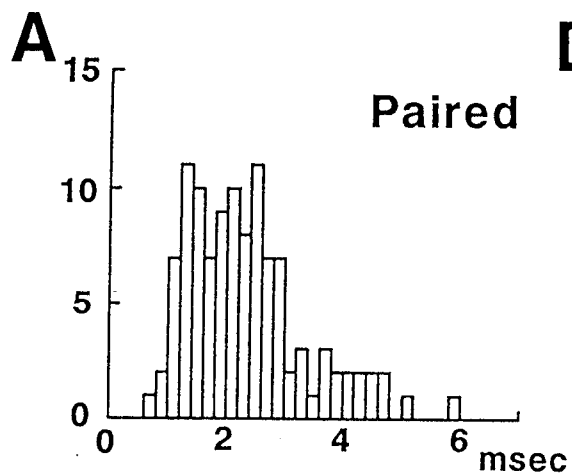


Fig. 5. Corticorubral EPSPs evoked by the CP stimulation (CP-EPSPs) in the five groups; (A) *normal*, (B) *naive*, (C) *random*, (D) *CS-alone* and (E-G) *paired*. Potentials with a faster time course in their rising phase were observed in the *paired* group as compared with other groups. F: Fast-rising potential followed by a second peak. Time to the latter peak resembled that of the CP-EPSPs in normal cats. G: Superposed potentials evoked by the CP stimulation with various intensities. The fast-rising potentials changed gradually in amplitude, but kept a similar time course for the various stimulus intensities. Upper traces are intracellular potentials; lower traces are corresponding extracellular field potentials. Calibration is common for A-G. Voltage calibration is 1.6mV for C and 2mV for others.



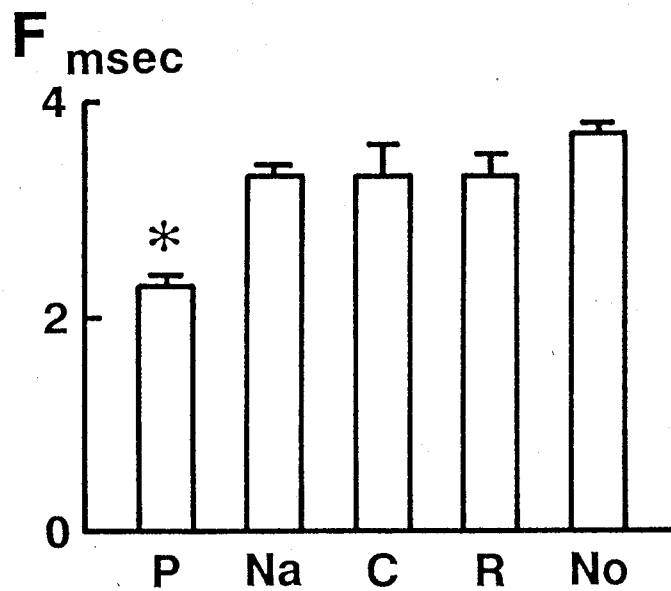
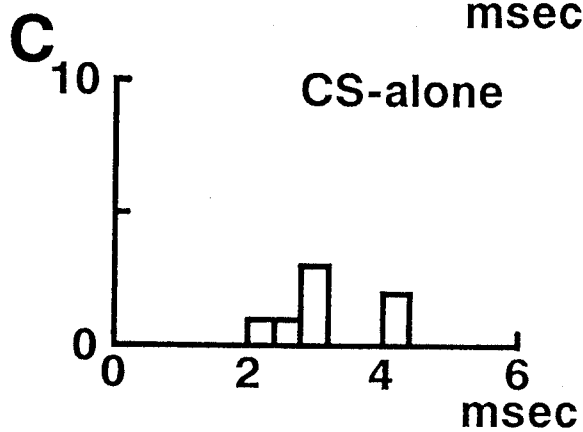
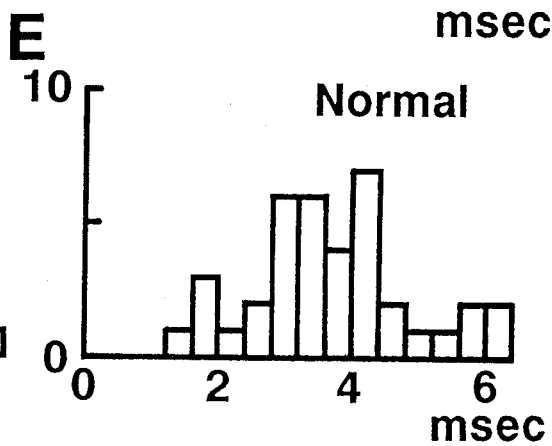
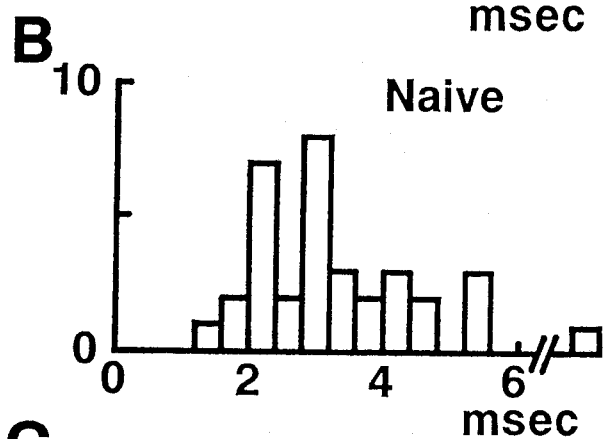
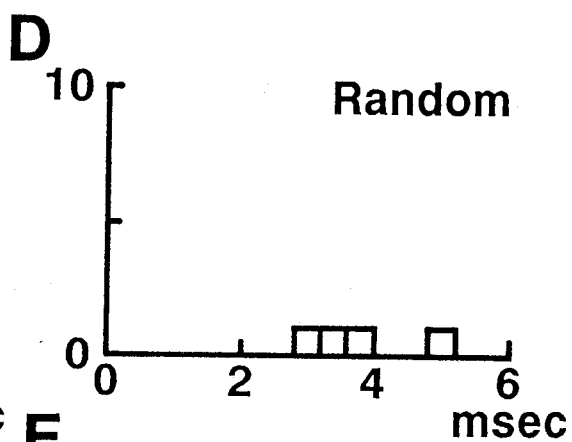
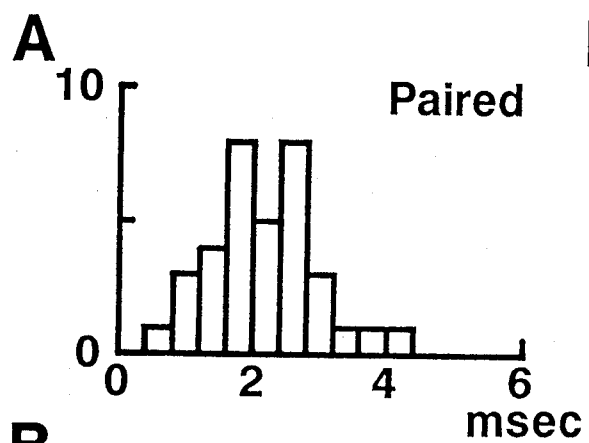


Fig. 6. Time to peak of the corticorubral EPSPs. A-E: Frequency distribution of times to the first peak of the CP-EPSPs in the five groups of cats; (A) *paired*, (B) *naive*, (C) *CS-alone*, (D) *random* and (E) *normal*. The number of cells is shown on the ordinate, the time to peak on the abscissa. The CP-EPSPs with short time to peak, for instance, less than 2msec, were often observed in the *paired* group. F: Summarized data representing the mean ( $\pm$ S.E.M.) time to peak of the CP-EPSPs in the five groups of animals; *paired* (P), *naive* (Na), *CS-alone* (C), *random* (R) and *normal* (No) groups. \*  $p < 0.001$  (ANOVA).



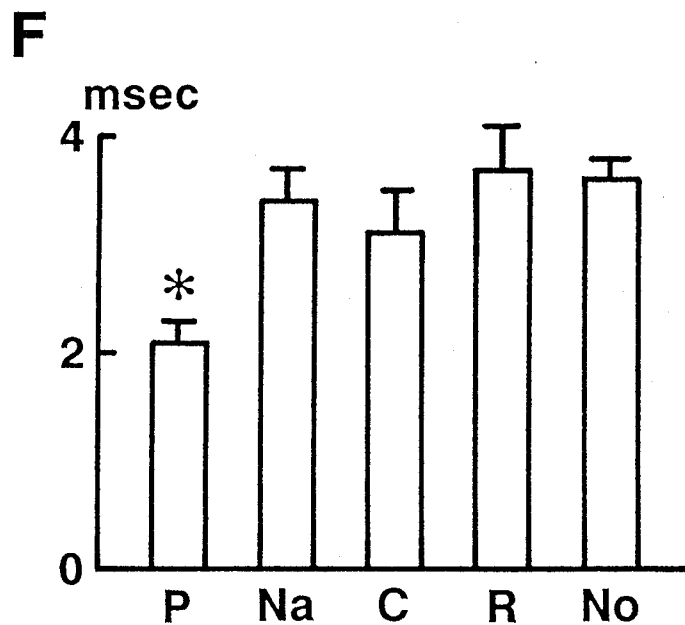


Fig. 7. Time to peak of the corticorubral EPSPs on an animal basis. Mean time to peak was calculated in each cells. A-E: Frequency distribution of times to the first peak of the CP-EPSPs in the five groups of cats; (A) *paired*, (B) *naive*, (C) *CS-alone*, (D) *random* and (E) *normal*. The number of cells is shown on the ordinate, the time to peak on the abscissa. The results were quite similar to those shown in figure 6. F: Summarized data representing the mean ( $\pm$ S.E.M.) time to peak of the CP-EPSPs in the five groups of animals; *paired* (P), *naive* (Na), *CS-alone* (C), *random* (R) and *normal* (No) groups. \*  $p < 0.001$  (ANOVA).

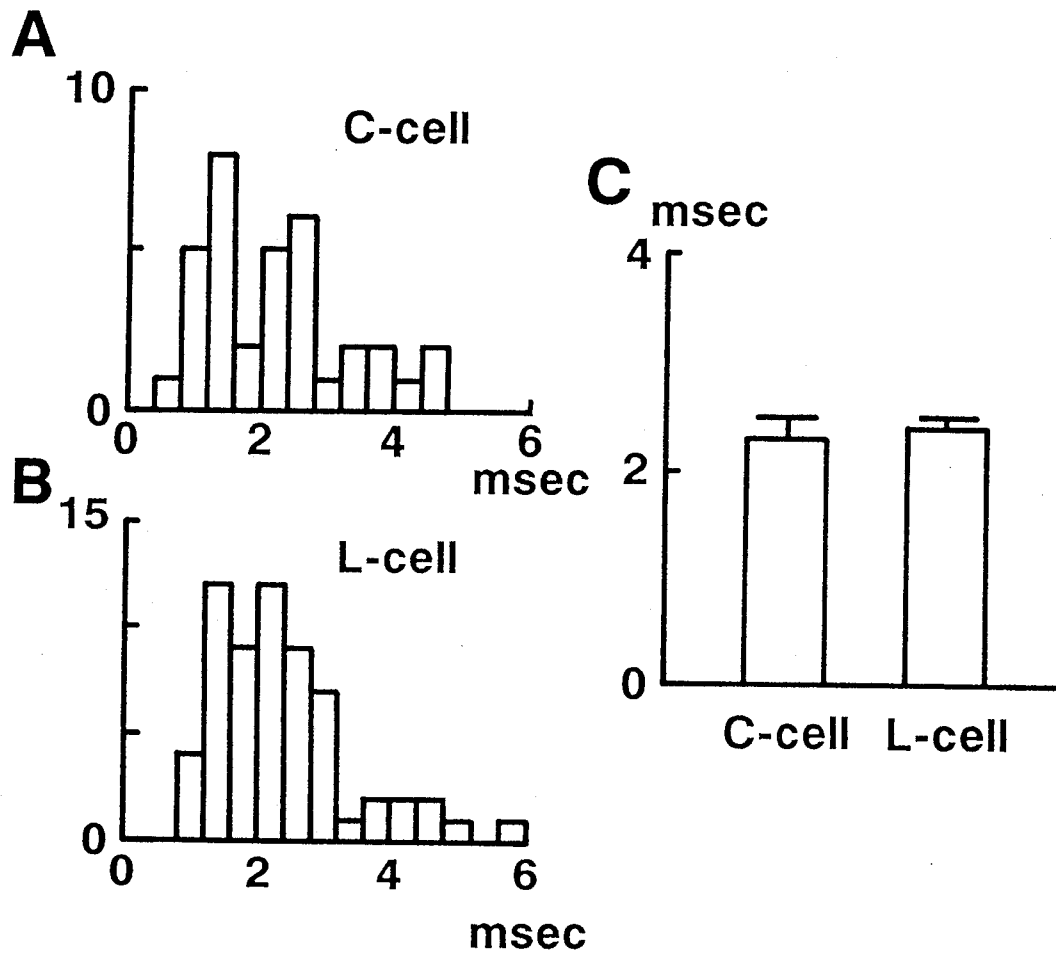


Fig. 8. Time to peak of the CP-EPSPs in the C- and L-cells. A,B: Frequency distribution of times to peak of the CP-EPSPs in the C-cells (A) and the L-cells (B) in the *paired* group. C: Summarized data representing the mean ( $\pm$ S.E.M.) time to peak of the CP-EPSPs.

## DISCUSSION

The present study has shown that the time to peak of the corticorubral EPSPs was shortened in associative manner and in parallel with acquisition of the conditioned forelimb flexion in the cats. The present results excluded non-associative contribution of the CS and/or the US. The results obtained here were consistent with previous studies which showed that the unit activity of the RN neurons in response to the CS enhanced in the *paired* group, not in other groups of *naive*, *CS-alone*, *random* (Oda et al. 1981, 1988). This is consistent with results obtained from cat spinal motoneuron which showed that the slope of the rising phase of the EPSPs is correlated with the frequency of spike discharge induced by the EPSPs (Fetz and Gustafsson 1983; see discussion in Oda et al. 1988). Our results infer that the appearance of the fast-rising corticorubral EPSP contributes to the increased firing frequency of RN unit discharge in response to the CS after the paired conditioning. Since the RN unit activity precedes the onset of electromyogram (EMG) activity of target muscles, the RN neurons are considered to be involved in the initiation of the motor response in the cat (Amalric et al. 1983; Martin and Ghez 1988, 1991) and the monkey (Kohlerman et al. 1982; Gibson et al. 1985; Kennedy 1987; Cheny et al. 1988). We also observed that onset of discharge in each RN neuron preceded the onset of EMG activity in forelimb muscles by 6.5-8.0ms by using the spike-triggered averaging method (Oda et al., unpublished observation). This time interval is consistent with that required for impulse conduction along the rubrospinal pathway to the muscles (8ms, see in Tsukahara et al. 1981). Therefore, the change in the waveform of corticorubral EPSPs possibly results in the increased firing probability of RN neurons and then induces forelimb muscle contraction.

In the extinction experiment (Tsukahara et al. 1981), repeated application of the CS alone reduced performance of the acquired forelimb flexion. The unit

activity in response to the CS rather decreased in the *CS-alone* group (Oda et al. 1988), while no significant change was found as for the time course of the corticorubral EPSPs (Fig.5-7). The decreased responsiveness of RN units could be due to another factor, for example, a decrease in the size of the corticorubral EPSP, which however was not tested in the present study.

Although the time to peak of the CP-EPSP did not show a significant difference among the control groups, a few RN neurons exhibited fast-rising CP-EPSPs in the *naive*, *CS-alone* and *random* groups (Fig.5-7, 11). It is known that several weeks after bilateral pyramidotomy, a monkey may exhibit a surprisingly high degree of coordinated voluntary control of the hand and forelimb muscle. These recovered control was severed by additional section of the rubrospinal pathway. The rubrospinal tract is thought to be crucial for the maintenance and/or recovery of forelimb control after the bilateral lesions of the corticospinal tract (Lawrence and Kuypers 1968; Kuypers 1981). Elimination of the corticofugal fibers induced interruption of voluntary control through the corticospinal pathway as well as retrograde denervation of corticorubral synapses from collateral of the corticospinal fibers. Formation of the corticorubral synapses could be partly induced by elimination of corticofugal fibers alone or associated with compensation of the behavioral deficits caused by the lesions. Even if this were the case, the effect of the paired conditioning on the waveform of corticorubral EPSPs was apparently larger than that of surgical operations alone.

#### Pathway for the US information

For the classical conditioning of the forelimb flexion, most effective interstimulus interval was 100ms and the conditioned response was not acquired with interstimulus interval of 30 or 400msec (Tsukahara et al. 1981). This restricted time window of interstimulus interval indicates that there must be an associative interference of CS information and US information at the neuronal level. In order to



examine specific molecular mechanisms for classical conditioning, it is necessary to identify the separate source of critical CS and the US information and the site of convergence which must be critical for storing information. In the invertebrate *Aplysia* (Walters and Byrne 1983; Hawkins et al. 1983), a CS-specific pathway is contacted by a second pathway conveying the US information. The CS pathway can be potentiated if the US pathway is stimulated after activation of the CS pathway. This principle may be used by vertebrate nervous systems, however, in more complex forms. In the classical conditioning of the forelimb flexion, although the CS pathway was restricted mainly to the corticorubrospinal pathway in the present study, pathway for the US information from the forelimb skin to the RN remains unknown. One candidate is a peripheral somatosensory input from skin to the RN neurons mediated by the ascending spinorubral projections (Jeneskog and Padel 1984; Padel et al. 1988) or transcerebellar somatosensory inputs. It has been reported that the RN neurons innervating the upper spinal segments (C-cells) are driven most briskly by peripheral input from the forelimb, whereas RN neurons innervating the lower spinal segments (L-cells) from the hindlimb (Nishioka and Nakahama 1973; Eccles et al. 1975; Vinay and Padel 1990). Hence, if these peripheral inputs mediate US information, the US applied to only forelimb skin would mainly affect the C-cells and the fast-rising EPSPs would appear mainly in the C-cells. However, this was not the case. Pairing the CS with the US resulted in changes in the waveform of the EPSPs in both L- and C-cells. Therefore, pathways which is irrelevant to the somatotopy and which send some modulatory information such as noxiousness, may be involved. One of the candidates is the nucleus locus coeruleus which is the main source of central noradrenergic projections and is activated by skin shock. Actually, it has been demonstrated that conditioned forelimb flexion is acquired by pairing the CP stimulation with electrical stimulation of the nucleus locus coeruleus (Oda et al. 1987). Thus, the nucleus locus coeruleus might be involved in the US pathways

## CHAPTER 3.

### Electrophysiological studies for the cellular mechanisms of the appearance of the fast-rising EPSPs

## INTRODUCTION

The corticorubral system in the cat provides us with a unique system in which to study changes in the distribution of synapses along the soma-dendritic membrane of the RN neurons. The corticorubral synapses are localized distally on dendrites (Brown 1974; King et al. 1972; Murakami et al. 1982). Due to the cable properties of the dendrites of RN neurons, corticorubral excitatory postsynaptic potentials (EPSPs) recorded in the somata have a slow-rising phase (Tsukahara and Kosaka 1968; Tsukahara et al. 1975b). Appearance of fast-rising EPSPs after elimination of input from the nucleus interpositus or after the cross-union of the peripheral nerves suggested that new corticorubral synapses has been formed on the somata or the proximal dendrites of the RN neurons (Tsukahara et al. 1975a, 1982; Fujito et al. 1982). This suggestion was supported by morphological examination of the distribution of corticorubral synapses on the soma-dendritic membrane of the RN neurons (Murakami et al. 1982, 1984).

As mentioned in the previous chapter, the CP stimulation induced fast-rising potentials after the classical conditioning of the forelimb flexion. The waveform of the fast-rising potentials resembled that of the fast-rising corticorubral EPSPs observed after the cross-union or lesion of the nucleus interpositus (Tsukahara et al. 1975, 1982). Therefore, the results were ascribed to formation of new corticorubral synapses on the somata or the proximal dendrites of the RN neurons (Tsukahara and Oda 1981; Oda et al. 1988). In order to confirm this view, we studied several electrophysiological properties of the corticorubral EPSPs. We characterized the newly appearing fast-rising potentials to show that they are

EPSPs induced by monosynaptic inputs from the sensorimotor cortex. The cable properties of the RN neurons were also investigated to test whether changes in the time course of the corticorubral EPSPs were attributed to modification of postsynaptic membrane properties.

## MATERIALS AND METHODS

Materials and all experimental procedures were as same as described in the previous chapter.

To examine the passive electrical membrane properties of the RN neurons, a step current was injected through the recording electrode (resistances 5-8 M $\Omega$ ). Bridge balance was carefully adjusted to minimize artifacts due to the electrode resistance. Current intensity of less than 1nA was used to avoid contamination of voltage dependent conductance. Potentials were digitized every 50 $\mu$ sec and stored by a microcomputer for further analysis.

## Results

### Physiological properties of the fast-rising corticorubral EPSPs which appeared after the classical conditioning

The fast-rising EPSPs had latencies within the monosynaptic range of the corticorubral EPSPs observed in normal animals (Fig.9) (ANOVA test,  $F=1.25$ ,  $p>0.25$ ). The mean latencies of the CP-EPSPs were  $0.88\pm0.02\text{msec}$  (*paired*,  $n=102$ ),  $0.93\pm0.02\text{msec}$  (*naive*,  $n=93$ ),  $0.88\pm0.04\text{msec}$  (*CS-alone*,  $n=29$ ),  $0.87\pm0.05\text{msec}$  (*random*,  $n=17$ ) and  $0.90\pm0.02\text{msec}$  (*normal*,  $n=109$ ). There was no correlation between the latency and the time to peak of the CP-EPSPs ( $\gamma=0.014$ ,  $t=0.14$ ,  $p>0.20$ ). Furthermore, the time course of the fast-rising CP-EPSPs did not change over various stimulus frequencies ranging from 2 to 100Hz, as shown in Fig.10. Therefore, the newly appearing fast-rising EPSP is likely to be monosynaptically evoked through the corticorubral synapses and not contaminated with polysynaptic responses.

The dual-peaked EPSPs were often observed in the *paired* group (42 of 111 neurons, 38%), while it was rarely seen in the *naive* (15 of 104 neurons, 14%), *CS-alone* (2 of 29 neurons, 7%), *random* (3 of 25 neurons, 12%) and *normal* (none of 109 neurons) groups (Fig.11A). The second peak was rather broad, which looks like a bump or a bulge appearing on the falling phase of the first EPSPs as shown in Fig.5E. It should be noted that ratio of the dual-peaked EPSPs may be underestimated especially in the *paired* group, because we excluded broad bump following the early summit in which peak time was not measurable. Since the late component was not eliminated by increasing stimulation frequency up to 70Hz, it is likely that the late component was also a monosynaptic potential. The times to the first and second peak from onset of the dual-peaked EPSPs in the *paired* group were  $1.7\pm0.09\text{msec}$  and  $3.6\pm0.17\text{msec}$  on average ( $n=42$ ), respectively. The latter

value corresponded to the time to peak of the CP-EPSPs in the normal group. Hence, it is likely that the shortening of rise time is due to the appearance of an additional fast-rising EPSP which is superposed on the slow-rising EPSPs.

It is known that the corticorubral EPSP shows frequency facilitation in amplitude following double shock stimulation in normal animals (Murakami et al. 1977). Similar property was observed in the fast-rising corticorubral EPSPs in the *paired* group. As shown in Fig.12A-C, the amplitude of the second EPSP was larger than that of the preceding one, but no difference in onset latency or the peak time was found. Figure 9D illustrates the time course of facilitation studied in the fast-rising EPSPs in the *paired* group (n=13). The facilitation was observed with the interval ranging from 2 to 10ms. The time course resembled that found in normal animals (Murakami et al. 1977).

We next investigated whether the newly appearing fast-rising EPSPs originated from the sensorimotor cortex (SM) as in the case of the slow-rising EPSPs observed in normal animals. In the *paired* group, stimulation of the SM also induced fast-rising EPSPs (SM-EPSPs) similar to the CP-EPSPs (Fig.13A,B). The mean latency from onset of the SM stimulation was  $1.7(\pm 0.08)$ msec, ranging from 1.0 to 3.2msec (n=55). The SM-EPSPs were much depressed when they were paired with preceding CP-EPSPs at short intervals, as shown in Fig.13. The interaction between the CP-and SM-EPSPs at these short intervals is explained by blockade of their presynaptic impulses due to collision or refractoriness along the same line of fibers which connect the SM and the RN through the CP. Figure 13D shows a facilitation in amplitude which may be a similar phenomenon to that induced by the double CP-stimulation as mentioned above. Therefore, the newly appearing fast-rising EPSPs originate, at least in part, from the sensorimotor cortex.

The fast-rising corticorubral EPSPs were also observed in the unitary EPSPs. As shown in Fig.14A-E, during threshold SM stimulation, potentials appeared in an all-or-none fashion. An EPSP appearing in this fashion is considered to be

activated by a single corticorubral fiber projecting onto the recorded RN neuron and is referred to as a unitary EPSP. The time to peak of the unitary EPSP was measured from more than 30 consecutive traces. That of the neuron shown in Fig.14A-E was 0.9msec and slightly shorter than that of the CP-EPSP (1.5msec) which was induced with a larger stimulus intensity. The unitary EPSPs showed similar or slightly shorter time to peak than that of the compound EPSPs in the same neuron (Fig.15A), presumably because of temporal dispersion of arrival time of each unitary component included in the compound EPSP (Tsukahara and Kosaka 1968). In the *naive* group, however, slow-rising unitary EPSPs were observed as exemplified in Fig.14F-I. As shown in Fig.15, the mean time to peak of the unitary corticorubral EPSPs in the *paired* group was  $1.6 \pm 0.14$ msec ( $n=20$ ) which was shorter than that in the *naive* group ( $2.5 \pm 0.19$ msec,  $n=21$ ) (t-test,  $t=3.79$ ,  $p<0.001$ ). Therefore, the result further supports the view that the appearance of the fast-rising EPSP is due to a change in the rising time course of individual unitary components and not due to induction of a regenerative potential.

It is known that after lesion of the nucleus interpositus or after the cross-union of peripheral nerves the newly formed corticorubral projections are arranged in a somatotopical manner similar to normal projections; from the forelimb SM area through dorsomedial part of the RN to the upper spinal segments, and from the hindlimb SM area through the ventrolateral part of the RN to the lower spinal segments (Tsukahara and Kosaka 1968; Padel et al. 1972, 1973; Tsukahara et al. 1975a, 1982). It was of interest to see whether the somatotopical organization was preserved after classical conditioning. As shown in Fig.16, stimulation of the medial part of the SM, but not of the lateral part, evoked fast-rising EPSPs with large amplitude in L-cells. By contrast, in C-cells, the corticorubral EPSPs were produced predominantly from the lateral part of the SM. We observed such a somatotopical organization in 45 out of 50 neurons (90%) in the *paired* group. Therefore, it is concluded that there is still a tendency toward somatotopy.

### No difference in the cable properties of the red nucleus neurons after the classical conditioning

The time course of EPSPs could be altered as a consequence of a change in dendritic cable properties as well as a change in location of synapses along the soma-dendritic membrane (Rall 1969). However, frequent occurrence of dual-peaked EPSPs implies that the former possibility is unlikely. To confirm this view, we examined whether cable properties of the RN neurons are different between the *paired* group and other control groups. We measured transient voltage responses to intracellularly injected step currents and estimated the electrotonic length, which is a useful parameter for comparing the cable properties of the RN neurons (Tsukahara et al. 1975b). We chose those RN neurons which maintained stable membrane potentials and with spike amplitudes exceeding 60mV for further study. The transient voltage response was approximated by the sum of exponential curves with different time constants. Figure 17A illustrates a transient response in a cell recorded in an animal of the *paired* group. The transient response, except within 5msec of the onset of current injection, could be fitted by an exponential curve with a time constant of 8.0msec ( $\tau_0$ ), which was consistent with that of the falling phase of the CP-EPSPs recorded in the same neuron (8.0msec). Then the difference between the transient response and the fitting curve was again fitted by an exponential curve with a second time constant of 1.5msec ( $\tau_1$ ). The input resistance of this neuron was 7.8M $\Omega$ . As shown in Fig.17C,D, time constants did not differ among the five groups (ANOVA test,  $F=0.79$ ,  $p>0.25$  ( $\tau_0$ ) and  $F=1.65$ ,  $p>0.10$  ( $\tau_1$ )). The mean and S.E.M. of  $\tau_0$  were  $6.8\pm0.38$ msec (*paired*,  $n=20$ ),  $7.3\pm0.56$ msec (*naive*,  $n=20$ ),  $7.4\pm0.26$ msec (*CS-alone*,  $n=5$ ),  $5.9\pm0.59$ msec (*random*,  $n=5$ ) and  $6.1\pm0.66$ msec (*normal*,  $n=9$ ). Those of  $\tau_1$  were  $1.1\pm0.12$ msec (*paired*,  $n=20$ ),  $1.4\pm0.22$ msec (*naive*,  $n=20$ ),  $1.4\pm0.20$ msec (*CS-alone*,  $n=5$ ),  $1.3\pm0.10$ msec (*random*,  $n=5$ ) and  $0.9\pm0.12$ msec (*normal*,  $n=9$ ). The mean input



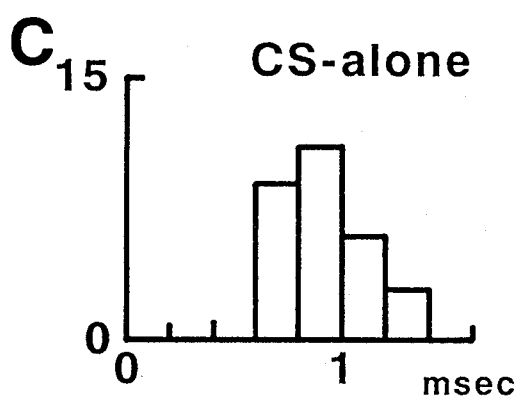
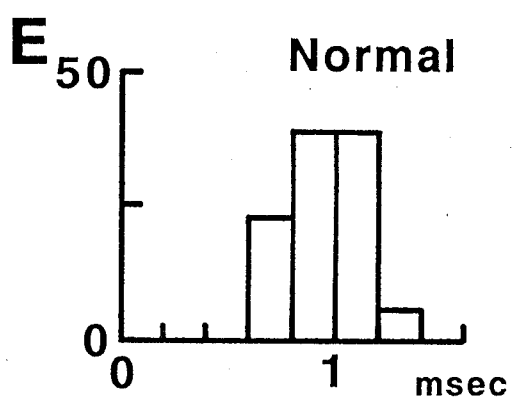
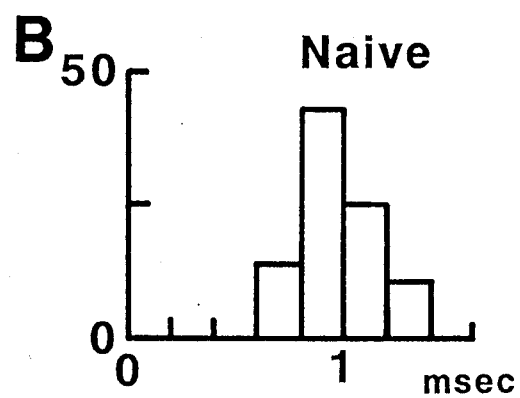
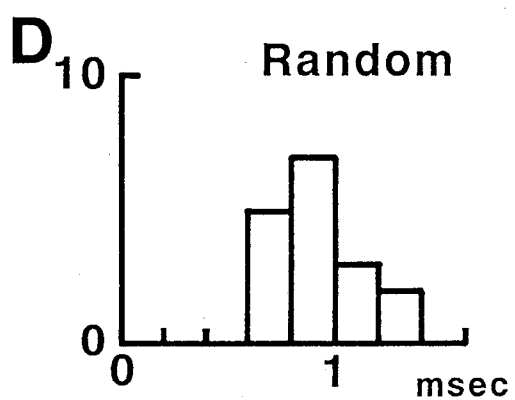
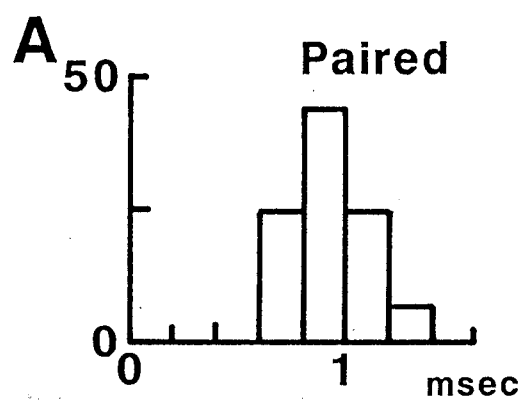
resistance of the RN neurons did not differ among the five groups (ANOVA test,  $F=0.62$ ,  $p>0.25$ ):  $4.8\pm0.54\text{M}\Omega$  (*paired*,  $n=20$ ),  $4.4\pm0.48\text{M}\Omega$  (*naive*,  $n=20$ ),  $3.3\pm0.46\text{M}\Omega$  (*CS-alone*,  $n=5$ ),  $4.7\pm1.87\text{M}\Omega$  (*random*,  $n=5$ ) and  $4.3\pm0.54\text{M}\Omega$  (*normal*,  $n=9$ ) (Fig.17E). From the ratio of  $\tau_0$  to  $\tau_1$ , the electrotonic length (L) was estimated by the following equation based on the equivalent cylindrical model (Rall 1969):

$$L = \frac{\pi}{\sqrt{(\tau_0/\tau_1) - 1}}.$$

The electrotonic length of the neuron shown in Fig.17A was 1.5. The mean values of each group were  $1.3\pm0.10$  (*paired*,  $n=20$ ),  $1.5\pm0.09$  (*naive*,  $n=20$ ),  $1.5\pm0.11$  (*CS-alone*,  $n=5$ ),  $1.7\pm0.11$  (*random*,  $n=5$ ) and  $1.3\pm0.12$  (*normal*,  $n=9$ ) (Fig.17F).

There was neither intergroup difference (ANOVA test,  $F=0.61$ ,  $p>0.25$ ) nor correlation between the electrotonic length and the time to peak of the CP-EPSPs in the *paired* group ( $\gamma=0.154$ ,  $t=0.60$ ,  $p>0.20$ ). These results show that the cable properties of the RN neurons are not altered by the conditioning procedure.

Therefore, the appearance of the fast-rising EPSP is likely to be due to a change in location of the corticorubral synapses on the soma-dendritic membrane of the RN neurons, rather than to a change in the cable properties of them.



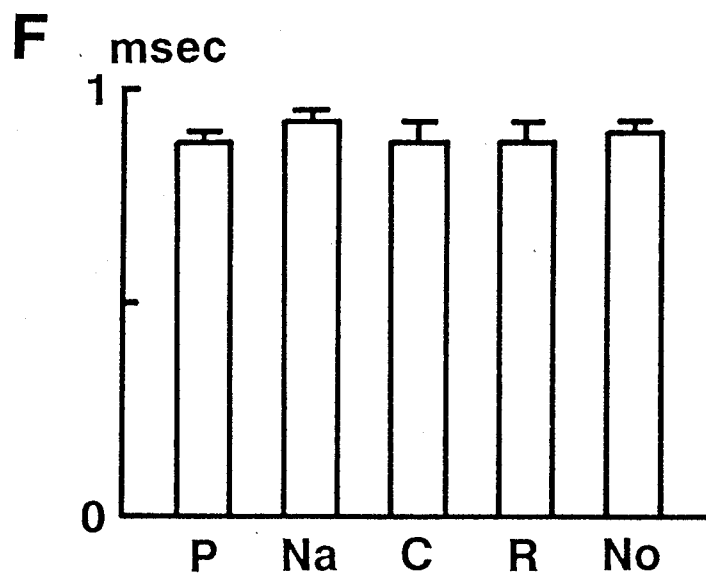


Fig. 9. Latency of the corticorubral EPSPs. A-E: Frequency distribution of the latencies of the CP-EPSPs in the five groups of cats; (A) *paired*, (B) *naive*, (C) *CS-alone*, (D) *random* and (E) *normal*. The number of cells is shown on the ordinate, latency on the abscissa. F: Summarized data representing the mean ( $\pm$ S.E.M.) latencies of the CP-EPSPs in the five groups of animals; *paired* (P), *naive* (Na), *CS-alone* (C), *random* (R) and *normal* (No) groups. There was no difference among the five groups.

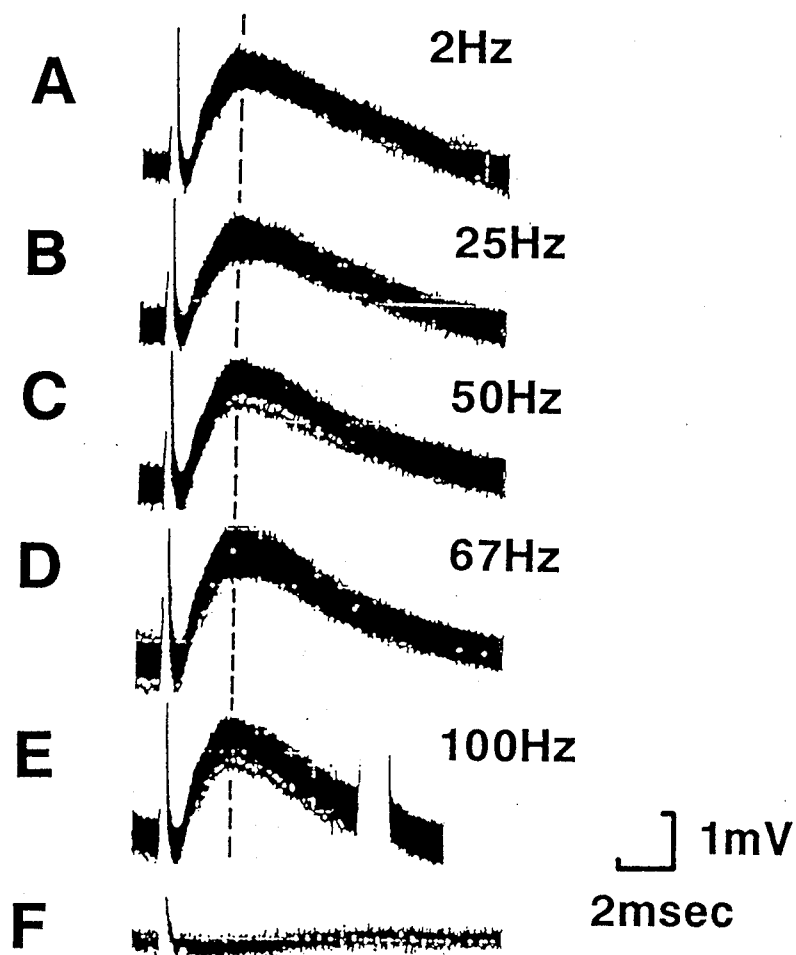


Fig. 10. The fast-rising corticorubral EPSPs followed high-frequency stimulations. A-E: The fast-rising CP-EPSPs maintained the same latency and peak time at various frequency of stimulation as denoted in a cat of the *paired* group. F: Corresponding extracellular potentials at the stimulation frequency of 2Hz. Calibration is common for A-F. Broken line shows peak time of the CP-EPSPs. The response to 100Hz stimulation (E) was interrupted by a ramp signal preceding the next stimulation.

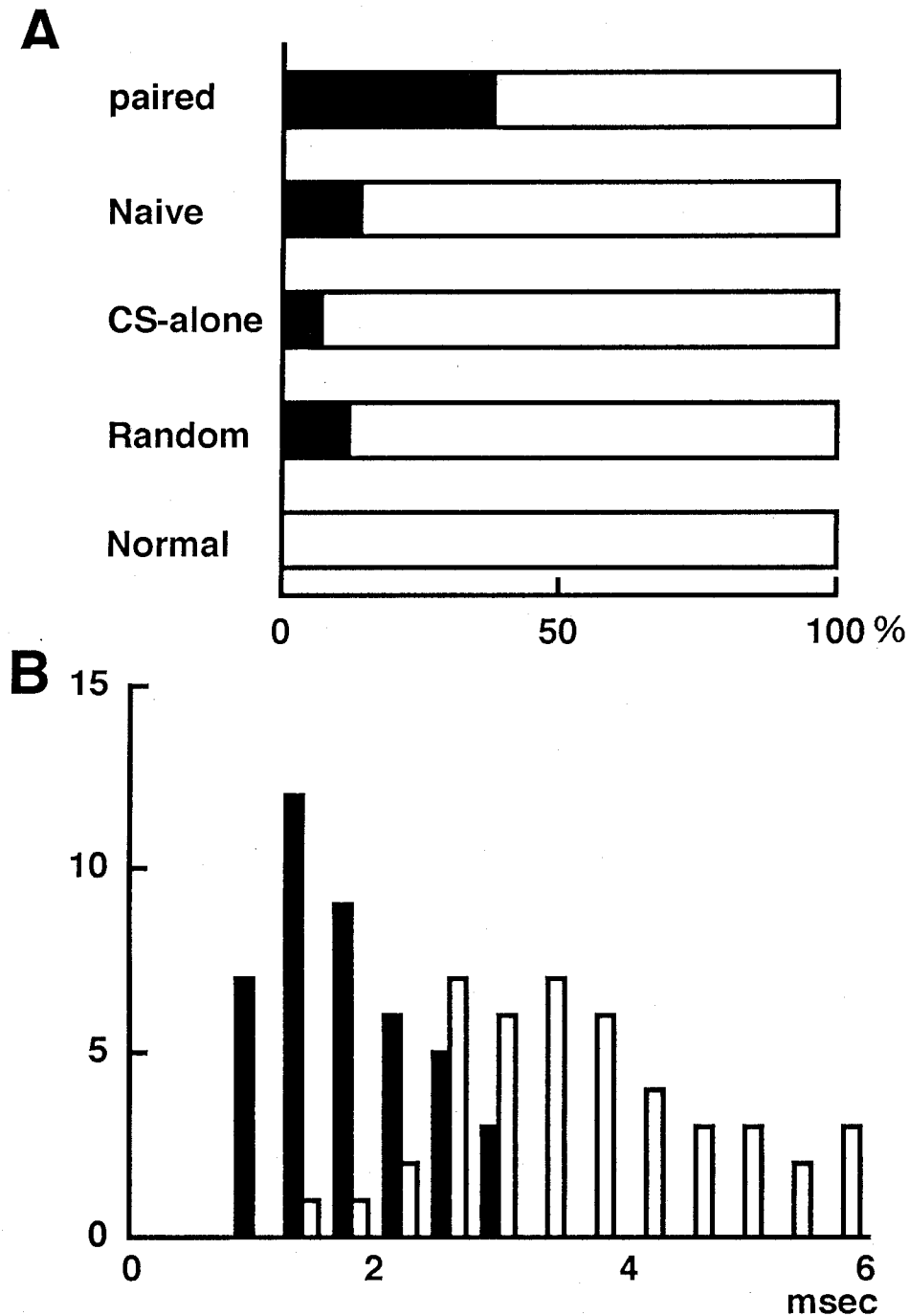


Fig. 11. Dual peaked EPSPs. A: Ratio of the dual peaked EPSPs (black area) observed in the five groups of animals; *paired*, *naive*, *CS-alone*, *random* and *normal*. The dual peaked EPSPs are frequently observed in the *paired* group. B: Frequency distribution of the times to the first (black column) and second peak (white column) of the CP-EPSPs in the *paired* group. Those of the second peak resembled the time to peak of the *normal* group.

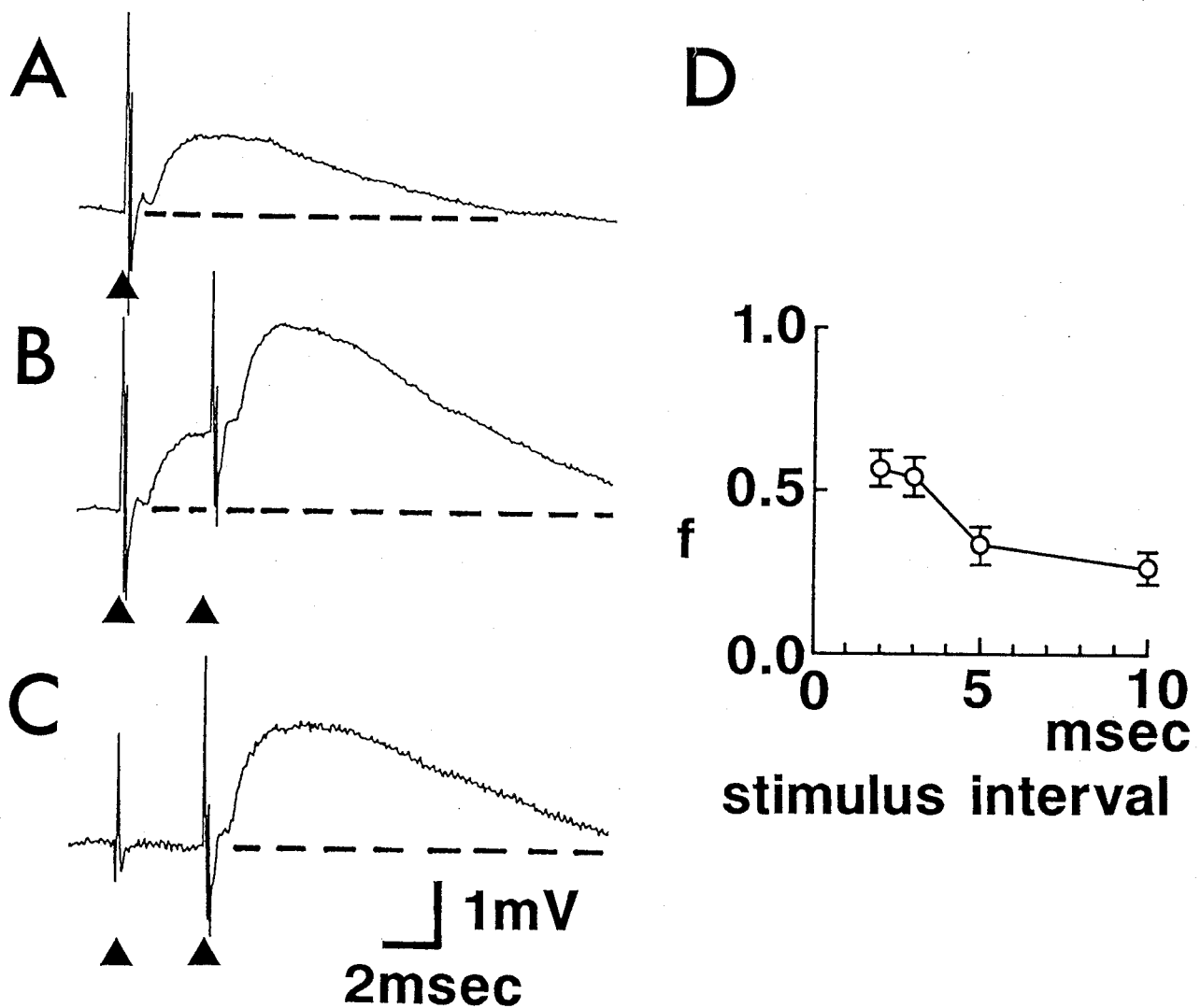


Fig. 12. Facilitation of the fast-rising corticorubral EPSPs. A,B: Averaged corticorubral EPSPs evoked by single (A) and double CP stimulation with an interval of 3 msec (B). C: The second EPSP is shown by subtraction of A and B. Calibration is common for A-C. Arrowheads indicate onset of stimuli. D: Mean time course of the facilitation in the *paired* group ( $n=13$ ). Ordinate: The degree of facilitation,  $f=(V_2-V_1)/V_1$ , where  $V_1$  and  $V_2$  are amplitude of the first and the second EPSP, respectively. Abscissa: stimulus interval.

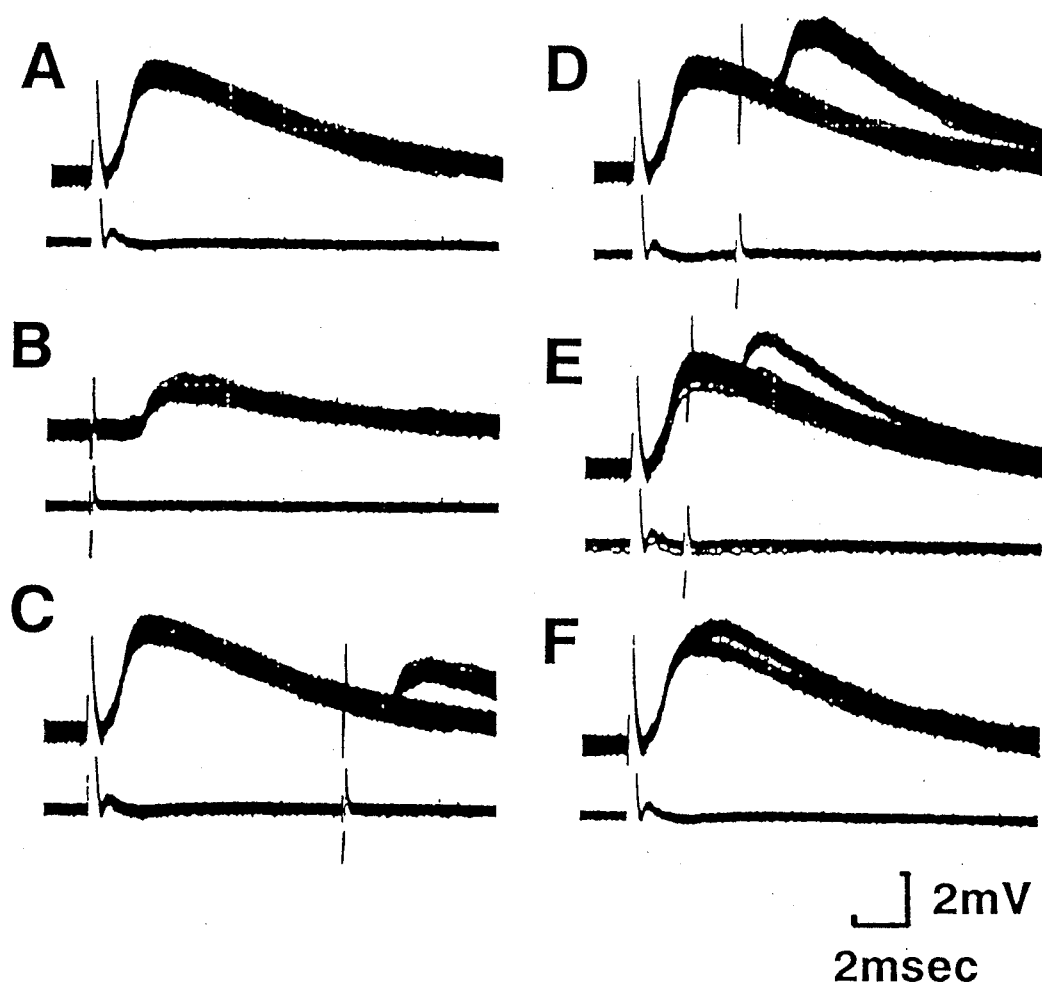


Fig. 13. Collision of the fast-rising corticorubral EPSPs induced by stimulation to the sensorimotor cortex and the cerebral peduncle in a cell of the *paired* group. A,B: Control EPSPs evoked by single CP (A) and SM (B) stimulation, respectively. Both represented the fast-rising time course. C-F: Records of collision experiments for the fast-rising corticorubral EPSPs. The SM stimuli were applied after preceding CP stimulation at various time intervals (10msec (C), 4msec (D), 2msec (E), 0msec (F)). Upper traces are intracellular potentials; lower traces are corresponding field potentials. Calibration is common for A-F.

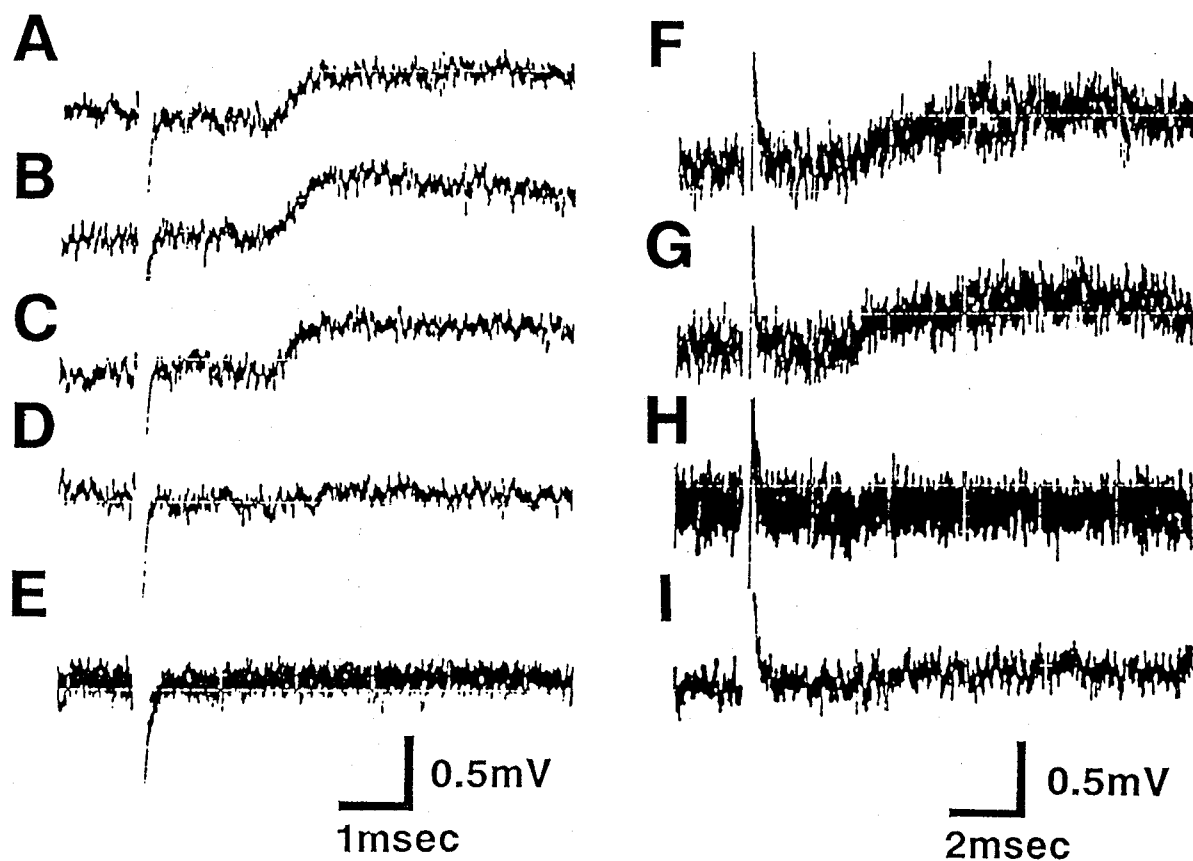


Fig. 14. Unitary corticorubral EPSPs. A-E: Threshold stimulation of the sensorimotor cortex induced unitary EPSPs (A-C) and a failure response (D) in the cell of the *paired* group, which is shown in Fig.13. Single traces in consecutive recordings are shown. F-I: Unitary corticorubral EPSPs (F,G) and failure responses (H) in a cell of the *naïve* group. Two traces were superimposed. E,I: Corresponding field potentials just outside of each neuron. Calibration in E is common for A-E and that in I for F-I.



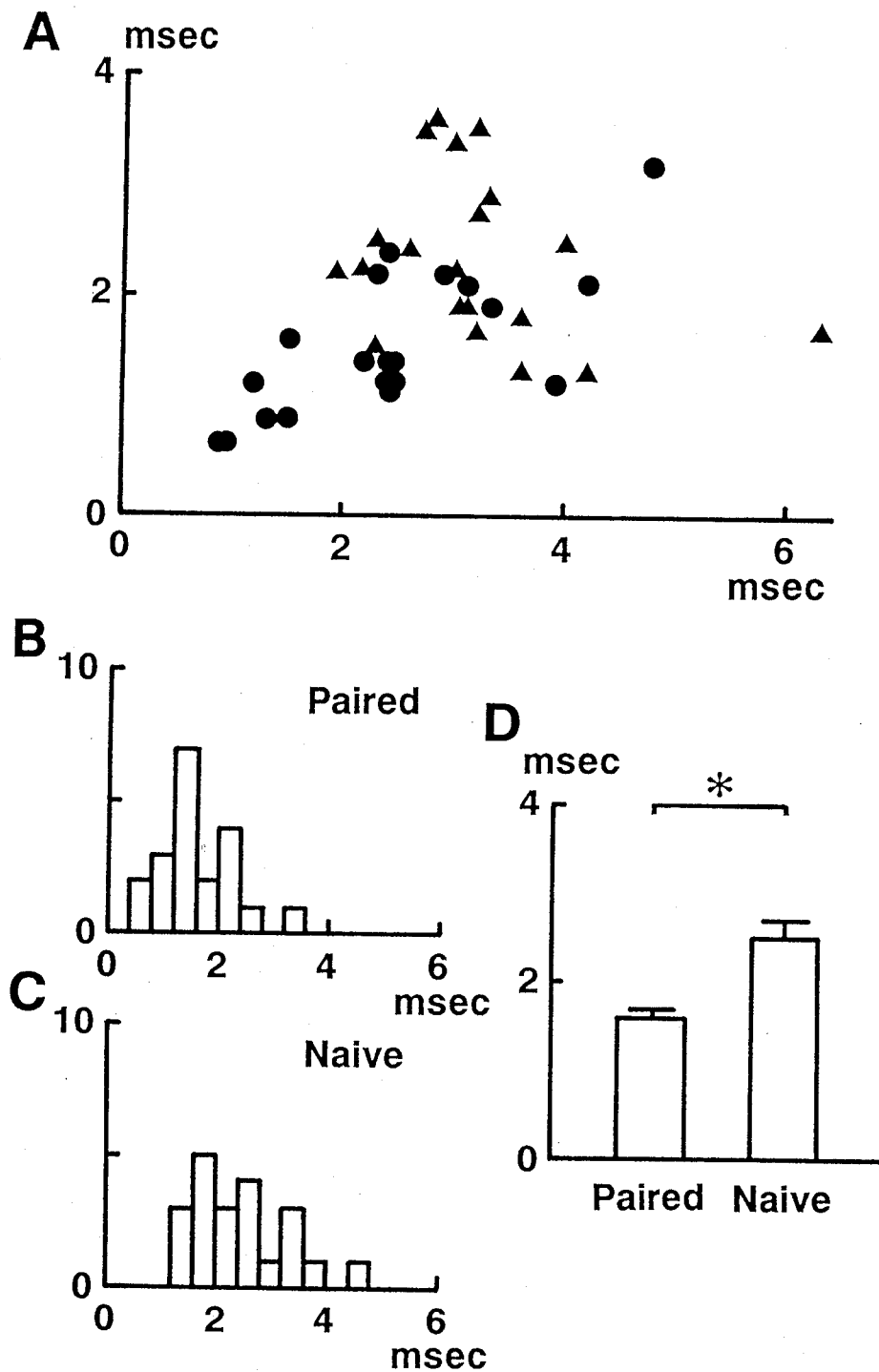


Fig. 15. Unitary corticorubral EPSPs. A: Relationship between the time to peak of the unitary EPSPs (ordinate) and that of the evoked EPSPs (abscissa). B-C: Frequency distribution of the times to peak of the unitary corticorubral EPSPs in the *paired* (B) and *naive* (C) groups. D: Each bar represents the mean ( $\pm$ S.E.M.) times to the unitary corticorubral EPSPs. \*  $p < 0.001$  (t-test).

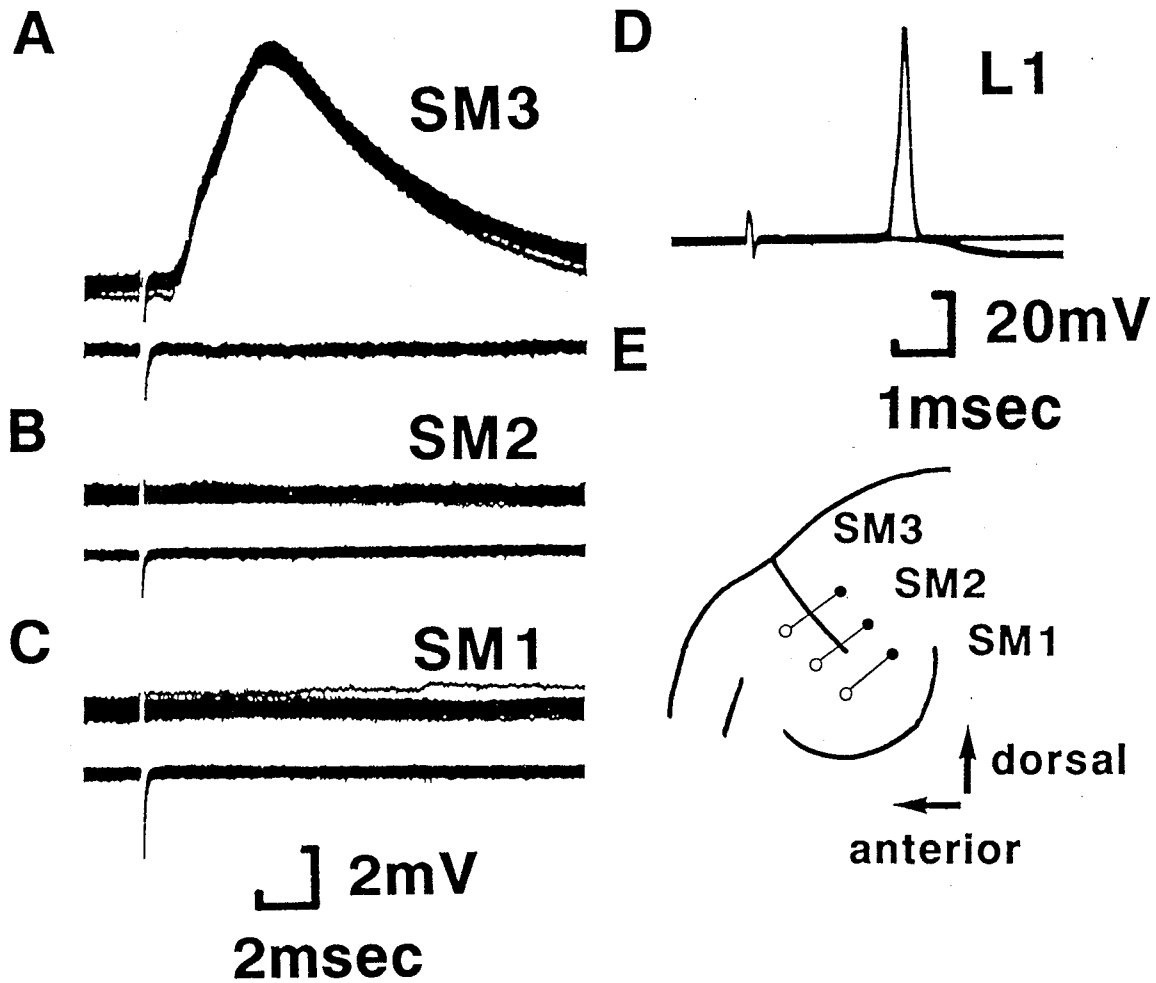


Fig. 16. Somatotopical organization of the origin of the fast-rising corticorubral EPSPs. A-C: EPSPs evoked by stimulation with the same intensity at three sites of the sensorimotor cortex as indicated in E. Calibration is common for A-C. D: Antidromic spike evoked by stimulation to the L<sub>1</sub> spinal cord indicated that the neuron was an L-cell. E: Lateral view of the left cruciate gyri showing positions of the stimulating electrodes (circles). Bipolar electrodes (SM1, SM2, SM3) were arranged on lateral to medial surface of the sensorimotor cortex. Lines between circles on both pre- and postcruciate gyri indicate pair of bipolar electrodes.

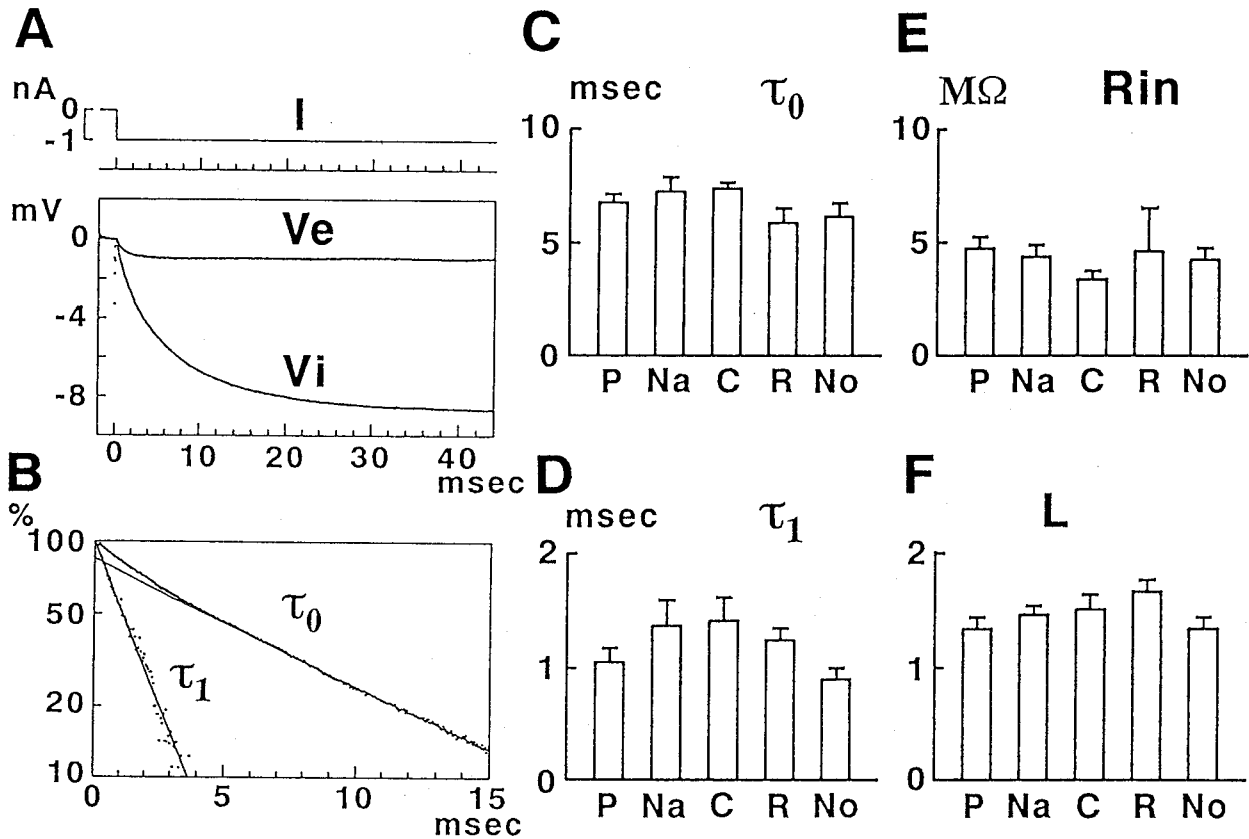


Fig. 17. Cable properties of RN neurons. A: The averaged membrane transient responses of an RN neuron in the *paired* group. They were induced by step current pulse (I) (duration, 50msec) applied through the recording electrode. The response was obtained by taking the difference between intracellular (Vi) and corresponding extracellular (Ve) potentials. They were fitted by the sum of two exponential curves with different time constants according to the Newton method approximation. B: Semilogarithmic plotting of the transient response shown in A and the fitting exponential curve ( $\tau_0$ ) with the largest time constant, 8.0msec. The difference between the membrane transient response and the fitting curve was also plotted. It was also fitted by the exponential curve ( $\tau_1$ ) with the second time constant, 1.5msec. C-F: Summarized data of time constants ( $\tau_0$ ,  $\tau_1$ ), input resistance ( $R_{in}$ ) and the electrotonic length (L). Each bar represents the mean $\pm$ S.E.M. There was no difference among the five groups of animals; *paired* (P), *naive* (Na), *CS-alone* (C), *random* (R) and *normal* (No) groups.

## DISCUSSION

### The cellular mechanism underlying the appearance of the fast-rising component

Since the present study has shown that the fast-rising component is a monosynaptic EPSP, a straightforward explanation for generating the fast-rising EPSP is sprouting from the intact corticorubral fibers and subsequent formation of new synaptic contacts with proximal dendrites or somata of RN neurons. Two alternative possibilities, however, should be considered: (1) reduction of the electrotonic length of dendrites (Rall1969), (2) shortening of time course of the corticorubral synaptic currents (Fig.18). The first possibility could occur if the dendrites shrunk or swelled or if the specific membrane resistance increased in the dendrites. However, we found no significant change in the electrotonic length or in the input resistance of RN neurons after the paired conditioning. In addition, changes in the electrotonic length could not account for the appearance of dual-peaked EPSPs. Therefore, the first possibility can be excluded. The second possibility could be due to a shortening in duration of synaptic conductance change or to the involvement of new synaptic currents having short duration. This possibility may be indicated from studies of hippocampal pyramidal cells in which two kinds of glutamate receptors, NMDA and non-NMDA receptors, co-localize and induce excitatory postsynaptic currents with different time courses (Bekkers and Stevens1989; Hestrin et al. 1990). We investigated how the time to peak of the EPSP depends on the time course of the synaptic conductance and location of the synapses, by simulating EPSPs (Fig.19). The model we used was based on the Rall's compartmental model (Rall 1964) which was demonstrated to be applicable to the RN neurons (Tsukahara et al.1975b; Sato and Tsukahara 1976). It consisted of 10 equal compartments. Synaptic conductance was applied to one of them. The

parameters were essentially the same as proposed previously (Tsukahara et al. 1975b). The waveform of an excitatory synaptic conductance change was represented by an alpha function (Jack and Redman 1971; Brown and Johnston 1983; Finkel and Redman 1983; Johnston and Brown 1983; Murakami et al. 1986). We used the peak time ( $1/\alpha$ ) of 350 $\mu$ sec obtained from a previous voltage clamp experiment (Murakami et al. 1986). The synaptic conductance applied to the most distal compartment (compartment 10) produced a slow-rising potential at the soma (compartment 1, Fig.19(c)). The time to peak of the calculated EPSP (3.6msec) was quite similar to that of the CP-EPSPs in normal cats in the present study and to that found previously (Tsukahara et al. 1975a). When synaptic conductance was applied to a proximal compartment (compartment 3 or 5), the time to peak of the EPSP was less than 2msec (Fig.19(a) and (b)). In contrast, even if conductance duration was shortened to about half of the control ( $1/\alpha=180\mu$ sec), time to peak of the EPSP decreased by only 0.5msec (Fig.19(d)). Figure 20 indicates relationship between time to peak of the calculated EPSPs and time course of synaptic transmission. Thus, the shortening of conductance duration cannot explain the presently observed fast-rising corticorubral EPSPs in the *paired* group. The simulation rather indicates that the time to peak was strongly affected by location of synapses and that a new corticorubral synapse is formed on the proximal half dendrite of the RN neuron.

Therefore, the electrophysiological evidence provided here supports the hypothesis that the fast-rising corticorubral EPSPs is due to the newly formed corticorubral synapses onto the proximal dendrites or somata of RN neurons.

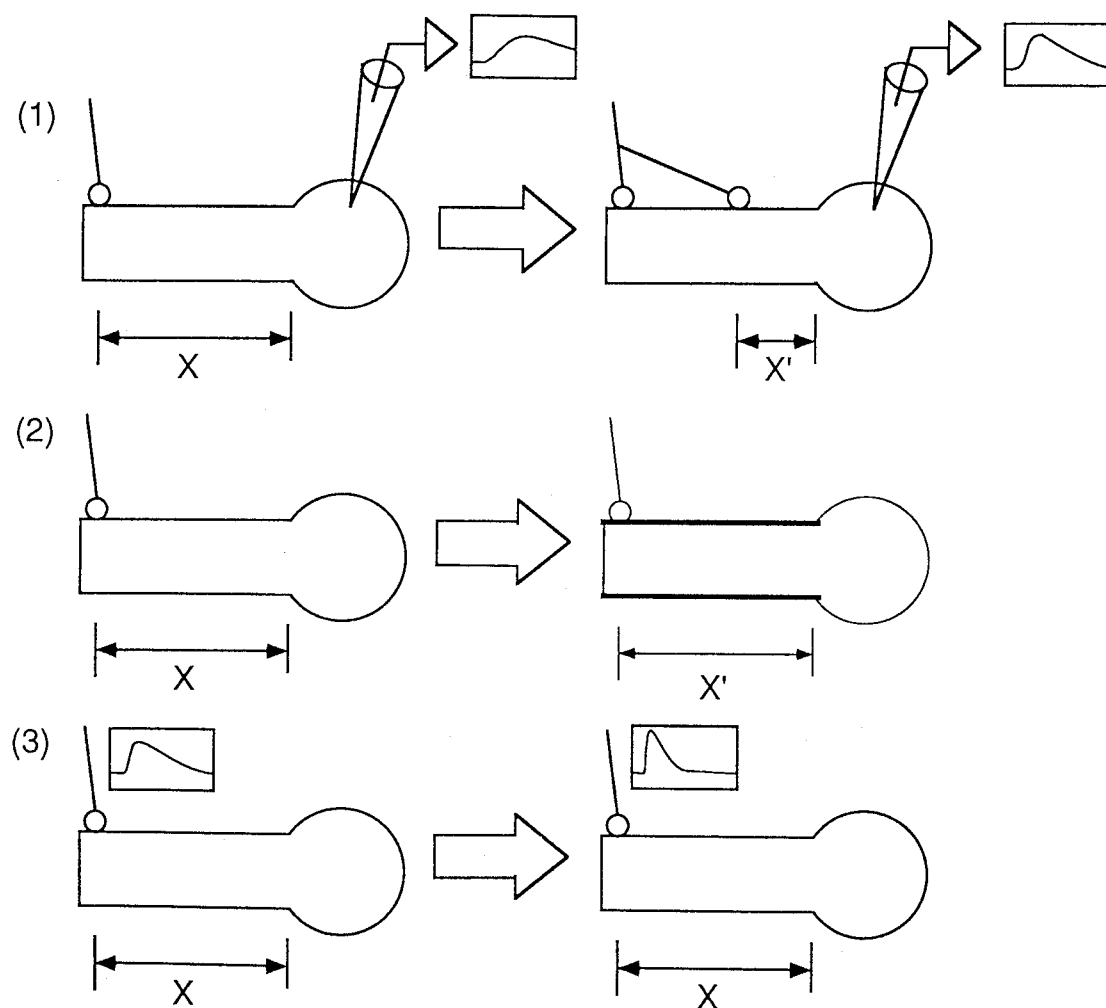


Fig. 18. Possible mechanisms for appearance of the fast-rising corticorubral EPSPs. (1) Sprouting of the corticorubral fibers and formation of new synapses on the proximal dendrites or somata of the RN neurons. Distance between the corticorubral synapses and the somata of the RN neuron is shortened after the paired conditioning ( $X > X'$ ). (2) Shortening of the electrotonic length of the dendrites of the RN neurons. Cable properties are changed and electrotonic length between the corticorubral synapses on the peripheral dendrites and the somata is shortened after the paired conditioning ( $X > X'$ ). (3) Shortening of waveform of postsynaptic current at the corticorubral synapses.

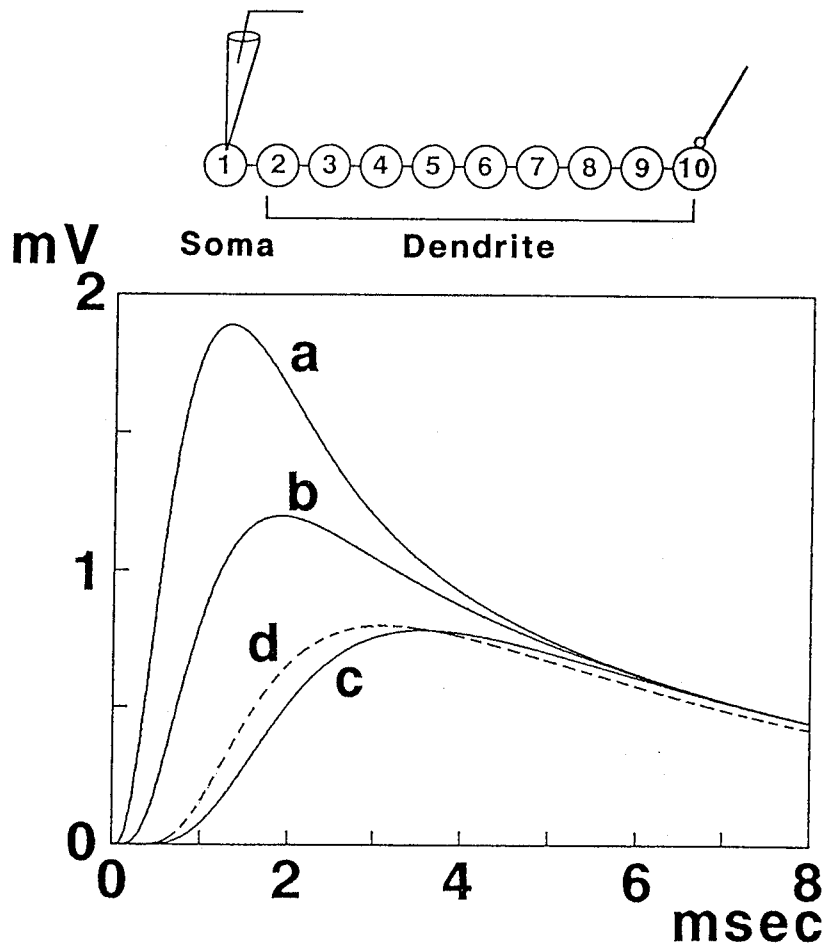


Fig. 19. Change in time course of simulated EPSP based on the Rall's compartmental model. The model consisted of ten equal compartments having the same electrical parameters. Parameters of the model; the electrotonic length of the cell ( $L$ ): 1.3, the membrane time constant: 6.1 msec, the specific membrane capacitance:  $1\mu\text{F}/\text{cm}^2$  and the specific membrane resistance:  $6.1\text{k}\Omega\text{cm}^2$ . Synaptic potential in the soma (compartment 1) was calculated by applying excitatory conductance change represented by an alpha function,  $g(t)=k\cdot t\cdot\exp(-t/\alpha)$ , to one of the dendrites (compartments 2 to 10). Reversal potential was assumed to be 60mV higher than the resting potential. Peak time ( $1/\alpha$ ) of the conductance change was selected as  $350\mu\text{sec}$  as obtained for the corticorubral EPSPs (Murakami et al. 1986). The synaptic conductance was applied to compartment 3(a), 5(b) or 10(c). The time course of the calculated EPSP was drastically affected (see next page)

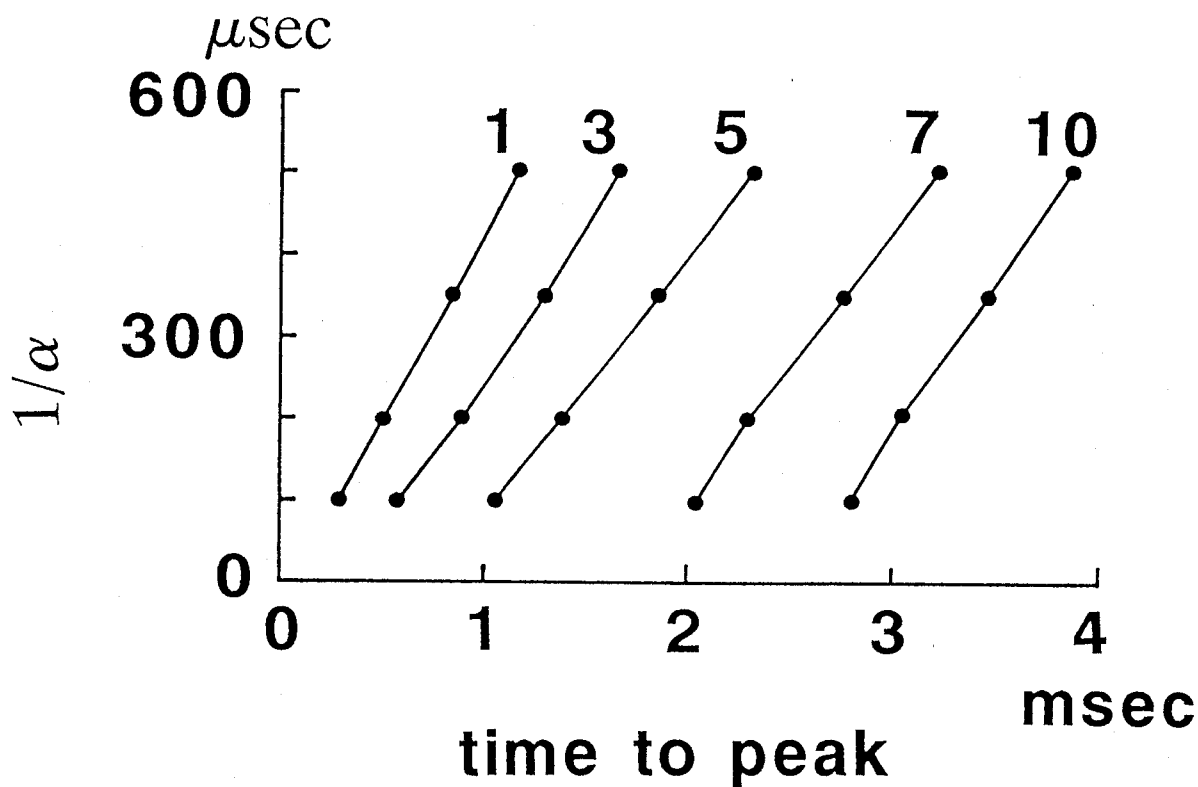


Fig. 20. Summarized figure of the simulation. Peak time ( $1/\alpha$ ) of the conductance change was shown on the ordinate, time to peak of the calculated EPSPs on the abscissa. Numbers shown above lines indicate the location where synaptic conductance was applied.

(Fig.19.) by the location of synaptic input. When the duration of the synaptic conductance applied to compartment 10 was shortened to about half of normal ( $1/\alpha=180\mu\text{sec}$ ), while the total amount of conductance change was kept constant, the calculated EPSP showed only a slight decrease in the time to peak (d, broken line). The time to peak values of the calculated EPSPs were 1.3msec (a), 1.9msec (b), 3.6msec (c) and 3.1msec (d), respectively.



## CHAPTER 4

### GENERAL DISCUSSION

#### Structural changes in synapses as a neuronal mechanism underlying learning

A series of experiments has been conducted to examine the neuronal mechanisms underlying the classical conditioning of the forelimb flexion. 1. A particular behavior, forelimb flexion, was modified in associative manner by the paired conditioning (Tsukahara et al. 1981). 2. Neural circuits responsible for the resulted behavioral changes was restricted to corticorubrospinal projection. The primary site was identified at the corticorubral synapses (Tsukahara et al. 1981; Oda et al. 1988). 3. Primary neuronal change which induced behavioral changes was shown to be the enhancement of the RN unit activity in response to the CS applied to the corticorubral fibers (Oda et al. 1981,1988). 4. The change in the waveform of corticorubral EPSPs was shown to be causally related with the acquisition of the classically conditioned forelimb flexion (present study, chapter2). 5. Electrophysiological evidences showed that appearance of the fast-rising EPSPs is due to the sprouting and subsequent formation of new corticorubral synapses onto the proximal dendrites or somata of RN neurons (present study, chapter 3). 6. In parallel with our study, electron microscopic observation demonstrated that a large proportion of corticorubral synapses was found on large diameter dendrites or somata of the RN neurons in the *paired* group than in the *naive* group (Murakami et al. 1987).

These data meet several criterion for identifying a particular neuronal changes underlying a particular behavioral modification by learning (modified from Byrne 1987). (1) Primary neural circuit responsible for a particular behavioral modification should be identified. (2) Primary site for neuronal changes should be identified on the primary neural circuit. (3) Neuronal correlates, changes in

neuronal activities, should be demonstrated electrophysiologically in the primary site. (4) Cellular or subcellular mechanisms for the neuronal correlates should be identified. (5) Necessary but insufficient factors relating with learning should not produce neuronal correlates. We have not yet tested the sixth criteria that neuronal changes should be related with the behavioral modification in time course of learning and in the extent and degree of learning. Combining these data (table 1), we suggest that the formation of functional corticorubral synapses onto the proximal dendrites or the somata of RN neurons is a neuronal mechanism underlying the classical conditioning of forelimb flexion. Slow time course of the acquisition of the classical conditioning requiring one week may be consistent with the slow process of synapse formation. One week is enough to induce changes in number of synapses or arborization of axon and dendrite, as shown after the monocular deprivation (Antonini and Stryker 1993), rearing in the complex environments (Kilman et al. 1988), long-term sensitization and habituation (Bailey and Chen 1983, 1988, 1989), long-term potentiation (Chang and Greenough 1984) and classical conditioning (Alkon et al. 1990). Preservation of the somatotopical organization in corticorubral connections after the paired conditioning indicates that the formation of new synapses contributes to enhancement of synaptic transmission without changing the wiring of the corticorubrospinal system.

It should be noted that we could not evaluate possible contribution of the synaptic transmission efficacy of pre-existing corticorubral synapses on the distal dendrites of the RN neurons, e.g., an increase in transmitter release or in sensitivity of receptors. As we mentioned in chapter 3, formation of new corticorubral synapses on the proximal dendrites or somata of the RN neurons provided satisfactory explanation for the increase in firing probability of the RN neurons in response to the CS.

There is an argument that the simplified model which we used in the present study is not model system of naive behavior and has risk of investigating the

neuronal changes which are not essential to the learning performed by intact animals (Squire 1987). However, the merit of the primary neural site being identifiable is large enough to examine neuronal mechanisms underlying the learning and memory.

Several anatomical studies have indicated that changes in the number or pattern of synaptic connections is related with memory formation and its maintenance (see review; Greenough 1984; Greenough and Bailey 1988). In mammals, however, how the observed morphological changes affect the neuronal activities responsible for the behavioral changes has not been made clear yet. But using an invertebrate simple neural circuit, the siphon withdrawal reflex arc of *Aplysia*, a change in the number of varicosities and/or active zones was suggested to be a neuronal mechanism underlying persistence of the long-lasting sensitization and the long-lasting habituation (Bailey and Chen 1983, 1988, 1989). Considering that newly formed corticorubral synapses were preserved for several months after lesion of the nucleus interpositus of the cerebellum (Tsukahara et al. 1975a) or cross union of the forelimb extensor and flexor nerves (Tsukahara et al. 1982), newly formed corticorubral synapses may contribute to retention of the conditioned forelimb flexion.

Studies in *Aplysia* show an interesting results that size and vesicle components of active zones also increased immediately after the training, however, were back to control level within one week after the training, while changes in number of varicosities and active zones were kept for at least 3 weeks (Bailey and Chen 1983, 1988, 1989). In general, memory can be consolidated gradually by repeating training trials in both strength and duration. An attractive hypothesis is that cascade of several molecular events undergoes transition from the short- to long-lasting plasticity in associative or non-associative learning. First, short-term memory formation is performed by modification of pre-existing proteins, e.g., phosphorylation through second messenger systems. This process is followed by

protein synthesis dependent process and stabilized by structural modifications of synapses. Application of protein synthesis inhibitors did not prevent short-term memory, while they prevented acquisition of long-term memory. Furthermore, they did not block once established long-term memory. Structural modification of synapse is started by a transient morphological rearrangement of pre-existing synapses in the active zones or spine apparatus, and is consolidated as a permanent memory according to the synapse formation and/or loss (Davis and Squire 1984; Staubli et al. 1985; Goelet et al. 1986; Montarolo et al. 1986; Greenough and Bailey 1988; Barzilai et al. 1989; Castellucci et al. 1989; Matthies 1991). In the classical conditioning of the forelimb flexion, acquisition of the conditioned response requires a week of training and several hundred trials in total. During the training period, behavioral score of performance did not show a rapid increase within a daily session which consisted of 50 trials and continued more than 30min. But it increased rather in cumulative manner day by day. Thus, it is of interesting whether immediate enhancement of the transmission efficacy at the corticorubral synapses corresponding to the short-term memory is involved in individual RN neurons. Alternative hypothesis is that synapse turnover occurred constantly and change in synapse number is induced by memory related change of stability of synapses. However, this is not enough to explain a shift in distribution of synapse location. Transient mechanisms which results in sprouting and synapse formation onto a proximal dendrite or somata of the RN cell is remained to be studied.

In conclusion, the present studies provide evidence that formation of synaptic connections works as a neuronal mechanism underlying learning and probably memory persistence in the mammalian central nervous system. Our results suggest that the location of synapses on the soma-dendritic membrane is another important factor for the establishment of learning.

Table 1. Comparison of data.

|          | Behavioral<br>modification | Enhancement<br>of unit activity | Appearance of<br>fast-rising<br>EPSPs | Anatomical<br>evidence for<br>sprouting |
|----------|----------------------------|---------------------------------|---------------------------------------|-----------------------------------------|
| Paired   | O                          | O                               | O                                     | O                                       |
| Naive    | —                          | X                               | X                                     | X                                       |
| Random   | X                          | X                               | X                                     | —                                       |
| CS-alone | X                          | X                               | X                                     | —                                       |
| Normal   | —                          | —                               | X                                     | (X)                                     |

O: positive changes. X: negative changes or no difference. ~: not examined.

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## ACKNOWLEDGEMENT

I'm very grateful to the late Professor Nakaakira Tsukahara for giving me a chance to investigate neuronal plasticity and mechanisms underlying learning in his laboratory. I wish to great thank to Dr. Yoichi Oda for tough and patient instruction, constant encouragement, support, and critical discussion. I'm grateful to Drs. Fujio Murakami, Son Wen-Jie, Nicholas Hartell and Masao Ito for their critical reading and useful advises in preparing original articles. I wish to thank to Drs. Keiji Tanaka and Ichiro Fujita for encouragement for preparing the thesis. I wish to thank following students who collaborate with me during this work: Fumikazu Wanibuchi, Takashi Shimada, Hiroshi Sumikawa, Tatsuya Nakai, Akiyoshi Shimada.