主論文

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Long-term Potentiation and N-methyl-D-aspartate receptors in the Visual Cortex of Young Rats

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SUMMARY

1. Long-term potentiation (LTP) of synaptic transmission following tetanic stimulation of the white matter was studied by recordings of extracellular field potentials and intracellular synaptic potentials from layer II/III of visual cortical slices from young rats ranging in age from 21 to 40 days.

2. Single shocks applied to the white matter at 0.1 Hz, used as test stimuli, elicited field potentials that consisted of primary and secondary components. The removal of Ca^{2+} ions from the perfusate allowed identification of the secondary component as originating postsynaptically and the primary one as reflecting a mixture of antidromic and postsynaptic potentials.

3. Tetanic stimulation at 5 Hz for 60 s was delivered to the white matter and field potentials were observed for 20 min to 9 h after the tetanus. LTP was defined as being present when the response displayed more than a 20 % increase in amplitude of the Ca^{2+}-sensitive components 20 min after the tetanus. LTP was induced in 12 of 23 slices tested, and this potentiation lasted throughout the period of observation. The average magnitude of potentiation was 147.8 $\pm$ 28.4 % of the control value for the 12 slices.

4. Administration of D,L-2-amino-5-phosphonovalerate (APV), an antagonist selective for N-methyl-D-aspartate (NMDA)-preferring receptors, slightly reduced the amplitudes of Ca^{2+}-sensitive components of the field potentials. The average magnitude of reduction was 80.2 $\pm$ 15.3 % of the pre-drug control values. In the presence of APV, LTP was induced in only

one slice of 12 tested.

5. Stable intracellular recordings were obtained from 23 cells from layer II/III. Excitatory postsynaptic potentials (EPSPs) evoked by white matter stimulation had mean onset and peak latencies of 4.1 and 11.3 ms, respectively. In some cells these fast EPSPs were followed by another slow EPSP with a much longer latency and higher amplitude. Administration of APV revealed further that the fast EPSPs consisted of two components, i.e., early and late components.

6. Tetanization of the white matter induced long-lasting enhancement of EPSPs in 8 of 12 cells tested. In 5 of these 8 cells, fast EPSPs were enhanced in amplitude and in the remaining 3 cells, slow EPSPs appeared de novo after the tetanus.

7. APV reduced the amplitudes of the fast EPSPs and abolished the slow EPSPs if present. The average magnitude of reduction for the fast EPSPs was 65.6 $\pm$ 15.1 % and this reduction was due mainly to an elimination of the late component. This component had the anomalous voltage-dependent properties; i.e., an enhancement with depolarization and reduction with hyperpolarization. In Mg^{2+}-free media, however, this voltage relation became reversed and conventional. These results are taken as evidence for the suggestion that the late component is mediated through NMDA receptors.

8. In the presence of APV, tetanic stimulation failed to induce significant potentiation of EPSPs in all cells tested but one (n = 11). It is suggested that the activation of NMDA receptors plays a crucial role for the induction of LTP in the developing rat visual cortex.

INTRODUCTION

Long-term potentiation (LTP) of synaptic efficacy in the hippocampus has been studied extensively since it was first reported by Bliss & Lømo in 1973. This type of neural plasticity has attracted the attention of many investigators as it is thought to represent a synaptic model of learning and memory in the mammalian brain (see Teyler & DiScenna, 1987 for review). Experiments using antagonists of excitatory amino acid (EAA) receptors demonstrated that amongst at least three types of EAA receptors, those of the N-methyl-D-aspartate (NMDA)-preferring class play a crucial role in the induction of LTP in the CA1 area of the hippocampus (Collingridge, Kehl & McLennan, 1983b, Harris, Ganong & Cotman, 1984; Wigstrom & Gustafsson, 1984).

Another type of neural plasticity that also has been studied widely is the susceptibility of visual cortical neurones to various environmental modifications during a particular period of postnatal development, called the "critical period" (see Fregnac & Imbert, 1984 for review). For example, binocular responsiveness of visual cortical neurones in the kitten is so sensitive to the influence of monocular visual deprivation during this critical period that most neurones become monocularly responsive to the non-deprived eye (Wiesel & Hubel, 1963; Hubel & Wiesel, 1970). One mechanism which underlies this type of visual cortical plasticity is thought to be activity-dependent changes in synaptic efficacy in developing visual cortex (Blakemore & Van Sluyters, 1975; Singer, 1979). From this point of view, it is rather surprising that only a few studies have been

done in an attempt to induce LTP in the developing visual cortex and to elucidate further the mechanisms underlying LTP. In 1979 it was first reported that cortical field potentials evoked by test stimulation of the optic nerve were potentiated following tetanic stimulation at 2 Hz applied to the same optic nerve and this potentiation lasted longer than 9 h (Tsumoto & Suda, 1979). The induction of LTP has been demonstrated subsequently in slice preparations of the kitten's and young rat's visual cortex using current source density analysis of field potentials evoked by stimulation of the underlying white matter (Komatsu, Toyama, Maeda & Sakaguchi, 1981; Perkins & Teyler, 1988).

These results raise the question of whether NMDA receptors play a role in inducing LTP in the visual cortex, where EAA receptors are known to be involved in afferent synaptic transmission (Tsumoto, Masui & Sato, 1986). Using a slice preparation of rat visual cortex, Artola & Singer (1987) reported that excitatory postsynaptic potentials (EPSPs) recorded intracellularly in response to stimulation of the white matter were potentiated for 30-90 min after tetanic stimulation of the same site; the induction of this LTP was susceptible to antagonism by a selective NMDA receptor antagonist. It is not clear from this short note, however, how often LTP is induced, to what magnitude the EPSPs are enhanced, how long the LTP lasts, what components of the EPSPs are potentiated and through what mechanisms the NMDA receptor antagonist blocks the induction of LTP. Using slice preparations of the developing rat's visual cortex we attempted to answer these questions and found that LTP of field potentials and EPSPs can be induced in layer II/III of the cortex by a certain type of tetanic

stimulation of the underlying white matter and that this induction is probably mediated through the activation of NMDA receptors.

A preliminary account of some of these experiments has been presented (Kimura, Tsumoto, Nishigori & Shirokawa, 1988; Tsumoto, Kimura, Nishigori & Shirokawa, 1988).

METHODS

Preparation of the Slices

Thirty-eight Sprague-Dawley rats, ranging in age from 21 to 40 days were used in this study. In most of the experiments, animals were initially anaesthetized with urethane (i.p., 1.3 g/kg), and then were perfused trans-cardially with cooled (4° C) medium of the following composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.4; glucose, 10; $NaHCO_3$, 26. The perfusion medium was gassed with 95% O_2 and 5% CO_2. In the other experiments, animals were stunned by a heavy blow on the back with an iron bar. The cranium was opened quickly and two blocks of tissue containing primary visual cortex (area 17) were removed from both hemispheres according to Krieg's atlas (1946). The blocks were then placed in the cooled (4° C) medium and coronal slices of 300-400 um thickness were prepared with a hand-held knife under binocular microscopic observation. Slices were incubated in the warmed (32±1° C) medium for at least one hour, then were transferred to a recording chamber where they were perfused with the same solution as was used for incubation, at a rate of perfusion of 4 ml/min.

In some experiments, the standard medium was replaced with a Ca^{2+}-free solution or with a Mg^{2+}-free solution. The Ca^{2+}-free solution had the same composition as the standard medium except for an omission of Ca^{2+}, an

addition of ethylene glycol-bis-N,N,N',N',-tetra-acetic acid (EGTA, 10 mM) and an elevation of the concentration of Mg^{2+} to 6.3 mM. The Mg^{2+}-free solution had the same composition as the standard medium except for an omission of Mg^{2+}.

Stimulating and Recording Electrodes

For the activation of optic radiation fibres, a pair of tungsten wires, insulated except at the tips and attached side by side with a tip separation of 0.2 mm, was placed in the white matter just beneath layer VI of the cortex. These stimulating electrodes had mean resistances of about 1 Mohm. Stimulus intensity was within the range of 1 to 100 V for pulses of 0.1 ms duration. To record field or intracellular potentials evoked by test stimulation of the white matter, glass micropipettes filled with 0.5 M sodium acetate containing 2 % pontamine sky blue (resistance: <10 Mohm) or 4 M potassium acetate (resistance: 60-150 Mohm), respectively, were inserted into layer II/III of the cortex under visual control.

Intracellular signals were amplified by high-input impedance amplifiers, with built-in current injection and bridge balance facilities (MEZ 8201, Nihon Kohden). Extracellular single unit and field potentials were filtered at 0.05-3kHz. During the experiments, the signals were displayed on an oscilloscope (VC 11, Nihon Kohden), and some of the data were digitized and recorded on an audio tape recorder (PC-108M, Sony Magnoscale) for later analysis.

Initially, control responses to single shocks given to the underlying white matter at 0.1 Hz were recorded, and then repetitive stimulation (tetanus) was applied to the white matter with pulses of the same amplitude but double the duration. The parameters of the tetany were: 2 Hz for 1 and 5 min; 5 Hz for 30 s, 1 and 5 min; 50 Hz for 10 s; 100 Hz for 2 s and 400

Hz for 1 s. We did not test tetanic stimulation lasting longer than 5 min, because such time-consuming tetanization seemed inappropriate for an analysis of synaptic potentials recorded intracellularly. Among the parameters tested, we found that the tetanization at 5 Hz for 30 and 60 s was the most effective in terms of the probability of induction of LTP. In the present study therefore, we usually used repetitive stimulation at 5 Hz for 30-60 s. After tetany, test shocks at the same intensity as the pre-tetanic control ones were again given to the white matter at 0.1 Hz and the evoked potentials were observed at least for 20 min.

Drug Administration and Ionophoresis

D,L-2-amino-5-phosphonovalerate (APV) and kynurenic acid were delivered to the slices by switching the standard perfusion medium to one containing those drugs at known concentrations through three-way switching taps so as not to cause an alteration of the rate of perfusion. The standard and test solutions were exchanged completely in the recording chamber within a few minutes after switching the taps. D-APV is a selective antagonist for NMDA receptors (Davies, Francis, Jones & Watkins, 1981; Hagihara, Tsumoto, Sato & Hata, 1988) whilst kynurenic acid is a relatively non-selective antagonist for the three types of EAA receptors (Perkins & Stone, 1982; Tsumoto et al., 1986). The concentration of APV was usually 25-50 uM and that of kynurenic acid was 200-500 uM (see RESULTS).

In some of the experiments, multibarrel micropipettes were used for extracellular single unit recordings and for ionophoretic administration of agonists of EAA receptors, to check the effectiveness of the antagonists administered through the medium. The agonists employed were: NMDA (50 mM, pH 8.0), quisqualic acid (20 mM, pH 8.0) and kainic acid (20 mM, pH 8.0), which are relatively selective agonists for their respective types of EAA

receptors (see Watkins & Evans, 1981 for review). Usually, bicuculline methiodide, an antagonist of gamma-aminobutyric acid ($GABA_A$) receptors (Curtis, Duggan, Felix & Johnston, 1970) was added to the medium at a concentration of 0.5-2.0 uM.

In some slices, extracellular dye marks were produced by passing tip-negative current pulses of 5 - 10 uA through the recording pipette containing pontamine sky blue. This resulted in clear deposits of dye of 30 - 100 um diameter. Each slice containing the dye deposit was fixed overnight in a 4 % solution of formaldehyde. Then it was frozen, sectioned at 60 um and stained with cresyl violet.

RESULTS

Field Potentials Recorded from Layer II/III of the Cortex

To test whether LTP of synaptic efficacy can be induced in slices of visual cortex by tetanic stimulation of the underlying white matter, and if so, what the most effective parameters of tetany in inducing LTP are, we first studied field responses recorded from layer II/III of the cortex to test stimulation of the white matter. In viable visual cortical slices, these layers can be recognized easily through a binocular dissection microscope as a translucent band parallel to the cortical surface. Thus, recording electrodes were inserted into layer II/III of the cortex under visual observation. In some of the experiments in which the slices were later examined histologically, the dye deposits ejected from the recording electrode confirmed that recordings had actually been made from layer II/III of primary visual cortex (Krieg, 1946).

Single pulses applied to the underlying white matter as control stimulation elicited field potentials consisting of two negative peaks (see Fig. 1A-1). These two potentials will be called the primary and secondary components, respectively. Sometimes, a third negative potential having a lower amplitude followed the secondary component. The intensity of the control shocks was set usually at two to three times the threshold for inducing clearly distinguishable primary components. In the 22 slices studied, the peak latencies of the primary component ranged from 2.9 to 5.7 ms (mean $\pm$ standard deviation = 4.1 $\pm$ 0.8) and those of the secondary component, from 4.6 to 7.8 ms (6.0 $\pm$ 1.1). Increasing stimulus intensity often shortened the latencies of the secondary component so that in some cases it fused with the primary component.

Identification of Postsynaptic Components of Field Potentials

Field responses evoked by stimulation of the underlying white matter may include potentials of afferent fibres and/or antidromically activated potentials of projection neurones. To test this possibility, Ca^{2+} was removed from the perfusion medium. Figure 1A shows an example of results

(Place Figure 1 here)

obtained from such a trial. When Ca^{2+} was removed from the perfusate, the primary component was reduced in amplitude and duration only slightly, while the secondary component was abolished completely (Fig. 1A-2). The extent of the insensitivity of the primary component to Ca^{2+}-free media varies from slice to slice; in some slices the primary component was reduced in amplitude dramatically, but in others it was not notably changed. By contrast with the primary component, the secondary component

was always sensitive to Ca^{2+}-free medium. These results suggest that, to variable degrees, the primary component consisted of antidromic responses of projection neurones and/or responses of presynaptic fibres but that the secondary component was entirely postsynaptic in origin.

This suggestion was supported by a latency analysis of the spikes recorded extracellularly from 70 neurones from layer II/III (Fig. 1B). The latency histogram of these cells had two peaks; the mode of short- and long-latency groups corresponded approximately to the peak latency of the primary and secondary components of the field potentials, respectively (compare B with A-1 in Fig. 1). In the short-latency group, about half the cells had antidromic responses (hatched columns of Fig. 1B). The details of these findings using extracellular single cell recordings will be described elsewhere. Thus, the variability in sensitivity of the primary component to Ca^{2+}-free media may be due to a variability in the contribution of antidromic responses to this component, from slice to slice. In most slices, therefore, LTP of the field potentials was assessed by measuring the amplitudes of the secondary component. In the other slices in which the secondary component was not of sufficient size and the primary component was clearly sensitive to Ca^{2+}-free medium, the primary component was used for the assessment. Slices which had no Ca^{2+}-sensitive components in the field potentials were omitted from the present study.

Induction of LTP of Field Potentials

In most slices, the Ca^{2+}-sensitive components of the field potentials were clearly enhanced in amplitude after tetanic stimulation of the underlying white matter. LTP was defined as a more than 20 % increase in

amplitude of the Ca^{2+}-sensitive component lasting at least 20 min after the tetany. Figure 2 shows an example of LTP and its time course observed in

(Place Figure 2 here)

a slice from a 33-day-old rat. Control shocks given at 0.1 Hz to the white matter elicited field responses with the two components (record a). One min after tetany, both components were enhanced remarkably (record b) and this enhancement enlarged further until 20 - 30 min subsequent to the tetany. The amplitude of the primary component increased to 166 % of the control value at 20 min and that of the secondary component to 208 % of the control at 30 min (graph in Fig. 2). Such a strong potentiation lasted without any significant reduction throughout the period of observation (40 min after tetany). As for record c, notice that the vertical amplification is halved compared to the others. In one of the slices, we continued the observation as long as 9 h after tetany and observed that the LTP lasted for the duration of the monitoring.

To evaluate the magnitude of the LTP, amplitudes of the Ca^{2+}-sensitive components at 20 or 30 min after tetany were expressed as percentages of the pre-tetanic control values. The mean value was 147.8 $\pm$ 28.4 % for the 12 slices in which significant LTP was induced.

Selectivity of Effects of the NMDA Receptor Antagonist

To test whether or not NMDA receptors are involved in the induction of LTP, we employed the pharmacologically selective antagonist for NMDA receptors, APV. As APV is known to antagonize NMDA receptors selectively only within a limited range of concentrations in the spinal cord and hippocampus (Davies et al., 1981; Collingridge, Kehl & McLennan, 1983a),

it was of importance to control the range of concentrations with which APV was used in the present preparation. For this purpose, we recorded the spike discharges of cortical cells extracellularly with multibarrel micropipettes and tested the effects of the superfusion of APV on the excitations induced by ionophoretic NMDA and quisqualate. At concentrations of 1 and 10 uM, APV was ineffective in blocking excitations induced by either agonist. At concentrations of 25-100 uM, APV eliminated completely the excitations evoked by NMDA but did not block those by quisqualate. At 200 uM or more, APV suppressed significantly or blocked completely excitations produced by quisqualate as well as those by NMDA. This indicates that the antagonistic action of APV is selective for NMDA receptors only at concentrations between 25 and 100 uM. In the present experiments therefore, we usually used APV at a concentration of 25 or 50 uM.

Effects of APV on Field Potentials and on the Induction of LTP

The administration of APV reduced slightly the amplitude of the Ca^{2+}-sensitive components of field potentials in 6 of 11 slices in which comparisons were made to values prior to administration of the antagonist. The average amplitudes during APV administration for the 6 slices was 80.2 $\pm$ 15.3 % of pre-drug control values. Three other slices showed no changes in field potential amplitude and the remaining two were rather exceptional in the sense that Ca^{2+}-sensitive components were enhanced in amplitude slightly during APV presentation.

For 12 slices, tetanic stimulation was given to the white matter at least 15 min after beginning administration of APV. In all slices except

one, LTP of field potentials was not induced by the tetany. Figure 3 shows

(Place Figure 3 here)

an example of field potentials before and after tetany in the perfusate containing APV. The primary component had a prominent peak with relatively long latency (peak latency, 5.6 ms) whilst the secondary component was much smaller in amplitude. Both components disappeared in Ca^{2+}-free media, indicating that they were of postsynaptic in origin. In this case, administration of APV reduced the amplitude of the primary component to 92 % that of the pre-drug control response (records not shown). After tetanic stimulation of the white matter both components remained virtually unchanged for the duration of the observation.

As described already, the amplitude of the Ca^{2+}-sensitive components at 20 or 30 min after tetany was expressed as a percentage of the pre-tetanic control value. In the presence of APV, the average value was 91.2$\pm$ 13.3 % for the 12 slices, i.e., there were no significant changes after tetany.

EPSPs Recorded Intracellularly From Cortical Layer II/III Neurones

EPSPs evoked by stimulation of the white matter were recorded from neurones in layer II/III of the cortex. The present results deal with 23 cells from which stable intracellular recordings were made for at least 30 min after tetanic stimulation. These cells had resting membrane potentials in the range of -50 to -86 mV (mean $\pm$ S.D. = -69.3 $\pm$ 8.6). To measure the peak amplitudes of the EPSPs, the intensity of the control shocks was set lower than the threshold for spike generation (81 $\pm$ 16 % of the threshold). Such a weak stimulus usually elicited an EPSP consisting of a single peak

(see record a, in Fig. 4). The mean onset and peak latencies of these EPSPs were 4.1 ± 0.8 and 11.3 ± 3.4 ms, respectively, for the 16 cells in which measurements were taken in the absence of APV. At higher intensities of stimulation, another peak with a longer latency appeared during the falling phase of the EPSPs in some cells (see top record in Fig. 6). The EPSPs thus appeared to consist of both early and late components. When the stimulus intensity was increased further, the peak latency of the late component was shortened and it was often fused with the early component, resulting in larger EPSPs (see records in the left column of Fig. 6). This point will be described later. Following these EPSPs, another EPSP with a much longer onset and peak latencies (15-96 and 30-110 ms, respectively) appeared in an all-or-none manner in 5 of 17 cells. These EPSPs are called slow EPSPs, whereas the former are called fast EPSPs.

Long-term Potentiation of EPSPs

Following tetanic stimulation of the white matter, a long-lasting increase in amplitude of EPSPs was seen in 8 of 12 cells tested, the remaining 4 not showing any significant change. In 5 of these 8 cells, the amplitudes of the fast EPSPs were increased significantly, and in the other 3 cells, slow EPSPs appeared following tetany. Figure 4 shows an example

(Place Figure 4 here)

of a long-lasting enhancement of the fast EPSPs. The onset and peak latencies of the EPSPs evoked by control shocks given to the white matter were 4 and 12 ms, respectively (see record a, at top left). Before tetanic stimulation, control shocks at 4.7 V (threshold for spike generation was 5.5 V) evoked only fast EPSPs with a mean peak amplitude of 6.0 ± 0.7 mV.

Five minutes after tetanization these EPSPs were enhanced in amplitude significantly (record b). Thereafter, the amplitude was increased further and it became 8.5 ± 0.7 mV, 25 min following tetany (record d). The time course of this potentiation is shown graphically in Fig. 4 wherein the amplitude of the EPSPs is expressed as a percentage of the control values. The mean magnitude of LTP of the fast EPSPs was 137.4 ± 7.8 % for the 5 cells.

In the 3 cells without significant changes in fast EPSPs, slow EPSPs appeared after tetanization. The peak latencies of these slow EPSPs ranged from 30 to 100 ms. An example of the appearance of slow EPSPs after tetanization is shown in Fig. 5. Before tetany, test shocks at an

(Place Figure 5 here)

intensity of 1.75 V (threshold for spike generation was 1.9 V) elicited only fast EPSPs with low amplitudes (record a). After tetany, these fast EPSPs did not change in amplitude significantly, but large EPSPs with peak latencies of 50 to 75 ms were elicited consistently by every test shock (record b). These newly-appearing EPSPs were seen throughout the period of observation for as long as 60 min. These slow EPSPs triggered sporadic discharges of spikes 14 min after tetany and, later, more consistently.

NMDA-Receptor Mediated Component of EPSPs

As mentioned above, the administration of APV appeared to prevent the induction of LTP of field potentials in most slices tested. As the next step of analysis therefore, we attempted to determine whether APV would bring about a block of the induction of LTP of EPSPs. We could obtain stable intracellular recordings from 11 cells for a time sufficiently long

to follow the EPSPs at least 20 min following tetanic stimulation in APV-containing medium: i.e., at least 35 min after the initiation of the administration of APV. We found initially that the presentation of APV itself reduced the amplitudes of the fast EPSPs and eliminated the slow EPSPs, if they were present. The magnitude of the reduction was calculated as a percentage of the amplitude of the EPSP at least 10 min after drug administration, compared to pre-drug control values. The mean value for the fast EPSPs recorded from the 11 cells was 65.6 $\pm$ 15.1 %. It should be stressed however, that the rising slope of the fast EPSPs remained unchanged in almost all cells. This suggests that the early component of the fast EPSPs is not necessarily mediated by NMDA receptors, but that the late component is mediated at least partially by them.

To add support to this suggestion, we analysed the changes in amplitude and waveform of the fast EPSPs after application of APV. Figure 6 shows an example of results obtained from such an analysis. Test

(Place Figure 6 here)

shocks at the intensity of 3.0 V elicited fast EPSPs with an obvious notch, suggesting that they consisted of early and late components (record at top). The peak latency of the late component was 34 ms on average. When the intensity of the test shocks was increased to 4.0 V, the latency was shortened further (22 ms) so that the late component fused with the early one. Administration of APV eliminated the late component but did not affect the early component appreciably, because the rising slope of the EPSPs did not display any change (top record, right column). The amplitudes of the early components were assessed by measuring the height of

the notch from the baseline. These values were 14 ± 0.9 mV in the standard medium and were not reduced following APV. At an intensity of 40 V, the early component was observed to trigger spike activity and the peak latency of the late component was shortened further to 18 ms (record at bottom left). In the presence of APV, spike discharges were still triggered from the early component but the late one appeared to be eliminated (record at bottom right).

We then addressed the question of whether the late component has voltage-dependent properties which have been reported as being characteristic of NMDA receptor-mediated responses in cultured neurones (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984; Mayer & Westbrook, 1985). An example of the results obtained from such an analysis is shown in Fig. 7A. This neurone showed a significant enhancement of the amplitude

(Place Figure 7 here)

of the fast EPSPs, 20 min after tetany (127 ± 6 % of control value). Having observed the potentiation, we changed the membrane potential and measured the amplitudes of the early and late components of the fast EPSPs at various potentials. In tests with depolarization, weaker shocks were used than for those with hyperpolarization, to prevent the stimuli from triggering spikes. Measurements of the amplitudes of the late component were obtained in the following manner. First, an interpolation was made of the falling slope of the early component, using its expected height as judged from the prominent shoulder, and an exponential reduction curve. A point on the recorded EPSP corresponding to a latency fixed as being 27 ms was measured and the difference in amplitude from this point to the

interpolated amplitude was measured. This value was defined as a late component's amplitude. Unlike the early component, the late component was increased progressively in amplitude with depolarization (Fig. 7A-1, left) and decreased with hyperpolarization (Fig. 7A-1, right). This trend is seen in the graph wherein the normalized amplitudes of each component are plotted against membrane potential (Fig. 7A-2). The similar tendency was seen in all other 5 neurons tested. This distinctive voltage-dependency of the late component is known as a negative slope conductance and is mediated by NMDA receptors (MacDonald, Porietis & Wojtowicz, 1982; Nowak et al., 1984; Mayer & Westbrook, 1985). Since this negative slope conductance was reported to be ascribed to the voltage dependent block of NMDA receptors by Mg^{2+} ion (Nowak et al, 1984; Mayer & Westbrook, 1985), we then tested effects of removal of Mg^{2+} ion from the perfusate on the voltage-dependence of the late component, as demonstrated in the cingulate and sensorimotor cortices by Thomson (1986). In Mg^{2+}-free media the late component dramatically increased in amplitude and duration, and its voltage relation became reversed so as to be conventional; i.e., the amplitude increased with hyperpolarization and decreased with depolarization (triangles in Fig. 7B). This reversal of the voltage-dependency was seen in two neurones from which stable recordings were done during alterations of the membrane potentials in the normal and Mg^{2+}-free media. The resting membrane potentials did not remarkably change in the Mg^{2+}-free media. These findings further support the suggestion that the late component of the fast EPSP is mediated through NMDA receptors.

Block of Potentiation of EPSPs by APV

In the presence of APV, tetanic stimulation of the white matter did not induce a significant potentiation of EPSPs in all cells tested but one (n = 11). As described above, APV reduced substantially the amplitude of the fast EPSPs in most cells. There is a possibility therefore that the tetanic stimulation during the administration of APV might have been too weak to induce LTP. To test this possibility, the intensity of the test shocks was increased during APV presentation so that each shock could evoke fast EPSPs of about the same or higher amplitude than were obtained during the pre-drug control situation. Figure 8 illustrates an example of the

(Place Figure 8 here)

results obtained from such a trial. When the amplitude of the fast EPSPs was reduced to 88.1 % of pre-drug values, the intensity of the test shocks was increased to 3.0 V, so that each shock evoked EPSPs with a higher amplitude than the pre-drug control values. Then, the tetanus consisting of pulses of this amplitude and twice the duration was delivered to the white matter. Such an intensified tetanus, however, still was ineffective in inducing LTP in the medium containing APV (Fig. 8).

The probability of LTP induction, assessed with measurements of the EPSP amplitudes was only 9 % in the perfusate with APV whereas it was 67 % in that without APV. The difference in the probability for the induction of LTP is statistically significant between the two perfusion conditions (x^2-test, $P < 0.05$). These results, together with those obtained in the field potential studies, suggest strongly that NMDA receptors play a crucial role for the induction of LTP of synaptic transmission in the developing rat's visual cortex.

DISCUSSION

The present results demonstrate that field potentials and EPSPs recorded from layer II/III of developing rat visual cortex are potentiated for at least several hours following tetanic stimulation of the underlying white matter and that this potentiation is blocked by the selective NMDA receptor antagonist, APV. This type of synaptic plasticity, called LTP, has been studied extensively in the hippocampus (Bliss & Lømo, 1973; see Teylar & Discenna; 1987, for review) because slice preparations of this structure were found suitable for the investigation of synaptic mechanisms of LTP (Schwartzkroin & Wester, 1975). On the other hand, there have been only a few studies reporting LTP in the visual cortex and no systematic attempts have been undertaken to elucidate the mechanisms through which cortical LTP is induced (Tsumoto & Suda, 1979; Komatsu et al., 1981; Lee, 1982; Perkins & Teyler, 1988; Komatsu, Fujii, Maeda, Sakaguchi & Toyama, 1988). Accordingly, LTP observed in the developing visual cortex will be compared with that which has been reported in the hippocampus.

LTP in the Visual Cortex and Hippocampus

The time course of the LTP observed in the present experiments appears to be similar to that reported for the hippocampus, although we did not continue our observation longer than 9 h following tetany. However, the magnitude of the cortical LTP is substantially smaller than that of hippocampal LTP; the maximal potentiation in the present study was 208 %, whereas in the hippocampus of adult rats, it has been reported to be over

900 % (Schwartzkroin & Wester, 1975) and 1800 % in the developing hippocampus of the rat, at post-natal day 15 (Harris & Teyler, 1984). This difference in magnitude of LTP between the visual cortex and hippocampus may not be due to differences in parameters of tetanic stimulation, because previous field potential studies using tetanic stimuli with various parameters in rat visual cortex reported LTP of magnitudes similar to those in the present study (Lee, 1982; Perkins & Teyler, 1988).

Thus, it appears reasonable to conclude that LTP induced in layer II/III of the developing rat's visual cortex is rather moderate in magnitude than it is in hippocampus. This difference may be related to the degree of synchronization of afferent inputs to target neurones following electrical stimulation. In the hippocampus, impulses evoked by afferent fibre stimulation with an intensity strong enough to induce LTP may arrive essentially simultaneously at postsynaptic cells in the CA 1 area (Andersen, Silfvenius, Sundberg, Sveen & Wigstrom, 1978). In the visual cortex on the other hand, impulses evoked by white matter stimulation may arrive at layer II/III cells considerably less synchronously, since these are known to receive geniculate inputs through one or two interneurones in addition to direct input (Peters, 1985).

Afferent Pathways to Layer II/III Neurones

In the present study, the secondary component of the field potentials had mean onset and peak latencies of 5.2 and 6.0 ms (n = 19). In an analysis of potential sources in slices of rat visual cortex, Shaw and Teyler (1982) reported that the negative wave, called component 5, of the potentials evoked by white matter stimulation had its maximum amplitude in

layer II/III of cortex and its onset and peak latencies were about 4.5 and 6.5 ms, respectively. These values correspond roughly to those of our secondary component of field potentials. On the basis of conduction velocity analysis of afferent volleys, they suggested that component 5 may represent the primary postsynaptic responses of layer II/III neurones to white matter stimulation. Thus, the secondary component of the field potentials may be evoked for the most part, monosynaptically from white matter. There remains the possibility that this component might be elicited through recurrent collaterals of other pyramidal cells sending off associational and callosal efferents (Peters, 1985). This seems unlikely however, because the field potentials evoked by white matter stimulation are similar in waveform to those recorded in vivo in rat visual cortex to photic stimulation, which does not directly activate axon collaterals to layer II/III neurones (Creel, Dustman & Beck, 1970). Therefore, it is reasonable to assume that field potentials evoked by white matter stimulation in our slice preparation is not likely to include sizable potentials mediated through axon collaterals, but mostly reflect an activation of afferents from the lateral geniculate nucleus.

In the intracellular analysis of this study, the early component of the fast EPSPs had mean onset and peak latencies of 4.1 and 11.3 ms, respectively. These values correspond roughly to the peak latencies of components 5 and 6 of the evoked potentials reported by Shaw & Teyler (1982). Component 6 is considered to be polysynaptic in origin (Shaw & Teyler, 1982). Thus, the early component of our fast EPSPs may contain polysynaptically evoked potentials, in addition to monosynaptically evoked

potentials. The late component of our fast EPSPs had relatively long peak latencies to white matter stimulation at subthreshold intensities for generation of spikes. When the stimulus intensity was increased, the latencies were shortened dramatically but did not reach those of the early component. Also, these results establish that the late component is sensitive to the antagonistic action of APV. In slice preparations of sensorimotor cortex, Thomson (1986) reported that EPSPs mediated purely through NMDA receptors to layer II/III neurones may be polysynaptic in origin. Taking these results altogether, it is likely that the late component of fast EPSPs is mediated polysynaptically from the white matter.

Contribution of NMDA Receptor-Mediated Components to LTP Induction

The analysis of EPSPs with administration of APV suggested that the late component of the fast EPSPs are mediated through NMDA receptors. This supposition received support from the results showing that the late component has voltage-dependent and Mg^{2+}-sensitive properties characteristic of NMDA receptor-mediated events (MacDonald et al., 1982; Nowak et al., 1984; Mayer & Westbrook, 1985). This suggestion is consistent with recent findings that EPSPs recorded from cultured cortical neurones and from neurones in cortical slices contain an APV-sensitive component in the falling phase (Thomson, 1986; Huettner & Baughman, 1988). In a few cells, the slow EPSPs appeared de novo after tetany and persisted as long as the observations were continued. These EPSPs were also found to be sensitive to the effects of APV. Thus, from the present results that APV prevents induction of LTP, it is possible to suggest that the NMDA-receptor mediated components may contribute to the induction of LTP in

standard media.

The operation of NMDA receptors in inducing LTP in the present paradigm might be attributable, at least in part, to the presence of bicuculline in the bathing medium. According to several previous reports, the administration of convulsant drugs unmasks an NMDA receptor-mediated component of synaptic potentials which is not distinguishable in standard media during low frequency stimulation (Wigstrom & Gustafsson, 1985; Dingledine, Hynes & King, 1986; Thomson, 1986). It is thus possible that NMDA receptor mediated components may not exert their full actions under normal physiological conditions. This may not always be the case however, in layer II/III of developing visual cortex, because there is some evidence indicating that inhibition matures relatively late in these layers (Komatsu, 1983).

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FIGURE LEGENDS

Figure 1 Analysis of field potentials recorded from layer II/III of the visual cortical slice. A, field responses in cortical layer II/III to white matter stimulation. A-1, in normal perfusion medium. Field responses consist of 2 components, the peak latencies of which are 2.7 and 5.6 ms. A-2, in Ca^{2+}-free medium. In this and all subsequent figures, each record is the superimposition of five consecutive oscilloscope sweeps. Note that the time scale is the same as the abscissae of histogram in B. B, latency distribution of unitary responses of 70 layer II/III cells. Open and hatched columns represent cells with orthodromic and antidromic responses, respectively, to white matter stimulation. In inset is shown an example of spike discharges recorded extracellularly from a layer II/III cell in response to single shock stimulation of the white matter. Arrows indicate how to measure latencies of responses.

Figure 2 Time course of LTP of field potentials recorded from layer II/III of the visual cortex of a 33-day-old rat pup. Records at the top (a, b and c) show potentials recorded at the corresponding time point in the graph. Note that the vertical scale in c is half that in a and b. In the graph, amplitudes of primary and secondary components of evoked potentials are plotted against time after tetanus. Tetanic stimulation was applied to the white matter at the time indicated by the arrow. Each point and bar represents mean $\pm$ standard deviation (S.D.) of 5 responses.

Figure 3 Field potentials which were not potentiated by tetanic stimulation during administration of APV. The initial upward deflection in

each record was due to stimulus artifact. The concentration of APV in the perfusion medium was 25 uM. Time after stopping tetany is indicated at the left of each record.

Figure 4 Time course of LTP of EPSPs recorded from a cell in layer II/III of the visual cortex of a 29-day-old rat pup. Records a - d at top show potentials recorded at corresponding time points in the graph. The upward deflections in the records are mostly fast EPSPs. Resting membrane potential of this cell was -58 mV. In the graph, the amplitudes of EPSPs are plotted as a percentage of control value. Each point and vertical bar represents mean $\pm$ S.D. of 5 successive records. The shaded horizontal band in the graph indicates mean $\pm$ S.D. of the control value.

Figure 5 Time course of LTP of EPSPs recorded from a cell in layer II/III of the visual cortex of a 32-day-old rat pup. Records a - d at top show potentials recorded intracellularly at the corresponding time point in the graph. The small upward deflections in each record are the fast EPSPs and the larger ones in records b - d are slow EPSPs. Resting membrane potential of this cell was -84 mV. In the graph, the amplitudes of the fast (filled) and slow (open) EPSPs are plotted against time after tetany. At 24 - 25 min after tetany, three late EPSPs triggered spikes so that their amplitudes were not measured.

Figure 6 Effects of APV on EPSPs recorded from a layer II/III cell of a 38-day-old rat pup. Stimulus intensity of test shocks given to the underlying white matter are indicated at the left of each record. In the right column are records obtained from the same cell in the presence of APV

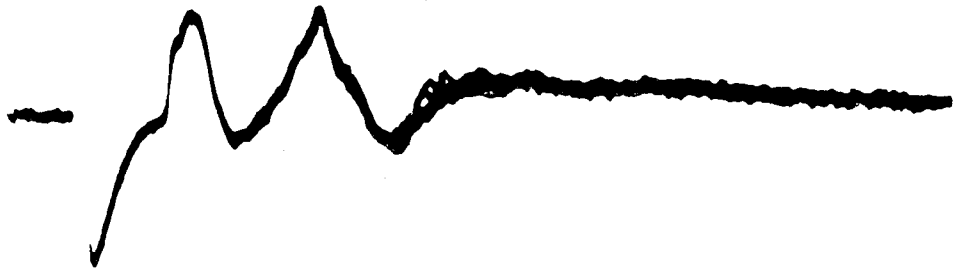
(50 uM) with the same stimulus intensity as at left.

Figure 7 Voltage dependence of early and late components of fast EPSPs recorded from layer II/III cells obtained from 36 day-old (A) and 37-day-old (B) rat pups. A-1, records showing fast EPSPs elicited by test shocks with intensities of 2.0 and 2.5 V, respectively, at membrane potential indicated at left. Single sweep for each record. At depolarized membrane potentials, weaker stimulus intensities were used so as to avoid spike generation (left column). A-2, amplitudes of early (filled circles) and late components (open circles) normalized to the values at -70 mV and plotted against membrane potential (abscissae). At -45 mV the late component triggered discharges of spikes so that its amplitude was not measured. Amplitudes of the early component were measured from the baseline to a notch in the rising phase of EPSPs. For details of the method of measuring amplitudes of the late component, see text. B, amplitudes of late components in Mg^{2+}-free (triangles) and normal (circles) media plotted against membrane potentials (abscissae). Note that decuple scales are used on the ordinate at left side for the values in the Mg^{2+}-free medium.

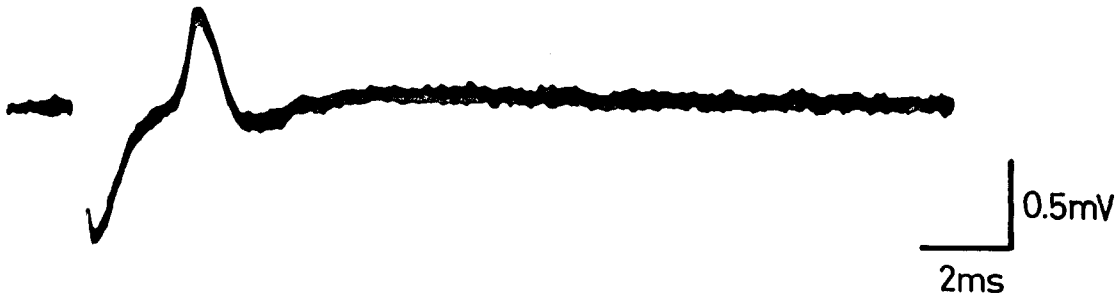
Figure 8 No induction of LTP during administration of APV even when intensified tetany was given to the white matter. Amplitudes of fast EPSPs recorded from a layer II/III cell obtained from a 35-day-old rat pup are plotted against time before and after tetany. 15 min after initiation of the APV presentation, intensity of test shocks increased from 2.5 to 3.0 V so that amplitudes of the EPSPs increased to 8-10 mV. Open horizontal bar indicates the duration during which APV was administered to this cell.

A Field Response

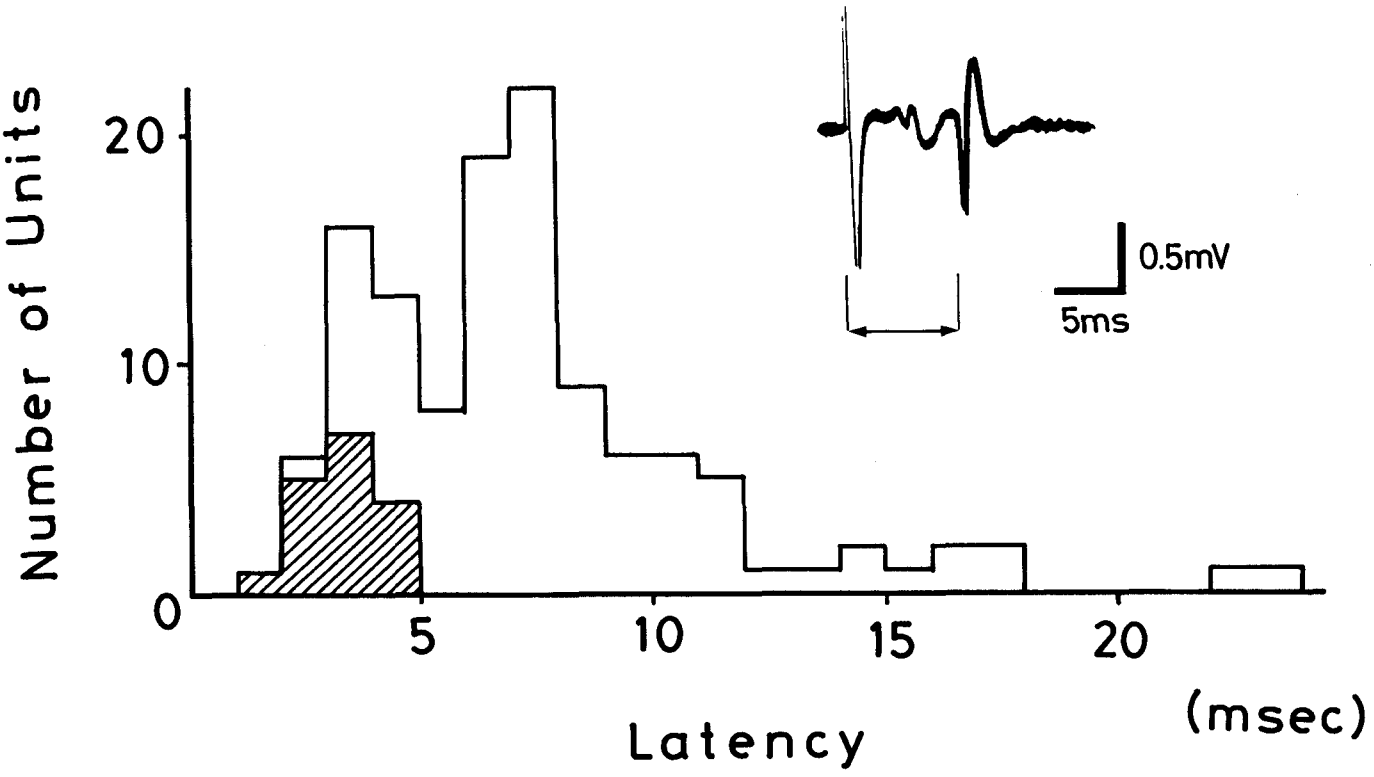
Normal

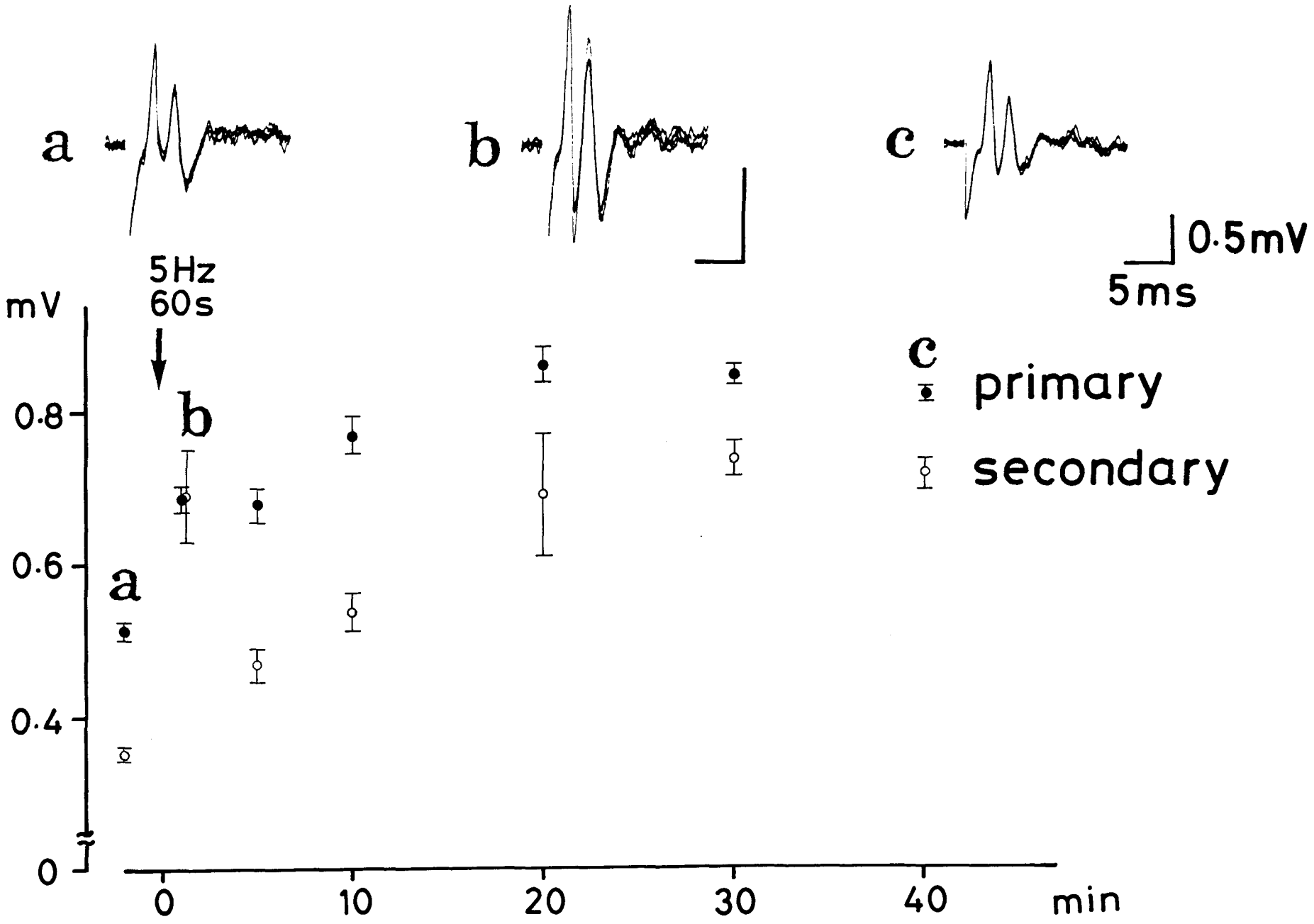


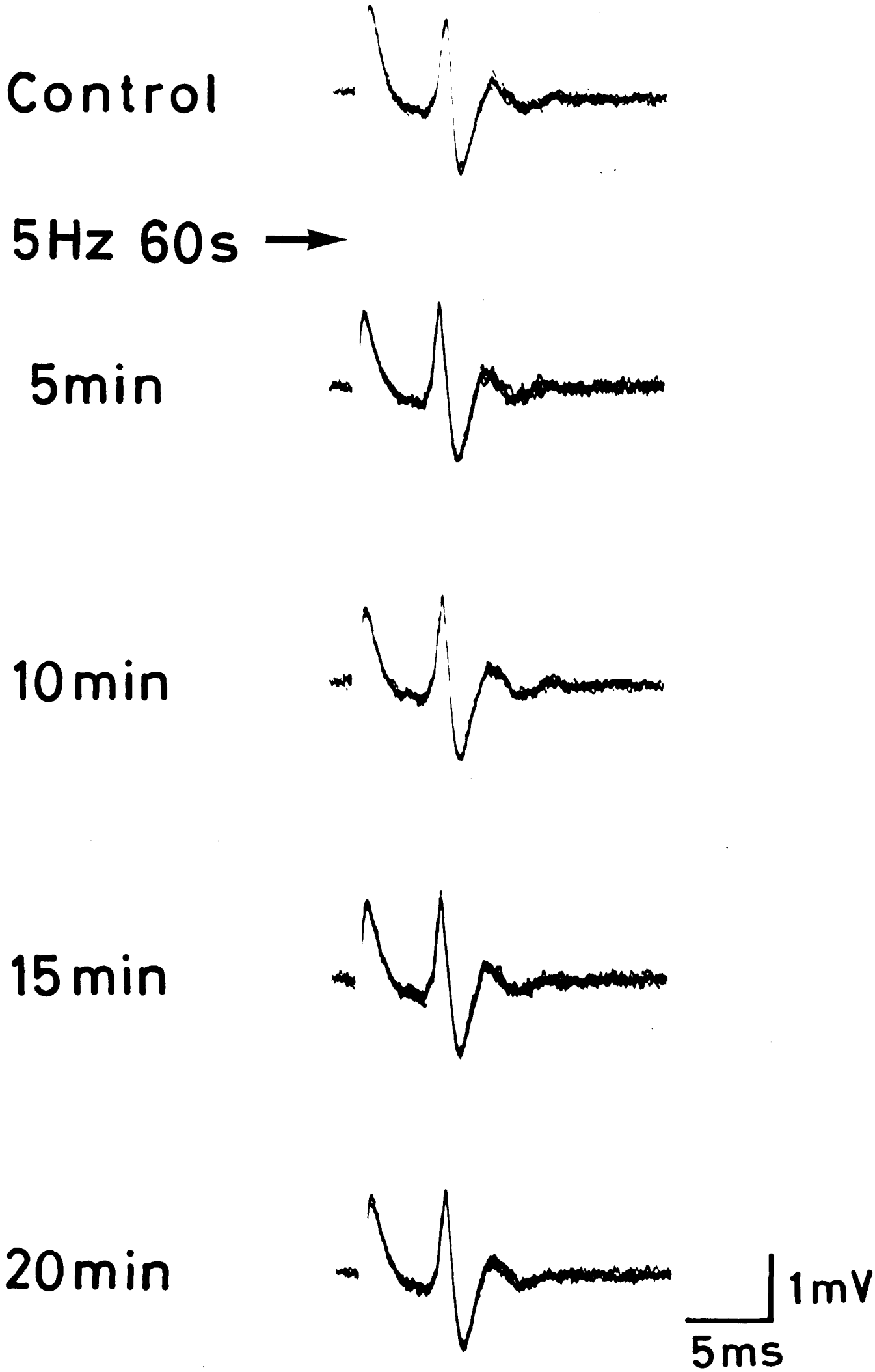
2) Ca^{++} free

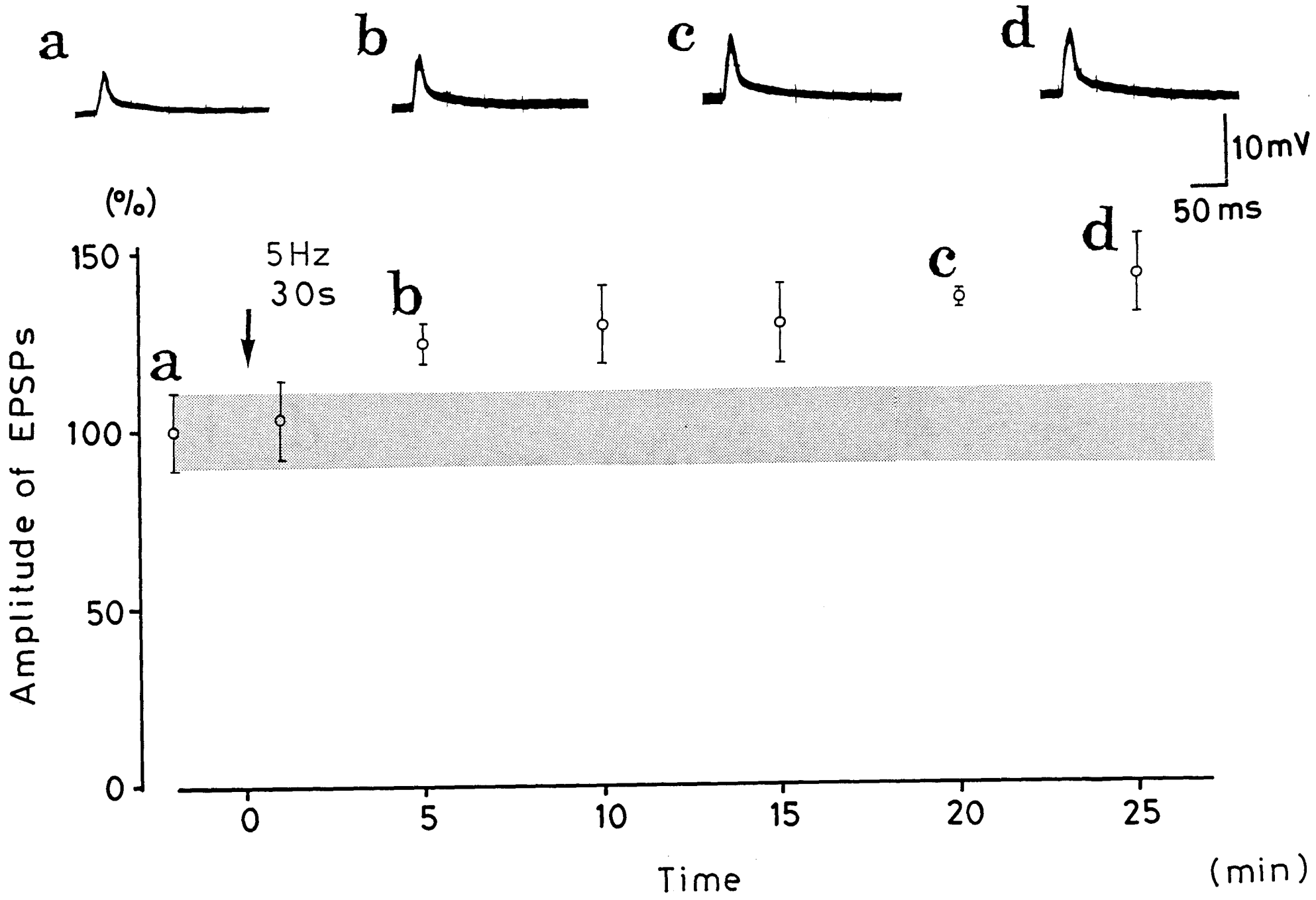


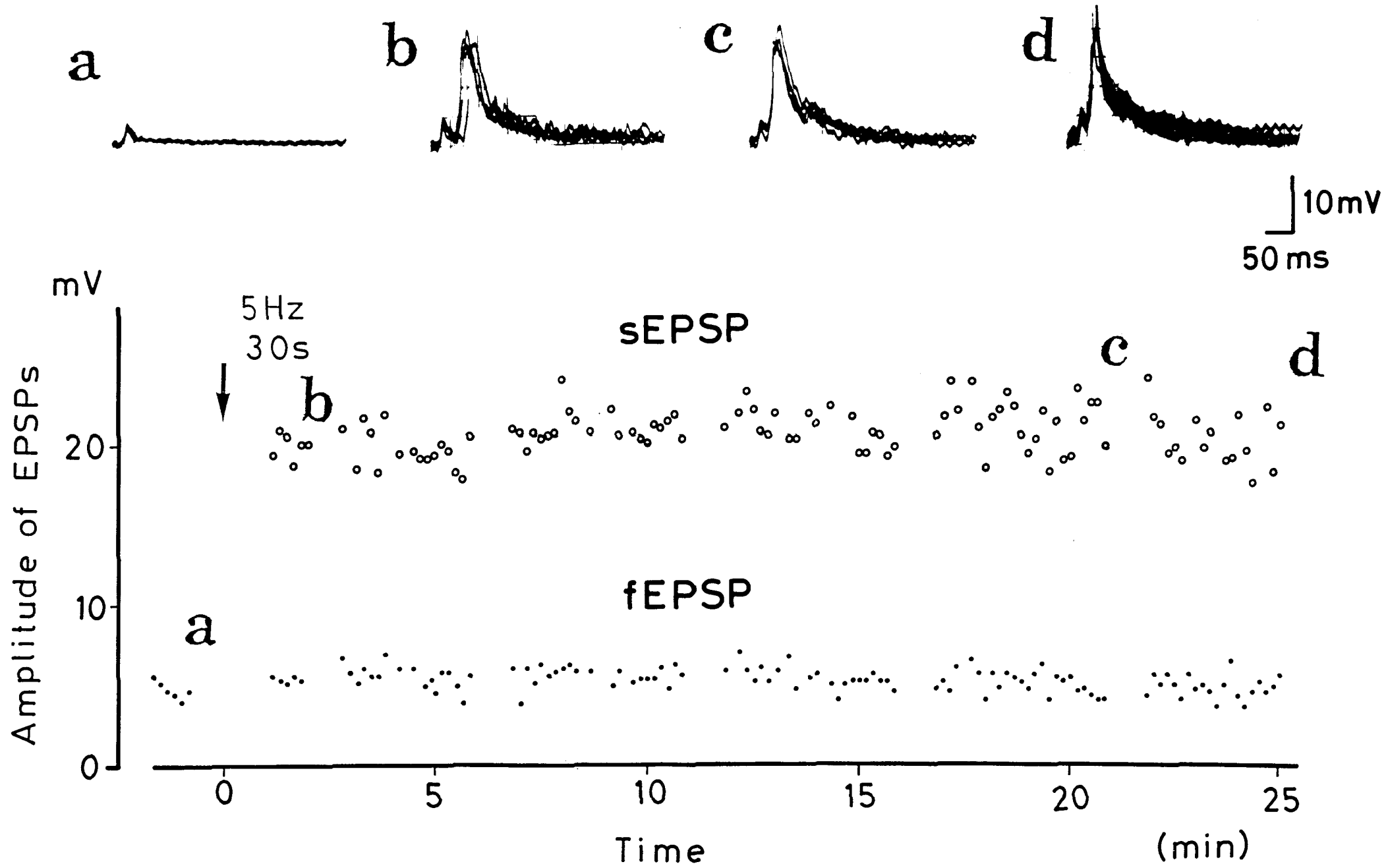
B Unitary Response

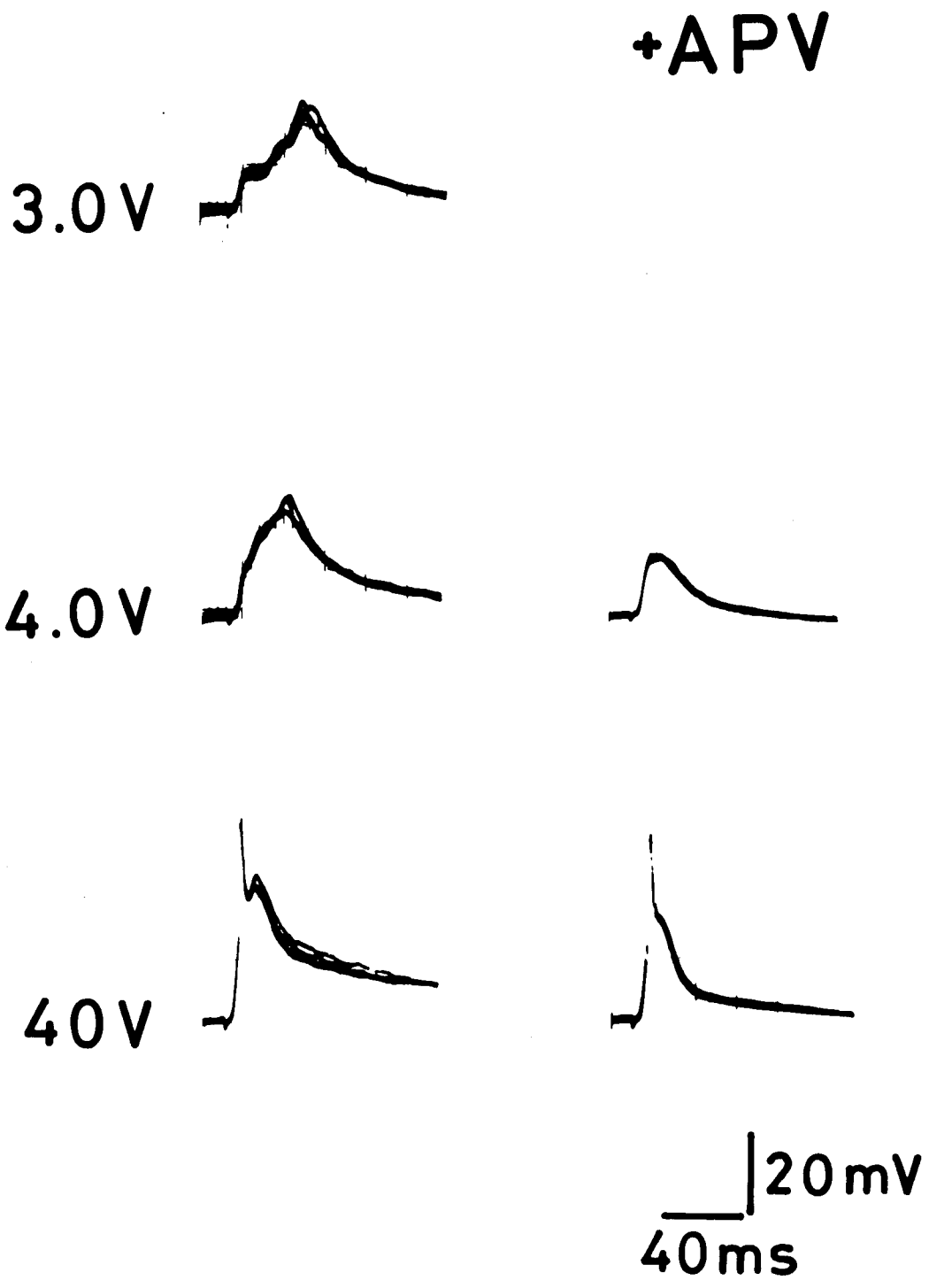


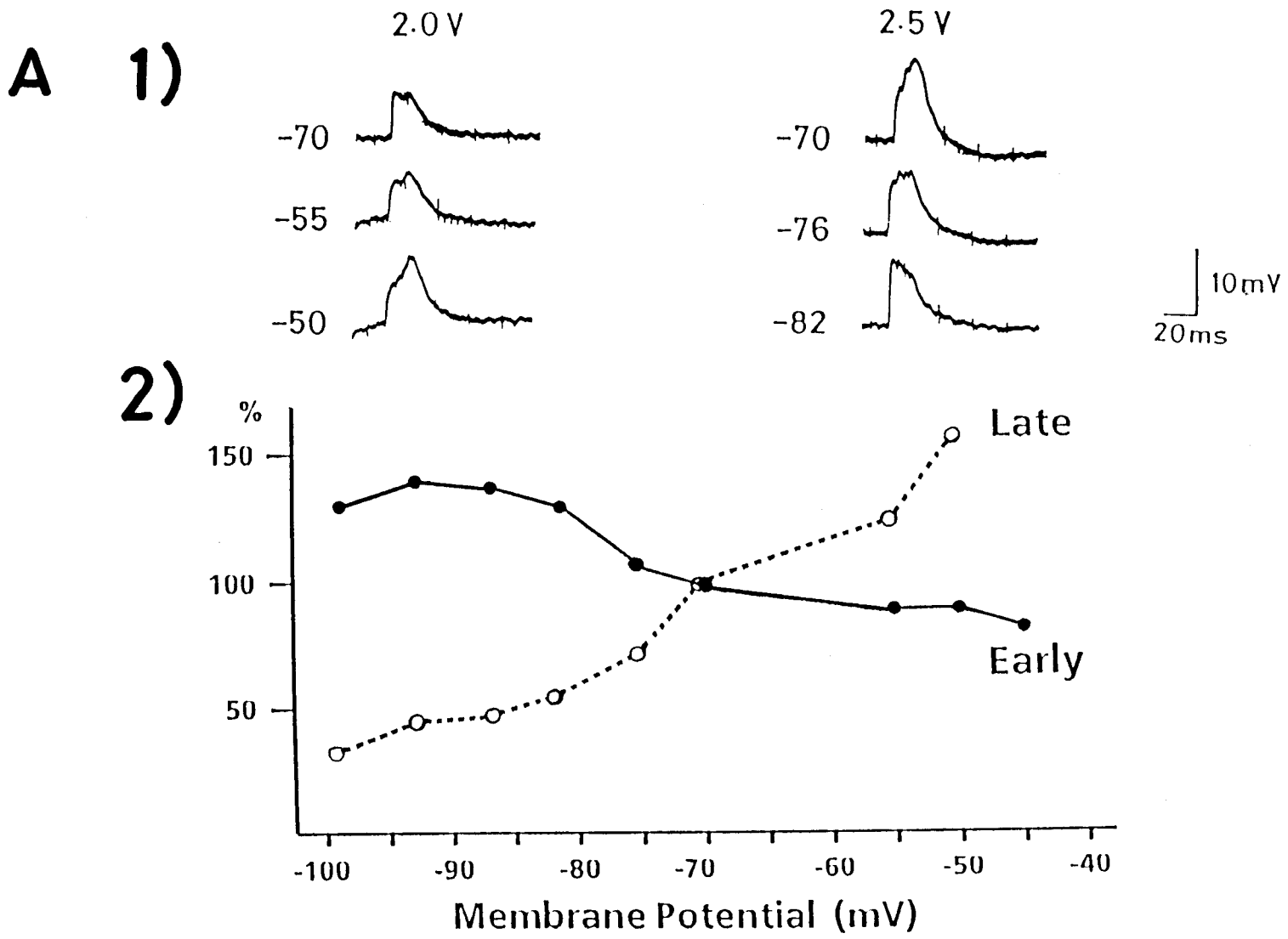

a
b
c
0.5mV
5ms
5Hz
60s
mV
0.8
0.6
0.4
0
a
b
c
primary
secondary
0
10
20
30
40
min

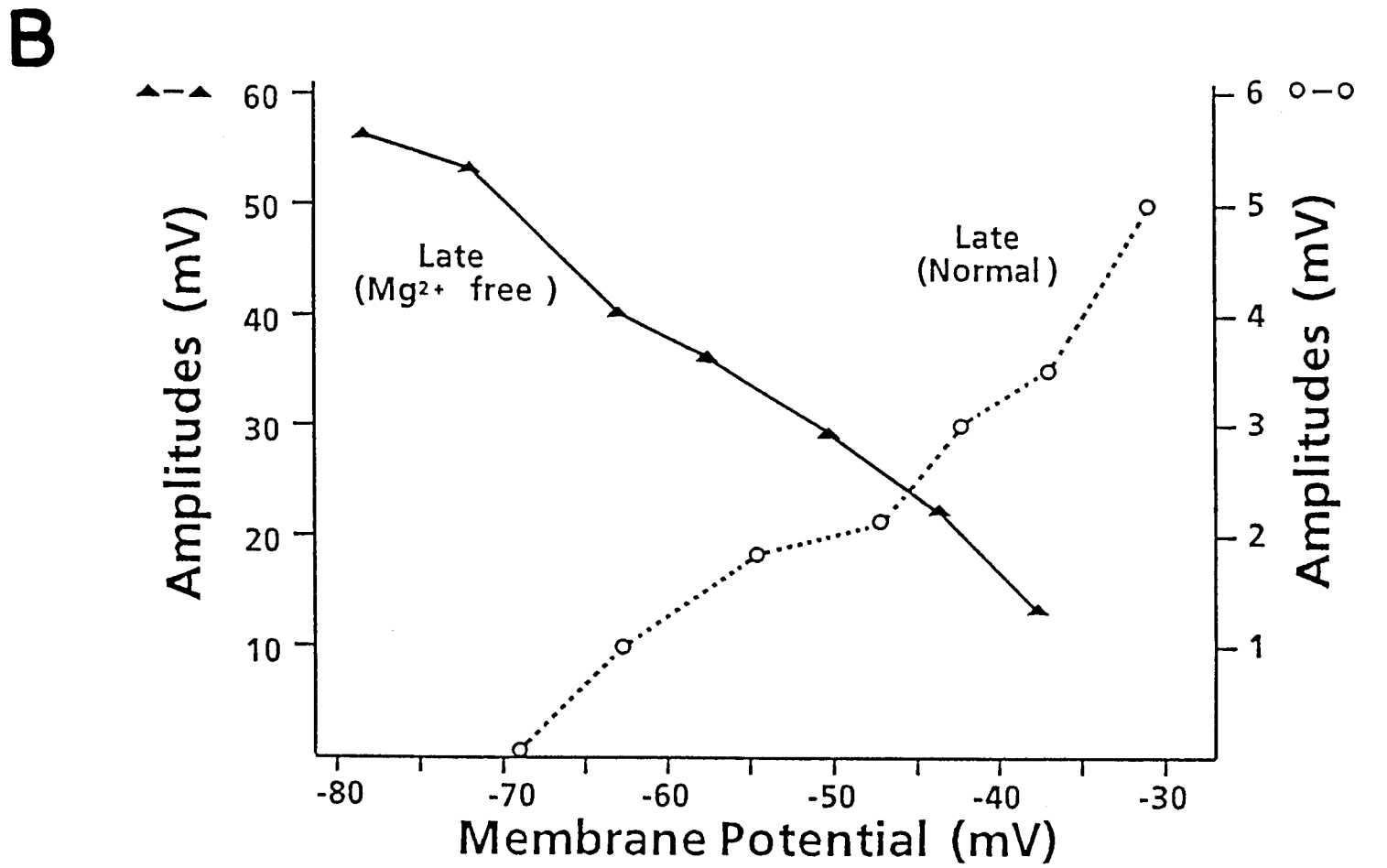

Control
5Hz 60s
5min
10min
15min
20min
1mV
5ms

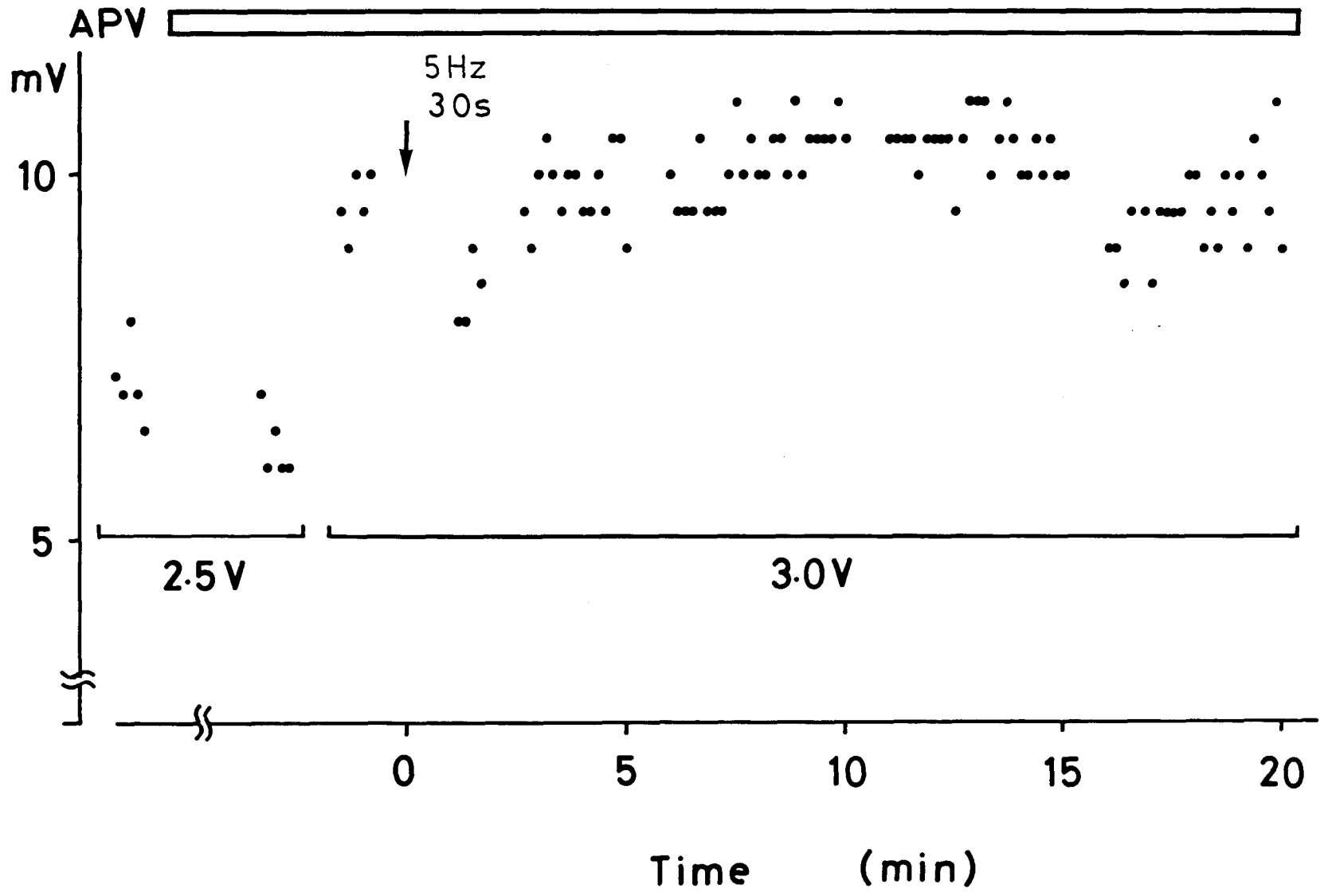

a
b
c
d
10mV
50 ms
(%)
150
100
50
0
Amplitude of EPSPs
5Hz
30s
a
b
c
d
0
5
10
15
20
25
Time
(min)


a
b
c
d
10mV
50 ms
mV
5Hz
30s
sEPSP
fEPSP
Amplitude of EPSPs
20
10
0
0
5
10
15
20
25
Time
(min)


+APV
3.0V
4.0V
40V
20mV
40ms


A 1)
2.0 V
2.5 V
-70
-55
-50
-70
-76
-82
10mV
20ms
2)
%
150
100
50
Late
Early
-100
-90
-80
-70
-60
-50
-40
Membrane Potential (mV)


B
Amplitudes (mV)
60
50
40
30
20
10
Late
(Mg2+ free)
Late
(Normal)
6
5
4
3
2
1
Amplitudes (mV)
-80
-70
-60
-50
-40
-30
Membrane Potential (mV)


APV
mV
5Hz
30s
10
5
2·5V
3·0V
0
5
10
15
20
Time (min)

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Postnatal development of immunohistochemically localized spectrin-like protein (calspectin or fodrin) in the rat visual cortex: its excessive expression in developing cortical neurons

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Summary

Postnatal development of the expression and localization of a membrane-associated cytoskeletal protein, calspectin (fodrin or brain spectrin), in the visual cortex, was immunohistochemically studied in newborn to adult rats, by using an anti-calspectin antibody. At birth, calspectin-immunoreactivity was already present at the plasma membrane and in the cytoplasm of neurons which were mostly pyramidal cells located in the upper part of the cortical subplate. Immature neurons located in the cortical plate were not stained by the antibody, suggesting that calspectin is expressed only in neurons which have differentiated or are differentiating.

At postnatal days 2 to 7, immunoreactive neurons were dramatically increased in layers V and VI and very intense labelling was seen in the apical dendrites of layer V pyramidal cells. Most of the stained processes of these and other neurons showed signs of rapid dendritic growth, i.e. non-terminal as well as terminal growth cones and filopodia. At days 10 to 17, dendrites of pyramidal cells in layers II and III became clearly detectable, although still slender. At days 24 to 34, the basal dendrites of pyramidal cells in layers II, III and V became intensely immunoreactive and dendritic spines were visualized by the antibody. In the adult, however, the calspectin immunoreactivity became very weak and spines were not recognizable. At all the ages, axons and neuroglia were unstained. Also, most of the neurons in layer IV of the cortex were not immunoreactive.

These results suggest that calspectin is most abundantly expressed in growing parts of the dendrites and spines. A hypothesis that calspectin may play a role in synaptic plasticity in the developing visual cortex is discussed.

Introduction

Calspectin (fodrin or non-erythrocyte spectrin) is a membrane-associated cytoskeletal protein found in various tissues including the brain (Goodman *et al.*, 1981; Kakiuchi *et al.*, 1981; Levine & Willard, 1981; Bennett *et al.*, 1982; Burridge *et al.*, 1982; Sobue *et al.*, 1982). While erythrocyte spectrin consists of α (M_r, 240 kDa) and β (220 kDa) subunits, calspectin is made up of α and γ (235 kDa) subunits (Bennett *et al.*, 1982; Glenney *et al.*, 1982a, b; Lazarides & Nelson, 1983). The brain is particularly rich in this protein, which is also referred to as brain spectrin (Zagon *et al.*, 1984). By analogy with erythrocyte spectrin, calspectin is supposed to control neuronal cell shape and lateral mobility of cell surface proteins in association with actin and other calcium-activated proteins (Burridge *et al.*, 1982; Levine & Willard, 1983; Goodman & Zagon, 1984). From the findings that calspectin and a calcium-activated protease to degrade it are concentrated in postsynaptic densities (Kakiuchi *et al.*, 1982b; Carlin *et al.*, 1983; Goodman *et al.*, 1983; Siman *et al.*, 1984; Levine & Sahyoum, 1986), calspectin is hypothesized to play a role in synaptic plasticity by inducing morphological changes in postsynaptic structures and/or positional changes in postsynaptic receptors (Goodman & Zagon, 1984; Lynch & Baudry, 1984; Siekevitz, 1985).

In the visual cortex of the monkey, cat and probably man also, it is well established that the degree of synaptic plasticity, estimated by susceptibility of binocular responsiveness of cortical neurons

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to monocular visual deprivation, changes with age and becomes almost null after the 'critical' or 'sensitive' period of postnatal development (Hubel & Wiesel, 1970; Awaya *et al.*, 1973; Von Noorden, 1973; Fregnac & Imbert, 1984). In morphological and behavioural studies of rats, it was also demonstrated that there is such a sensitive period in the development of their visual cortex (Rothblat *et al.*, 1978; Rothblat & Schwartz, 1979). If calspectin is somehow related to synaptic plasticity, its expression and localization in the visual cortex may change with age in a similar manner to the degree of plasticity. We tested this possibility by using immunohistochemical staining of cortical neurons with anti-calspectin antibody. We found that calspectin is expressed abundantly in the dendrites and somas of visual cortical neurons of the rat in the sensitive period, but its expression becomes rather faint in the adult.

Materials and methods

Purification of calspectin

A synaptic membrane-rich microsomal fraction of the bovine brain, prepared as described previously (Kakiuchi *et al.*, 1982a, b), was suspended in a warm (37°C) hypotonic medium consisting of 0.1 mM sodium phosphate (pH 8.0), 0.2 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM ATP, 0.25 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM diisopropylfluorophosphate (DFP) and 0.05 $\mu g\,ml^{-1}$ pepstatin A. The suspension was gently stirred for 15–30 min and then chilled on ice. It was then centrifuged at 105 000 *g* for 60 min. Calspectin extracted into the supernatant fluid was concentrated by ammonium sulphate fractionation (25–60% saturation). The concentrate, dissolved with a minimum amount of buffer I, consisting of 20 mM Tris–HCl (pH 7.5), 0.1 mM dithiothreitol and 100 mM KCl, was clarified by centrifugation for 30 min at 200 000 *g* and then applied to a column of Sepharose 4B which had been equilibrated with buffer I plus 0.1 mM EGTA. The column was eluted with the same medium and a fraction containing calspectin (tetramer) was collected. This fraction was further purified by calmodulin affinity column chromatography as previously described (Kakiuchi *et al.*, 1981). In short, calspectin in this fraction was absorbed on a calmodulin–Sepharose column in the presence of 0.2 mM $CaCl_2$ and eluted from the column with buffer I plus 1 mM EGTA. The eluted fraction containing calspectin was applied to a DEAE–cellulose column pre-equilibrated with buffer I. Calspectin in a concentrated form (up to $2\,mg\,ml^{-1}$) was eluted from the column with 20 mM Tris–HCl (pH 7.5), 0.1 mM dithiothreitol and 500 mM KCl.

Purification of anti-calspectin IgG

Antiserum to bovine brain calspectin was produced in rabbits. Calspectin (1 mg) in 0.5 ml of 20 mM phosphate-buffered saline (PBS) was emulsified with an equal volume of complete Freund's adjuvant and injected into the footpads of three rabbits. Two additional injections (0.5 mg protein with incomplete Freund's adjuvant) were given on days 28 and 42, and the rabbits were bled on day 56. The anti-calspectin IgG fraction was prepared from the serum by ammonium sulphate fractionation (48% saturation) followed by protein A affinity chromatography. The anti-calspectin IgG thus obtained from the three rabbits was diluted with PBS (1:100) for immunohistochemistry.

Immunohistochemistry

Sixteen Sprague–Dawley rat pups, ranging in age postnatally from days 0 to 34, and four adult rats were used for this study (Table 1). The day of birth was termed postnatal day 0. The animals were deeply anaesthetized with urethane and then perfused through the heart for 10–15 min with a solution of 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and placed in 30% sucrose in 0.1 M phosphate buffer until they sank completely. Sections (10–20 μm thick) were cut coronally in a cryostat at −20°C and washed in PBS. Every third to fourth section was stained with Cresyl Violet to identify cytoarchitectural details of the cortex. Other sections were incubated in 1% rabbit calspectin antibody solution containing a few drops of 0.2% Triton X-100 for 48 h. To test specificity of the antibody solution, an additional few sections were incubated in 1% rabbit calspectin antibody solution preincubated with calspectin. The sections were washed three times for 10 min each in PBS, then incubated in 1% goat anti-rabbit IgG for 12 h. They were again washed three times for 10 min each in PBS, then incubated in 1% HRP-conjugated rabbit IgG for 12 h. For HRP reaction, preincubation solution and reaction solution were prepared. The sections were first placed in the preincubation solution containing 0.05% diaminobenzidine and 0.002% nickel ammonium in 0.1 M phosphate buffer (pH 7.4). The tissues were then transferred to the reaction solution provided by adding 0.01% hydrogen peroxide to preincubation solution. Reaction product was developed within

Table 1. Age distribution of the animals studied.

Age (postnatal days)	*No. of animals*
0	2
2	1
3	1
5	1
6	1
7	1
10	1
12	1
13	1
15	1
17	1
24	1
26	1
30	1
34	1
Adult	4
Total	20

15–20 min before the reaction was stopped by quick rinses in PBS.

Results

Immunological characterization of the anti-calspectin IgG

Immunoblotting was performed to test whether our antibody raised in rabbits against purified bovine calspectin could specifically recognize the α and γ subunits of calspectin in the rat brain. The purified bovine calspectin and whole brain homogenates from a rat pup at postnatal day 2 and an adult rat were separated by SDS gel electrophoresis containing 5% polyacrylamide using the buffer system of Laemmli (1970). Two identical gels were run simultaneously (Fig. 1). One gel was stained with Coomassie brilliant blue and destained with 7% acetic acid (Fig. 1A). The other gel was subjected to immunoblotting using anti-calspectin antibody according to the method of Towbin *et al.* (1979) (Fig. 1B). From this figure it is obvious that our antibody against purified bovine calspectin is specific for calspectin, staining only α (240 kDa) and γ (235 kDa) subunits of calspectin in the rat brain. In Fig. 1B the intensity of staining between lanes 2 and 3 seemed to be about the same. This does not necessarily imply, however, that there were no significant differences in the levels of calspectin between neonatal and adult brains, since the linearity of quantification of protein by this method is defined within a limited range and the levels of calspectin were beyond this range.

Specificity of the antiserum

Specificity of the anti-calspectin IgG was immuno-

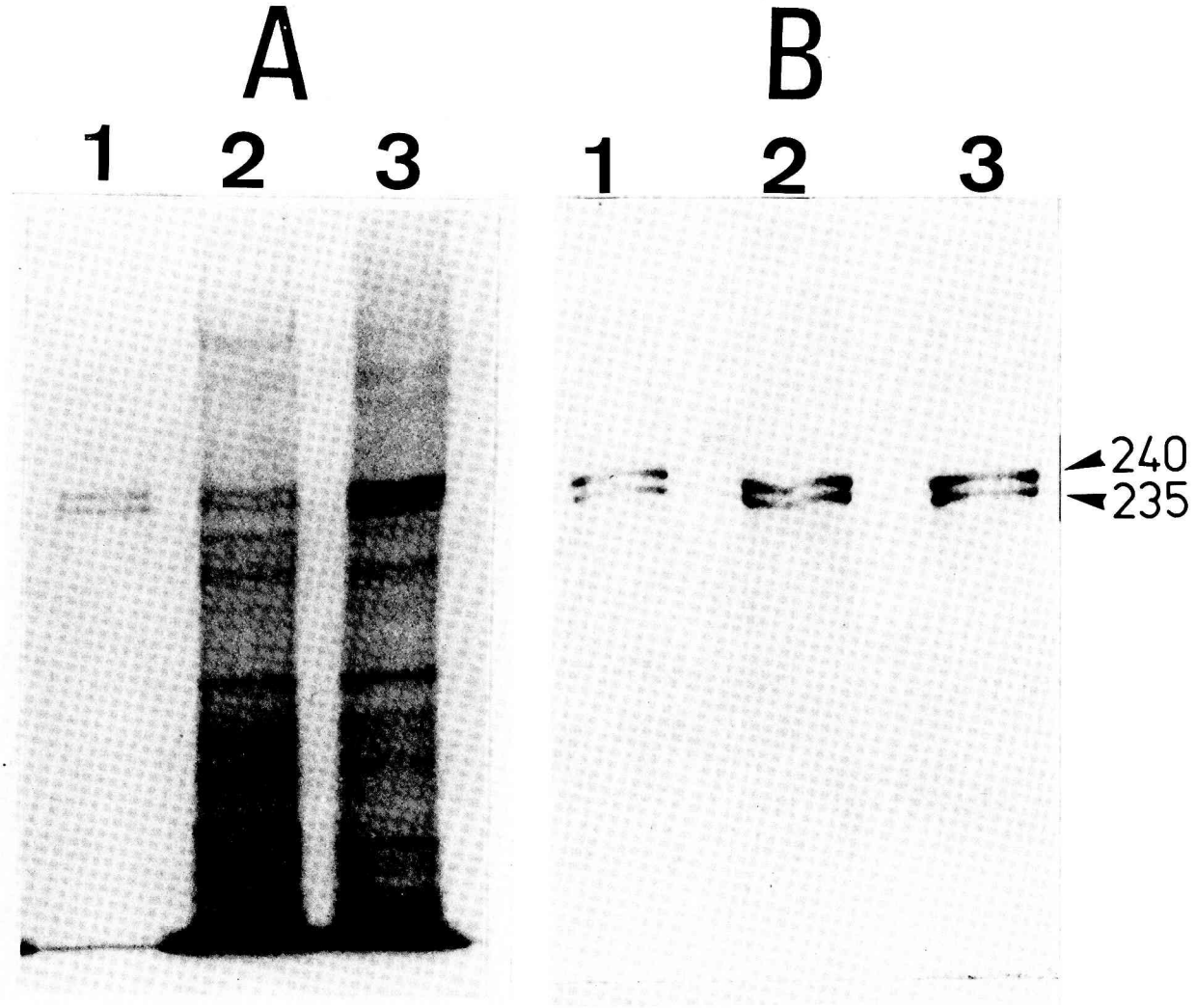


Fig. 1. Characterization of the anti-calspectin IgG by immunoblotting of bovine brain calspectin (lane 1), the total homogenate of neonatal rat brain (lane 2) and that of adult rat brain (lane 3). The neonatal rat was at postnatal day 2. (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) stained with Coomassie brilliant blue. (B) Immunoblotting using the anti-calspectin IgG. SDS–PAGE shows that both the neonatal and adult rat brain homogenates contain α (240) and γ (235) subunits of calspectin. This immunoblotting shows that the anti-calspectin IgG is specific to both the subunits of calspectin.

histochemically confirmed following three observations. No staining was observed when: (i) the anti-calspectin IgG solution was replaced by normal serum, (ii) the anti-calspectin solution was omitted from the immunohistochemical procedures, or (iii) the anti-calspectin IgG was pre-adsorbed with calspectin. Thus, our anti-calspectin solution could be used as a reliable probe for the immunohistochemical localization of calspectin in the brain. In a strict sense, the immunopositive structures identified in the present study should be described as showing calspectin-like immunoreactivity, but in this paper we will use the simpler term calspectin immunoreactivity instead.

Postnatal day 0

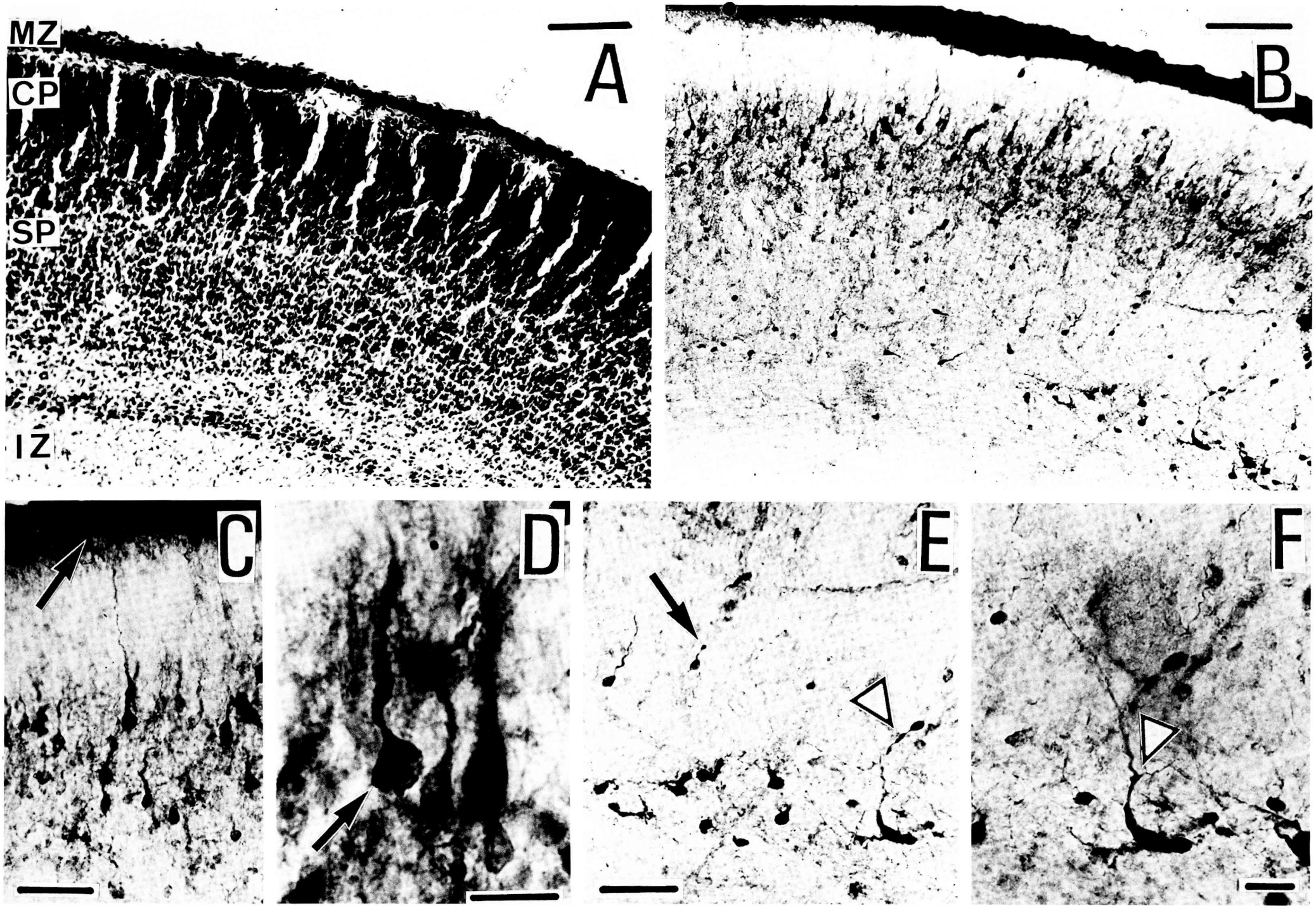
Just after birth the cortical area, corresponding to area 17 of the adult cortex (Krieg, 1946), is very thin and has four distinguishable layers: a marginal zone, a cortical plate, a subcortical plate and an intermediate zone (Fig. 2A). The deepest part of the subcortical plate is distinct from the rest of it. The cortical plate has a colonnade appearance and is filled with small, elongate cell bodies of uniform size, as described previously (Eayrs & Goodhead, 1959; Berry & Rogers, 1965; Lund & Mustari, 1977; Parnavelas *et al.*, 1978; Juraska & Fifkova, 1979; Miller, 1981). In the subcortical plate there are larger cells which are less densely packed and more mature in appearance. This layer is destined to become layers V and VI of the mature cortex (Berry & Rogers, 1965; Lund & Mustari, 1977; Juraska & Fifkova, 1979; Miller, 1981).

Calspectin-immunoreactive neurons were mainly located in the upper part of the subcortical plate, although they were also present in the lower part (Fig. 2B). In most of the immunoreactive cells, the cytoplasm was intensely stained, but in a substantial number of cells, only the plasma membrane seemed to be stained (arrow in Fig. 2D). In the majority of the reactive cells located in the upper subcortical plate, their apical dendrites were clearly stained and often seen to reach the pial surface (arrow in Fig. 2C). Usually at this age, no collateral processes were seen along the shafts of the apical dendrites. The basal dendrites were not visualized by the antibody. This may be due not to a failure of the staining but to the fact that the basal dendrites of immature pyramidal cells have not yet emerged (Berry & Rogers, 1965; Juraska & Fifkova, 1979; Miller, 1981). Long basal processes which may correspond to axons were not detected in the present preparations, although they are clearly visualized by the Golgi staining even in late rat foetuses (Peters & Feldman, 1973). An interesting finding at this age was that neurons located in the middle and bottom of the subplate had processes with growth cones which were intensely stained by the antibody (Fig. 2E, F). These growth cones included non-terminal varicosities or growth buds (Morest, 1969) as well as terminal growth cones (arrowheads and arrow in Fig. 2E and F). It is to be noted that neurons located in the upper part of the subplate had vertically oriented somas and dendrites while those located in the deepest part of the subplate often had horizontally or obliquely oriented main processes (Fig. 2B, E). In no layer of the cortex were there immunoreactive cells which could be classified as neuroglia or glioblasts (Smart & Leblond, 1961), although the migration of such cells towards the cortical plate may start at day 21 of pregnancy (Hicks & d'Amato, 1968). At no age were neuroglia stained by the anti-calspectin antibody.

Postnatal days 2 to 5

At this stage of development the thickened subcortical plate could be divided into layers V and VI, but the cortical plate was not yet differentiated into layers IV, III and II (see Fig. 3A). Examples of the visual cortex at these ages are shown in Figs 3 and 4. Compared with the cortex at birth, the immunoreactive cells were dramatically increased in layers V and VI (Fig. 3B, C). A particularly heavy labelling was seen in layer V in which stained cells were lined up in a broad horizontal zone (Figs 3B, 4A). These cells had prominent apical dendrites reaching the pial surface of the cortex and their somas were triangular or pyramidal in shape so that the cells were regarded as being pyramidal cells (Fig. 3C, middle zone). The shafts of apical dendrites were not smooth but uneven, and seemed to have a series of swellings or varicose enlargements. The terminal branches of

Fig. 2. Photomicrographs of coronal sections of the visual cortex stained by Cresyl Violet (A) and the anti-calspectin antibody (B–F). The rat pup used was just after birth (postnatal day 0). In (A): MZ, marginal zone; CP, cortical plate; SP, subcortical plate; IZ, intermediate zone. In (B–F) pia and immunoreactive cells are darkly stained. Pia was stained even when the anti-calspectin IgG was pre-absorbed with calspectin. In this and subsequent figures, therefore, the staining of pia was regarded as non-specific. (A) and (B) are from serial sections. (C) A higher magnification picture showing immunoreactive neurons located in the subcortical plate. Their apical dendrites are often seen to reach the pial surface (arrow). The darkly stained pia is seen at the top. (D) A high magnification picture showing preferential staining of the plasma membrane of neurons. (E) A higher magnification picture from the bottom right of (B) showing calspectin immunoreactive growth cones of two types, such as non-terminal (arrowhead) and terminal growth cones (arrow). Stained cells in the lower part are located at the border between the subcortical plate and the intermediate zone. (F) A higher magnification of (E). Scale bars: (A, B) 100 μm; (C, E) 50 μm; (D, F) 20 μm.


MZ
CP
SP
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these apical dendrites were intensely stained by the antibody and formed dendritic arches in layer I (Fig. 4A). Thus, a continuous interwoven arcade of the fine dendritic trees in layer I of the cortex was one of the most conspicuous of the stained structures at these ages. There was a tendency for the majority of the intensely stained dendritic shafts to be gathered into fascicles (Fig. 4B). Consequently, the strongly stained dendritic arcades seemed to form a series of inverse cones in layer I of the cortex. Immunoreactive swellings of processes, which were probably growth cones, were seen at the branching point of the apical dendrites. At higher magnification, spots or clusters of the intensely staining reaction products associated with the membrane were seen in the proximal shaft of apical dendrites (arrowheads in Fig. 4C).

Most of the cells located in layer VI of the cortex were also stained by the antibody (Fig. 3C, lower part). In particular, cells located at the bottom of the layer were intensely immunoreactive (Fig. 3B, C). These cells had processes showing signs of rapid dendritic growth, i.e. irregular enlargements, growth cones and filopodia (Fig. 3C, bottom) (Morest, 1969). Also, it is noteworthy that their main processes were not always vertically oriented. In the cortical plate, stained cells were relatively rare at postnatal days 2 to 5 (see Figs 3B, 3C, 4A). In the lower part of the cortical plate, which is destined to become layer IV, there were a few weakly immunoreactive cells with relatively large, oval somas (arrow in Fig. 3C), which appeared to be non-pyramidal cells.

Postnatal days 6 and 7

At these ages, pyramidal cells in layer V seemed to grow their basal dendrites very rapidly. The basal dendrites were intensely immunoreactive, although secondary and tertiary branches were not yet visible in most of the cells. Many of the basal dendrites had growth cones, from which slender processes passed in various directions (arrow in Fig. 4E). At the bottom of layer VI there were immunoreactive cells having horizontally or obliquely oriented main processes with varicosities or growth cones (Fig. 4D), indicating that they were still growing.

Postnatal days 10 to 13

At this stage of development, somas and dendrites of layer V pyramidal cells were intensely stained by the antibody (Fig. 5B–D). However, the trunk and fine distal branches of the apical dendrites were not completely stained so that they seemed to be relatively sparse in layers IV and I of the cortex (Fig. 5A). On the other hand, the basal dendrites of layer V pyramidal cells were more intensely immunoreactive than at postnatal days 6 and 7 (Fig. 5C, D). In layers II and III there were also immunoreactive cells with fairly well-stained apical dendrites (Fig. 5A, B). However, their basal dendrites seemed to be still slender and relatively poorly arborized (see cells in the upper part of Fig. 5B). At these ages, neurons in layer IV were not immunoreactive so that only parts of the apical dendritic shafts of layer V pyramidal cells were seen in this layer (Fig. 5A, B).

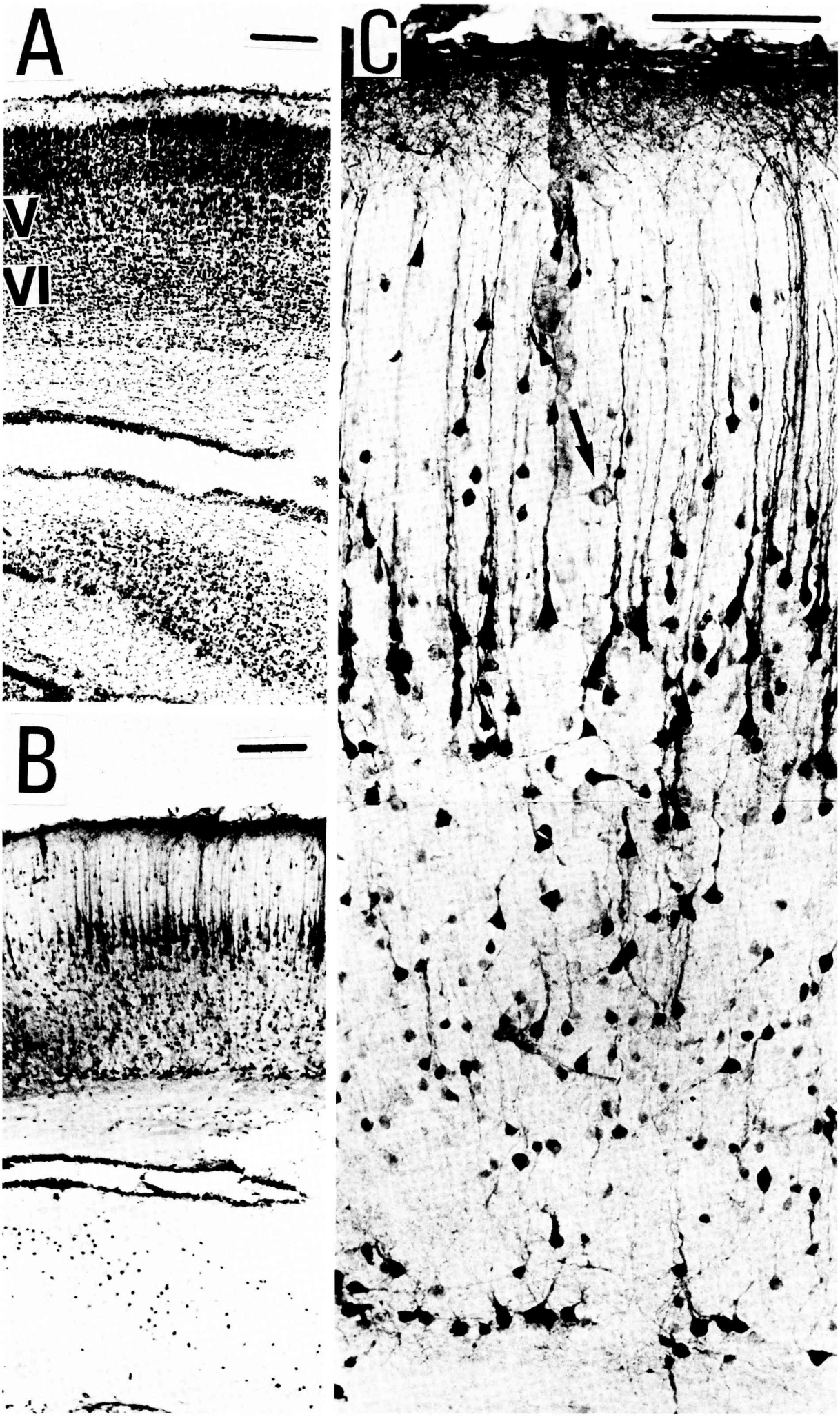
Postnatal days 15 and 17

At these ages, the staining pattern and laminar distribution of calspectin immunoreactive structures were essentially the same as those at postnatal days 10 to 13, although the basal dendrites of layer V pyramidal cells were slightly more complexly arborized and more intensely stained by the antibody. Also, most of the pyramidal cells in layers II and III had basal dendrites which were immunoreactive but still slender.

Postnatal days 24 and 26

At these ages, the basal dendrites of layer V pyramidal cells became more intensely immunoreactive than at days 15 to 17 (Fig. 6A, B, D, E). Quarternary to sexternary branches of the basal dendrites were clearly stained so that they made a firm network in layer V of the visual cortex (Fig. 6D). Somas and apical dendrites of layer V pyramidal cells were also immunoreactive. However, their apical dendrites were not thoroughly stained by the antibody. Only the shaft of the apical dendrites up to layer II was immunoreactive and, thus, its terminal arborization in layer I was not seen (Fig. 6A). Along the dendritic shafts the stained spines were recognizable (arrows in Fig. 6F). Pyramidal cells located in layers II and III were also stained by the antibody. Their somas and dendrites, basal as well as apical, were clearly visualized, although the staining was not so conspicuous as that of layer V pyramidal cells (Fig. 6C). A small number of non-pyramidal cells in layer IV were also immunoreactive. In layer VI of the cortex, immunoreactive cells were scattered and few of them had main dendrites which were obliquely or

Fig. 3. Photomicrographs of coronal sections of the visual cortex at postnatal day 5 stained with Cresyl Violet (A) and anti-calspectin IgG (B, C). In (A) and (B) the hippocampus is seen below the cortex. (C) A higher magnification picture of the cortex from (B). The bottom of this picture corresponds to the border between layer VI and the white matter. In addition to pyramidal cells in layer V (middle zone), cells in layer VI are also heavily stained. A non-pyramidal cell located in layer IV is weakly stained (arrow). Scale bars: (A, B) 400 μm; (C) 100 μm.


A
V
VI
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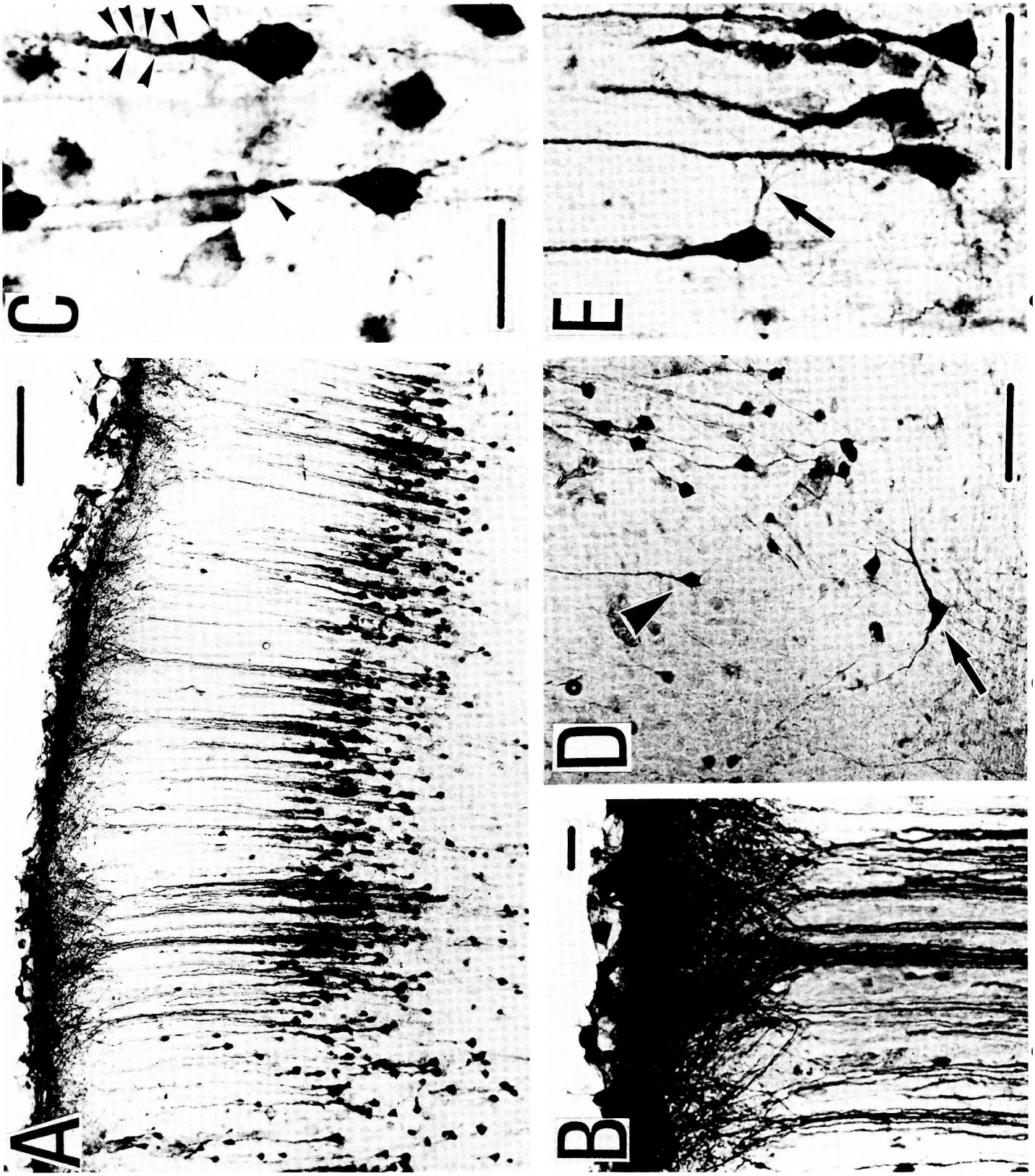
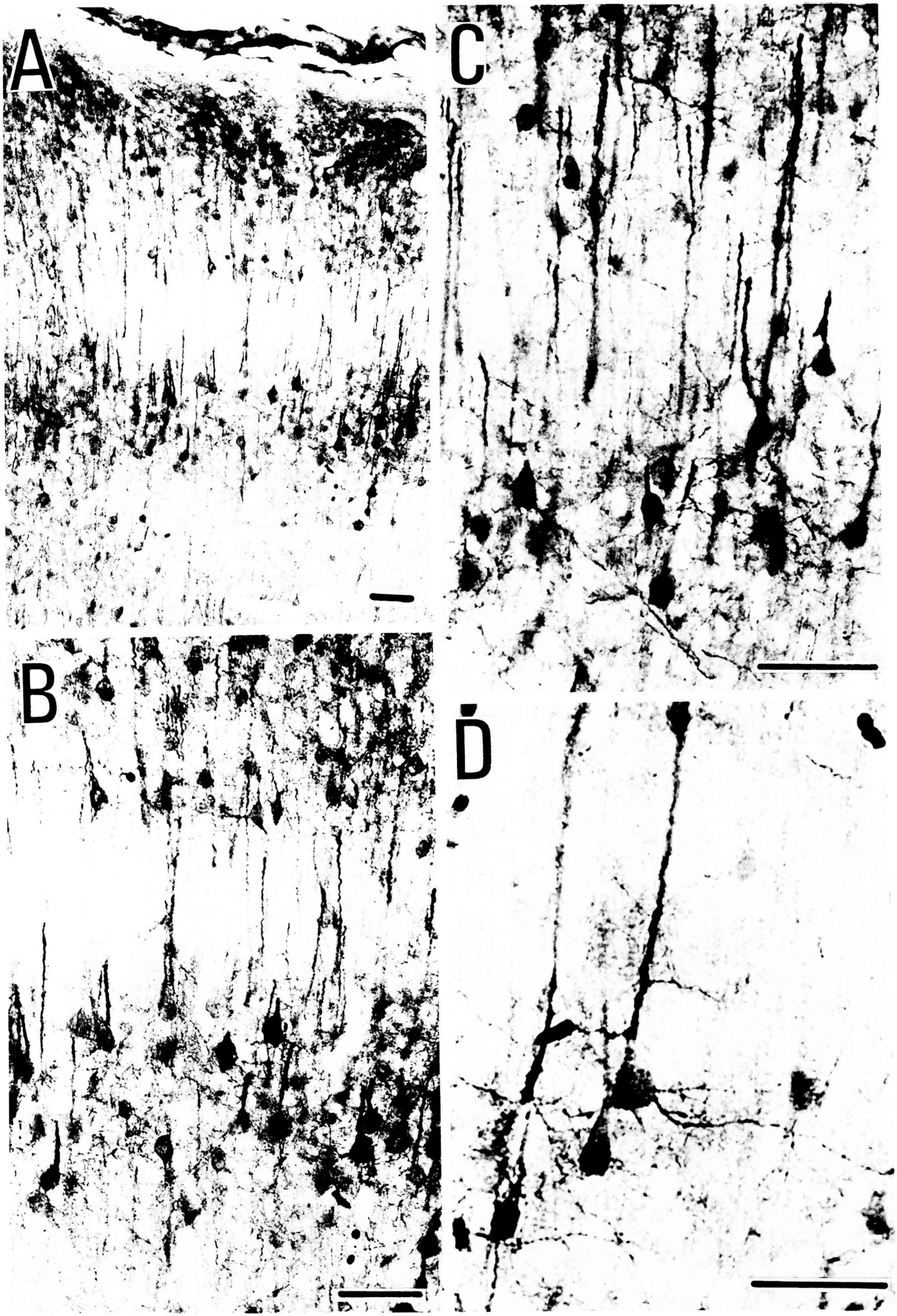


Fig. 4. (Above). Photomicrographs of coronal sections of the visual cortex at postnatal day 5 (A–C) and at postnatal day 7 (D, E) stained with the anti-calspectin antibody. (A) A low magnification picture showing intense labelling of pyramidal cell bodies in layer V and dendritic terminal arcades in layer I of the cortex. (B) A higher magnification picture from (A). (C) A high magnification picture of layer V pyramidal cell bodies. Arrowheads indicate immunoreactive spots associated with the plasma membrane of the trunk of apical dendrites. (D) A high magnification picture showing layer VI of the visual cortex at postnatal day 7. Neurons with horizontally or obliquely oriented dendrites (arrow) were seen at the bottom of layer VI and a neuron with long, vertically oriented apical dendrites (arrowhead) was seen at the upper layer VI. (E) A high magnification picture showing a layer V pyramidal cell with a prominent growth cone at the basal dendrite. Scale bars: (A) 100 μm; (B, C) 20 μm; (D, E) 50 μm.

Fig. 5. (Over page). Photomicrographs of coronal sections of the visual cortex at postnatal day 10. (A) A low magnification picture showing that neurons in layers II, III and V are intensely stained by the antibody. (B) A higher magnification picture from (A). The upper part is layer III. (C) A further higher magnification picture from (B). (D) A higher magnification picture showing layer V pyramidal cells with long basal dendrites extending in the horizontal direction. Scale bars: 50 μm.


A
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C
D

horizontally oriented as found at postnatal days 0 to 7.

Postnatal days 30 and 34

At this stage of development the basal dendrites of pyramidal cells in layer V were the most conspicuous of the stained processes in the cortex (Fig. 7A, B). The immunoreactivity of the extensively arborized basal dendrites seemed to be more intense than at any other age, although we did not quantitatively evaluate the degree of immunoreactivity of tissues. Even the fine branches of the basal dendrites were clearly stained so that they made up darkly stained, thick networks in layer V of the cortex (Fig. 7B). Such a network of basal dendrites was also seen in layers II and III, although the reactivity was less intense in these layers. At these ages, spines along almost all the branches of the apical and basal dendrites became clearly visible at higher magnification (arrows in Fig. 7C, D). The density of stained spines was much higher than that at postnatal days 24 and 26. This indicates that the immunoreactivity of spines became more intense than at postnatal days 24 and 26, since the size and density of spines, at least at bifurcating branches, were already adult-like before 21 days of age (Juraska & Fifkova, 1979; Miller, 1981; Juraska, 1982). Axons of the pyramidal cells in layers V, III and II were not recognizable. Most neurons in layer VI were also immunoreactive, while almost none of the cells in layer IV was stained by the antibody (Fig. 7A, B).

Adults

In the adult, immunoreactive neurons were mainly located in layers II, III and V of the visual cortex. However, their immunoreactivity was much weaker than at days 24 to 34 and the network of basal dendritic trees less conspicuous (Fig. 8A). Photomicrographs shown in Fig. 8 are examples of the most intensely stained sections among those from the four adult rats used in the present study. In the adult, spines of the basal and apical dendrites were not detectable. The apical dendrites of layer V pyramidal cells were only focally immunoreactive so that parts of the shaft appeared to be discontinuous (Fig. 8A). The apical and basal dendrites of pyramidal cells in layers II and III were also only patchily immunoreactive (Fig. 8A). It is to be noted that the apical dendrites of these and layer V pyramidal cells appeared to be spiral and became frayed near the end of the stained parts (Fig. 8B, top right). Axons of the pyramidal cells were not detectable at all. Most of the neurons located in layers IV and VI seemed not to be immunoreactive. Thus at all ages, neurons in layer IV of the rat visual cortex were for the most part unstained by the anti-calspectin antibody.

Discussion

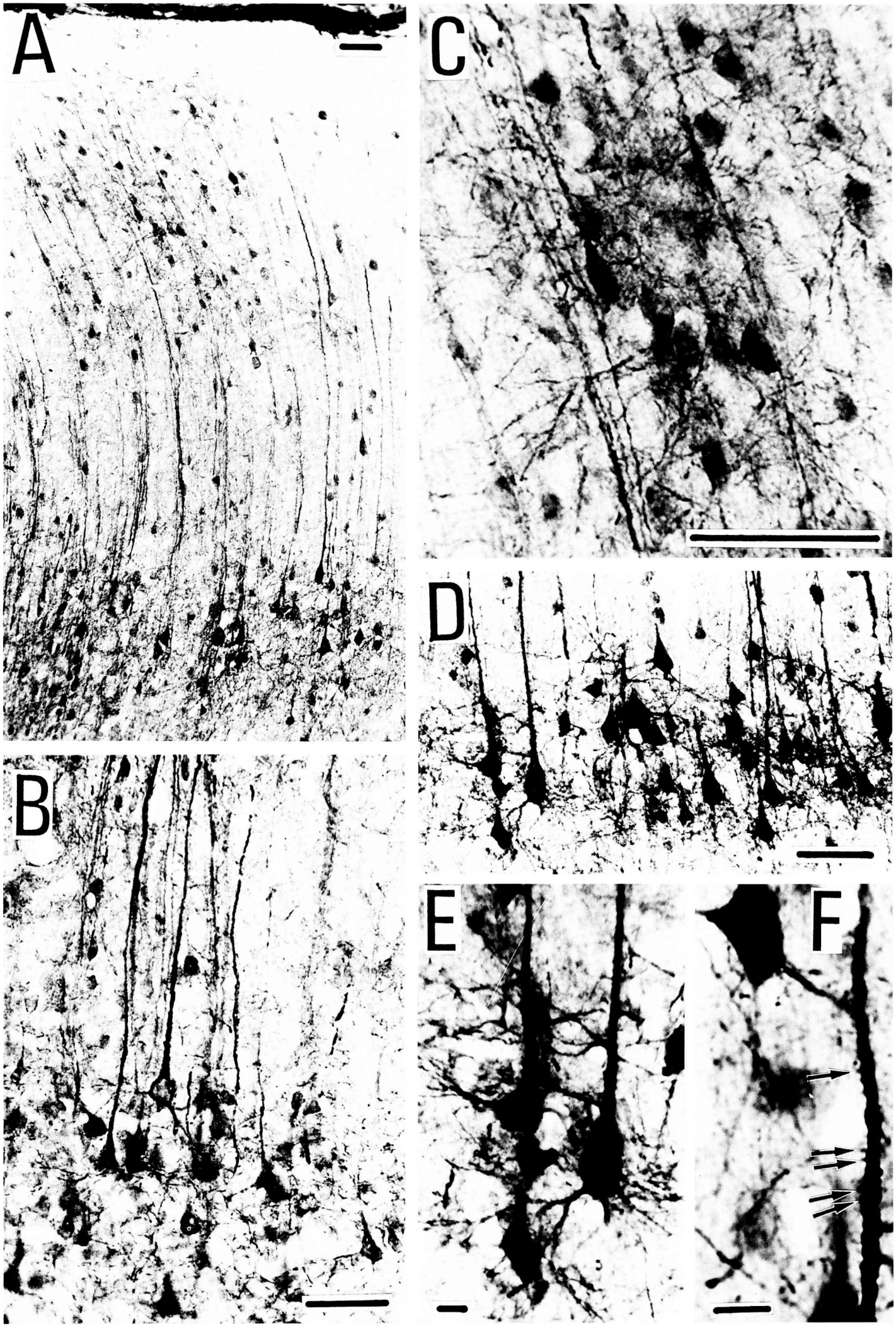
The present results have demonstrated that calspectin-immunoreactive neurons were already present at birth and they were mostly located in the upper part of the subplate of the occipital cortical area which corresponds to area 17 of the adult cortex. The labelled cells in the upper subplate had somas which were already triangular or pyramidal in shape and fine apical dendrites which were vertical to the pial surface. Thus, they could be classified as pyramidal cells, although at that stage the basal dendrites were not yet distinguishable.

On the other hand, none of the cells in the cortical plate was clearly stained by the anti-calspectin antibody. Even at postnatal days 2 to 5, most of the cells in the cortical plate were non-immunoreactive. Since neurons located in the cortical plate at these ages are immature and not yet differentiated (Eayrs & Goodhead, 1959; Berry & Rogers, 1965; Lund & Mustari, 1977; Parnavelas *et al.*, 1978; Juraska & Fifkova, 1979; Miller, 1981), the present results suggest that calspectin is expressed only in neurons which have differentiated or are differentiating. However, the already differentiated neurons were not always immunoreactive. Most of the neurons located in layer IV of the visual cortex were not stained by the antibody even at postnatal days 10 to 34 when neurons in other layers, particularly layers II, III and V, were intensely immunoreactive. Thus during all the stages of development layer IV of the visual cortex appeared empty in the immunohistochemically stained preparations. This point will be discussed later.

Regional distribution of calspectin within neurons and changes during development

Table 2 summarizes a qualitative estimate of the degree of calspectin immunoreactivity in different

Fig. 6. Photomicrographs of coronal sections of the visual cortex at postnatal day 26. (A) A low magnification picture showing immunoreactive cells located mostly in layers II, III and V of the cortex. The darkly stained pia is seen at the top. (B) A higher magnification picture showing intensely stained pyramidal cells in layer V. (C) A higher magnification picture from the upper part of (A) showing immunoreactive neurons in layer III. (D) A high magnification picture showing intensely stained somas and basal dendritic trees in layer V. (E) A higher magnification picture from the left side of (D). (F) A higher magnification picture showing intensely stained dendritic spines (arrows) along the apical dendrites of a pyramidal cell. Scale bars: (A–D) 50 μm; (E, F) 10 μm.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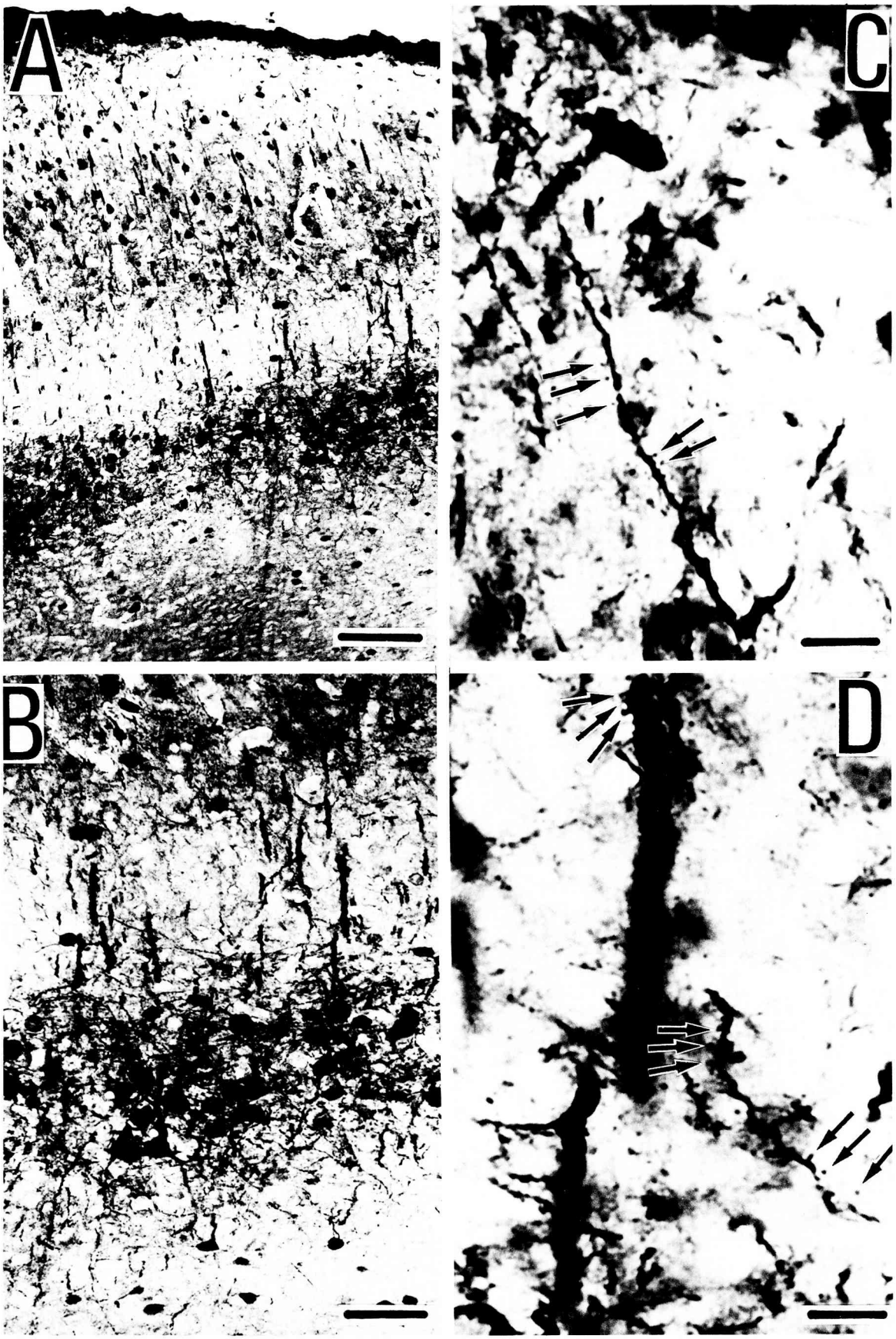

Fig. 7. Photomicrographs of coronal sections of the visual cortex at postnatal day 34, stained by the anti-calspectin antibody. (A) A low magnification picture showing the intensely stained neurons in layers II, III and V. (B) A higher magnification picture from (A). (C) A higher magnification picture showing dendritic spines (arrows) along branches of the apical dendrites of layer V pyramidal cells. (D) A higher magnification picture showing spines (arrows) along apical and basal dendrites of layer III pyramidal cells. Scale bars (A, B) 50 μm; (C, D) 10 μm.

Table 2. Qualitative estimate of calspectin immunoreactivity within pyramidal neurons in layers II+III and V.

	Soma		*Apical dendrites*		*Basal dendrites*		*Dendritic growth cones*		*Spines*	
	II+III	*V*	*II+III*	*V*	*II+III*	*V*	*II+III*	*V*	*II+III*	*V*
PD 0	–	++	–	+	–	–	–	+++	–	–
PD 2–5	+	+++	+	+++	+	+	++	+++	–	–
PD 6,7	+	+++	+	+++	+	++	++	+++	–	–
PD 10–17	++	+++	++	++	+	++	+	–	–	+
PD 24,26	++	+++	++	+	++	+++	–	–	+	++
PD 30, 34	++	+++	++	+	+++	+++	–	–	+++	+++
Adults	+	+	+	+	+	+	–	–	–	–

The reactivity showing very dark and complete staining is defined as +++, that showing less dark staining as ++, that showing faint and/or partial staining as +, and that showing no or little staining as –. PD, Postnatal day.

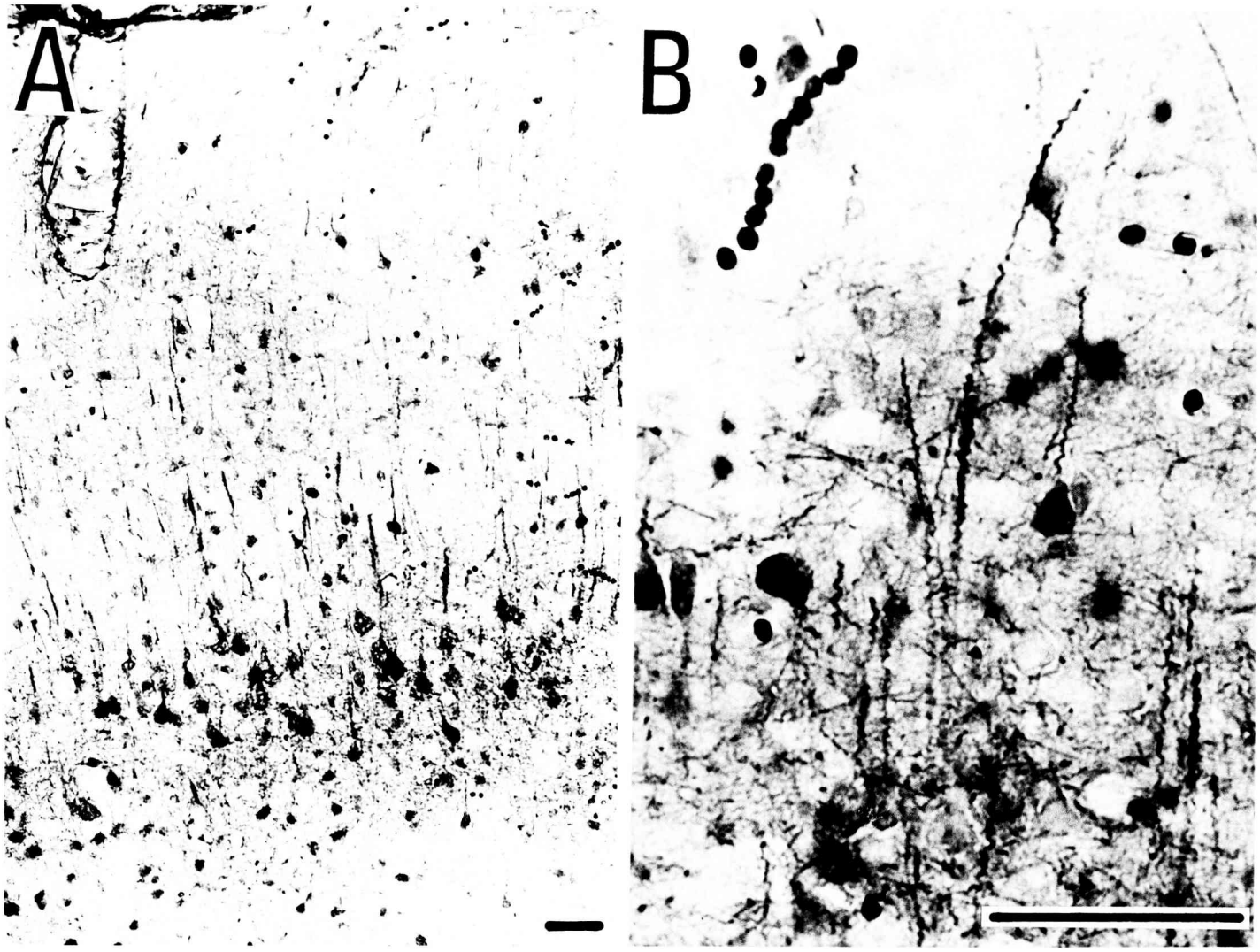

Fig. 8. Photomicrographs of coronal sections of the visual cortex of an adult rat, stained by the anti-calspectin antibody. (A) A low magnification picture showing weakly stained cortical neurons. (B) A high magnification picture showing relatively intensely stained neurons in layers II and III. Stained apical dendrites appeared spiral. Chains of dark circles seen in the upper half are residual red blood cells. Scale bars: 50 μm.

regions of neurons in layers II, III and V of the rat visual cortex. The somas and apical dendrites of layer V pyramidal cells were the structures first detected by the antibody in postnatal development of the visual cortex. In most of the stained cells the cytoplasm seemed to be intensely immunoreactive, but in a substantial number of cells only the plasma membrane of the soma and dendritic trunk were reactive. Throughout all the ages there was a tendency for the proportion of the cells in which only the plasma membrane was stained to be increased by using more dilute antibody solutions. These results suggest that calspectin may exist at the plasma membrane in a higher concentration than in the cytoplasm, or that its immunoreactivity at the membrane is stronger than in the cytoplasm.

In the present study the strongest immunoreaction was seen in the apical dendrites of layer V pyramidal cells at 2 to 7 days of age and in their basal dendrites at 24 to 34 days (Table 2). In most of the neurons in layers II and III, their apical dendrites were unambiguously visualized by the antibody after about 10 days of age, i.e. about 3 to 8 days later than those of layer V pyramidal cells. This seems consistent with the previous morphological and physiological results that pyramidal cells in layers II and III mature later than those in layer V of the visual cortex of the rat, cat and monkey (Berry & Rogers, 1965; Lund *et al.*, 1977; Juraska & Fifkova, 1979; Miller, 1981; Tsumoto & Suda, 1982).

Except for the adult, dendrites of cortical neurons, particularly those in layers II, III, V and VI, were intensely stained by the anti-calspectin antibody. Before postnatal day 7, the non-terminal as well as terminal growth cones and filopodia along these dendrites were the most conspicuous of the stained structures. This observation is consistent with a recent study which reported that neurite extensions and growth cones of neurons in culture were strongly stained by the anti-spectrin antibody (Hesketh *et al.*, 1986). Thus, it seems reasonable to conclude that calspectin is most abundantly expressed in growing parts of the dendrites. From this point of view, however, it is perplexing that the basal dendritic trees of pyramidal cells in layers II, III and V were most heavily stained by the antibody as late as days 24 to 34 when their morphological maturation has been reported to have almost finished (Eayrs & Goodhead, 1959; Juraska & Fifkova, 1979; Miller, 1981). In a quantitative study with the Golgi staining method, however, Juraska (1982) reported that pyramidal cells in layer III continue to grow their basal dendrites until at least day 30. This suggests that at days 24 to 34 the basal dendrites of pyramidal cells, at least layer III pyramidal cells, are still growing and calspectin is expressed sufficiently to be intensely stained by the antibody. As to layer V pyramidal cells, however, Juraska (1982) also reported that the total length of their basal dendrites was not changed after day 15. It is possible that the strong immunoreactivity of the basal dendrites of layer V pyramidal cells at days 24 to 34 may be related to the increase in spine density along the dendrites (Juraska, 1982). In fact, dendritic spines of neurons in layers V as well as II and III become very conspicuous at postnatal days 24 to 34.

In the adult, however, the dendritic spines are not stained by the antibody. Of course, this is not due to a complete loss of spines, because with Golgi staining, the spines are easily detected along dendrites of most of the adult cortical neurons (Valverde, 1970; Juraska & Fifkova, 1979; Miller, 1981). It is also known, however, that in the adult the density of spines becomes much lower than at day 30 in various parts of the dendritic tree, e.g. basal bifurcating branches of layer III pyramidal cells (Juraska, 1982). Such a decrease in spine density was also reported in the cat and monkey visual cortex (Cragg, 1975; Lund *et al.*, 1977; Boothe *et al.*, 1979). This indicates that there are excessive spines at a relatively late stage of postnatal development of the visual cortex. Thus, the calspectin immunoreactivity strong enough to be detected by the present methods may be present only in the spines which have budded out from the dendrites and are consolidating their structures.

Lack of staining of axons and immunological characterization of the antibody

Even at ages when the calspectin immunoreactivity of the dendrites and somas of cortical neurons, particularly pyramidal cells, became most intense, axons and glial cells were not stained by the antibody. This is consistent with previous results obtained with antibodies against erythrocyte spectrin in the mouse cerebellum, hippocampus and other brain regions (Zagon *et al.*, 1984). Recently, this group reported that the adult mouse brain contains at least two distinct spectrin subtypes, both consisting of 240 and 235 kDa subunits (Riederer *et al.*, 1986). According to them the brain spectrin (240/235) was found in neuronal cell bodies and axons but not dendrites, while the other brain spectrin (240/235E; E connotes its antigenically close relation to erythrocyte spectrin) was confined to somas and dendrites but was not present in axons. Although their observations were carried out mostly in the cerebellum and hippocampus and ours in the visual cortex, the location of immunoreactive structures suggests that our antibody may recognize 240/235E brain spectrin. There is still a possibility, however, that our antibody might also recognize both subtypes (235 and 235E), since we purified calspectin from the synaptic-membrane rich microsomal fraction of the bovine brain, which might also contain the 235 subunits of Riederer *et al.* (1986). There are differences, however, in methods and

animal species between the present study and that of Riederer *et al.* They used the mouse both for purification of their brain spectrin and for immunohistochemistry, while we used the cow for purification of calspectin and the rat for immunohistochemistry. Thus, it seems possible that in the bovine brain preparations the concentration of the 235 subunit is much lower than that in the mouse brain preparations, or the immunoreactivity to the antibody against the 235 subunit may be less intense in the rat visual cortex than in the mouse cerebellum and hippocampus.

Relevance to synaptic plasticity in the visual cortex

Recently Lynch & Baudry (1984) have proposed a hypothesis that calspectin (fodrin in their terminology) may play a role in synaptic plasticity, leading to a long-term potentiation of synaptic transmission in the hippocampus. They postulated that the long-term potentiation might be due to an increase in glutamate binding caused by an activation of a calcium-dependent protease, calpain, breaking down calspectin which is supposed normally to occlude the glutamate receptor. Thus, repetitive excitatory inputs might result in long-lasting changes in the synapse by breakdown of a membrane calspectin filamentous network. More recently, a similar but more morphology-oriented theory was put forward by Siekevitz (1985).

In the visual cortex of the cat and monkey during postnatal development, responsiveness of neurons to visual inputs can be changed by various experimental modifications of visual environments (for review see Fregnac & Imbert, 1984). For example, if kittens are deprived of vision in one eye by lid sutures, neurons in the visual cortex lose their responsiveness to visual inputs from that eye (Wiesel & Hubel, 1963). Such a change takes place only in young kittens between 3 weeks and 3 months of age, thus this period is called the 'critical' or 'sensitive' period (Hubel & Wiesel, 1970). The rat visual cortex has also been shown to have a similar type of plasticity to monocular visual deprivation by behavioural and morphological studies (Fifkova, 1968; Rothblat *et al.*, 1978; Rothblat & Schwartz, 1979). In these studies, the sensitive period of the rat visual cortex was assessed to end between 30 and 45 days of age.

Thus, the present results seem consistent with the hypothesis that calspectin plays a role in synaptic plasticity in the visual cortex, since the calspectin immunoreactivity of cortical neurons is intense up to 34 days of age and then becomes dramatically weak in the adult. Also, this view is supported by the results that the dendritic spines of neurons in layers II, III and V become clearly stained by the antibody only during 24 to 34 days of age when the rat visual cortex is supposed to be very plastic, as described above. Dendritic spines are considered as the postsynaptic structures which are most responsible for synaptic plasticity (Fifkova & Van Harreveld, 1977; Lee *et al.*, 1980; Crick, 1982). The above-mentioned hypothesis seems consistent also with the present findings that most of the neurons in layer IV of the visual cortex are not immunoreactive to the antibody throughout postnatal development, because these cells have been shown to be less susceptible to changes in afferent inputs than neurons in the other layers of the kitten visual cortex (Shatz & Stryker, 1978; Komatsu *et al.*, 1981; Fregnac & Imbert, 1984).

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Frontiers in Excitatory Amino Acid Research, pages 435-442

A ROLE OF NMDA RECEPTORS IN LONG-TERM POTENTIATION OF SYNAPTIC TRANSMISSION IN THE RAT VISUAL CORTEX

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INTRODUCTION

Recent pharmacological studies in the hippocampus and spinal cord have demonstrated that receptors for excitatory amino acids, such as glutamate (Glu) and aspartate (Asp), can be classified into at least three types, on the basis of their most sensitive agonists, i.e., 1) N-methyl-D-aspartate (NMDA)-preferring receptors, 2) kainate (KA)-preferring receptors and 3) quisqualate (QUIS)-preferring receptors (see Watkins and Evans, 1981; McLennan, 1983 for review). The last two receptors have been implicated in the mediation of synaptic transmission while NMDA receptors have been shown to play a role in a form of synaptic plasticity, i.e., long-term potentiation (LTP) of synaptic efficacy in CA1 area of the hippocampus (Collingridge et al., 1983). In the cat visual cortex, Tsumoto et al. (1986,1987) have reported evidence suggesting that Glu and/or Asp may be excitatory transmitters at geniculo-cortical synapses and receptors involved may mainly be of QUIS and KA type.

Functional properties of visual cortical neurons can be changed by various experimental modifications during postnatal development (see Fregnac and Imbert, 1984 for review). These changes are believed to be due to plasticity of synapses in the developing visual cortex. In fact, it was demonstrated that LTPs could be induced in the kitten visual cortex by 2 Hz repetitive stimulation applied to the optic nerve in in vivo preparations (Tsumoto and Suda, 1979) and to the white matter underlying the cortex in slice preparations (Komatsu et al.,1981). In connection with the proposed role of NMDA receptors in the hippocampus,

these results raise a question of whether or not NMDA receptors play a role in synaptic plasticity in the visual cortex. In the present study, we attempted to answer this question by using visual cortical slices of the rat.

METHODS

Visual cortical slices were obtained from Sprague-Dawley rat pups aged 4-6 weeks except a 3-month-old-rat. In most of the experiments, the animals were initially anesthetized with urethane (i.p., 1.3 g/kg) and then infused through the heart with cold oxygenated medium. In some experiments, the animals were stunned by a blow on the back of the neck with the iron bar. They were then decapitated, and coronal sections (0.4-0.5 mm) were dissected from the visual cortex. Procedures for preparing the medium and for incubating the slices in it were essentially the same as those described by Yamamoto (1972). The slices were then transferred into a recording chamber. An arrangement of

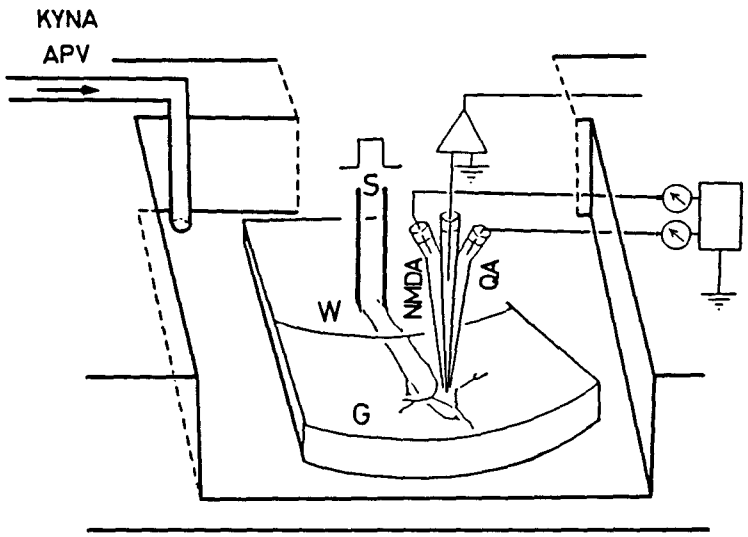

Figure 1. Schematic drawings of arrangements of stimulating and recording electrodes, and of procedures for applying agonists and antagonists of excitatory amino acids to neurons in visual cortical slices. KYNA, kynurenic acid. APV, D,L-2-amino-5-phosphonovaleric acid. NMDA, N-methyl-D-aspartate. QA, quisqualic acid.

recording and stimulating electrodes is shown in Fig. 1. Activities of neurons in layers II/III of the cortex were recorded extracellularly as field potentials or intracellularly as synaptic potentials evoked by electrical stimulation of the underlying white matter. In most of the experiments, single-barrelled micropipettes filled with 0.5 M sodium acetate containing potamine sky blue or with 4 M potassium acetate were used for extracellular or intracellular recordings, respectively.

In some of the experiments, multi-barrelled micropipettes were used for ionophoretic application of agonists to check effectiveness of antagonists applied through the medium, as shown in Fig. 1. Agonists employed were NMDA (50 mM, pH 8.0), QUIS (20 mM, pH 8.0) and KA (20 mM, pH 8.0). Antagonists added to the perfusion medium were D,L-2-amino-5-phosphonovaleric acid (APV) and kynurenic acid (KYNA). D-APV is an antagonist selective for NMDA receptors (Davies et al., 1981) while KYNA is a relatively non-selective antagonist of three types of receptors (Perkins and Stone, 1982; Hagihara et al., 1988). Usually, bicuculline methiodide, an antagonist of gamma-aminobutyric acid (GABA) receptors of A type, was added to the perfusion medium at the concentration of 0.5-2.0 μM.

RESULTS

Concentration of APV

APV is known to antagonize NMDA receptors selectively only within a limited range of concentration in the spinal cord and hippocampus (McLennan, 1983). In another word, its action is not selective for the three types of excitatory amino acid receptors if the concentration is too high. So, we analysed effects of APV applied through the medium with various concentrations on excitations of cortical neurons induced by ionophoretic application of the agonists, as shown in Fig. 1. At the concentrations of 1 and 10 μM, APV was found to be ineffective in blocking excitations induced by NMDA. At the concentrations of 25, 50 and 100 μM the excitations by NMDA were completely blocked by APV, but those by QUIS were not. At the concentration of 200 μM, the excitations by QUIS were significantly suppressed by APV, and at 400 μM those by QUIS as well as by NMDA were completely blocked. This indicates that the antagonistic action of APV is selective for NMDA receptors only at the concentrations between 25 and 100 μM. In the present experiments, therefore, we usually used APV at the concent-

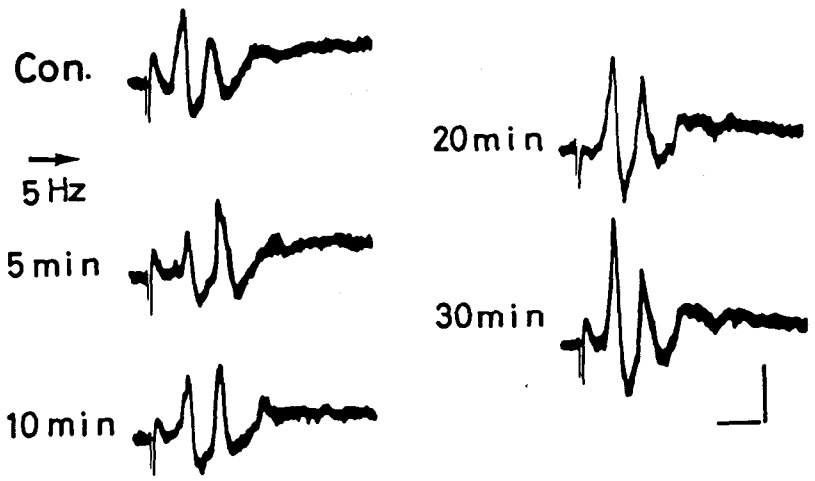

Figure 2. Cortical field responses evoked by test shocks applied to the underlying white matter. Five records are superimposed. Scales at the right bottom indicate 5 msec and 0.5 mV. Time after stopping the tetanic stimulation is indicated at the left of each record.

ration of 50 μM.

Induction of LTPs in the Rat Visual Cortex

In most of the slices tested, LTPs of the field responses were induced in layers II/III of the cortex by tetanic stimulation (5 Hz for 30-60 sec) of the underlying white matter. The records shown in Fig. 2 illustrate field responses of the cortex elicited by test shocks applied to the white matter in a 27 day-old rat pup. The test shocks given at 0.1 Hz evoked field potentials with two components (top left). Five minutes after stopping the tetanic stimulation the late component was significantly albeit slightly enhanced, and this enhancement lasted throughout the observation period. Twenty minutes after stopping the tetanus the early component was remarkably potentiated, and this potentiation was also seen 30 min after the tetanus. Such a significant and long-lasting potentiation of the field potentials was seen in more than half of the slices tested.

Excitatory post-synaptic potentials (EPSPs) evoked by white matter stimulation were recorded intracellularly from

20 cortical cells and their amplitudes and shapes were measured before and after the tetanic stimulation. Cells which were lost before having recorded EPSPs at 20 min after the tetanus were discarded from the present analysis. Stimulus intensity was adjusted so that each shock elicited EPSPs which were just subthreshold for inducing spikes. Usually, it was 1-5 μA for 0.1 msec pulses. For the tetanic stimulation, the intensity was doubled by using 0.2 msec pulses with the same current intensity. Usually, a weak test stimulus elicited EPSPs which appeared to consist of single peaks. Onset and peak latencies of these EPSPs were 2.5-6 and 8-23 msec, respectively. These EPSPs are called early EPSPs. When the intensity of the test stimulus was increased, EPSPs with longer latencies (late EPSPs) appeared in most of the cells. When the intensity of test shocks was further increased, the latency of late EPSPs was shortened and they were sometimes fused into the early EPSPs, resulting in much larger EPSPs.

In more than half of the cells, early EPSPs were significantly enhanced after the tetanic stimulation, and this enhancement lasted as long as the recordings were continued (usually 30 min after the tetanus). In some of the cells the late EPSPs became manifest only after the tetanic stimulation and they were often seen to fuse with the early EPSPs. In the cells which already had obvious late EPSPs before the tetanus, these EPSPs were enhanced so that each test shock elicited spikes after the tetanic stimulation.

Blockade of LTPs by APV Application

The application of APV prevented the induction of LTPs in all the slices in which field responses were measured. Also, EPSPs were not potentiated after the tetanic stimulation during the APV application in almost all the cells from which stable intracellular recordings were done longer than 20 min after stopping the tetanus. An example for this is shown in Fig. 3. This cell was obtained from a young rat aged 3 months. Test shocks given to the white matter elicited EPSPs with onset latencies of 5 msec and amplitudes of 9 to 14 mV. After starting the application of APV, these EPSPs were slightly decreased in amplitude and duration so that their shapes became thinner. During the APV application the tetanic stimulation was given to the white matter, but it did not induce significant enhancement or depression of EPSPs (left half of Fig. 3). Then, APV was completely withdrawn from the infusion medium. Twenty-five minutes after stopping the APV application, the

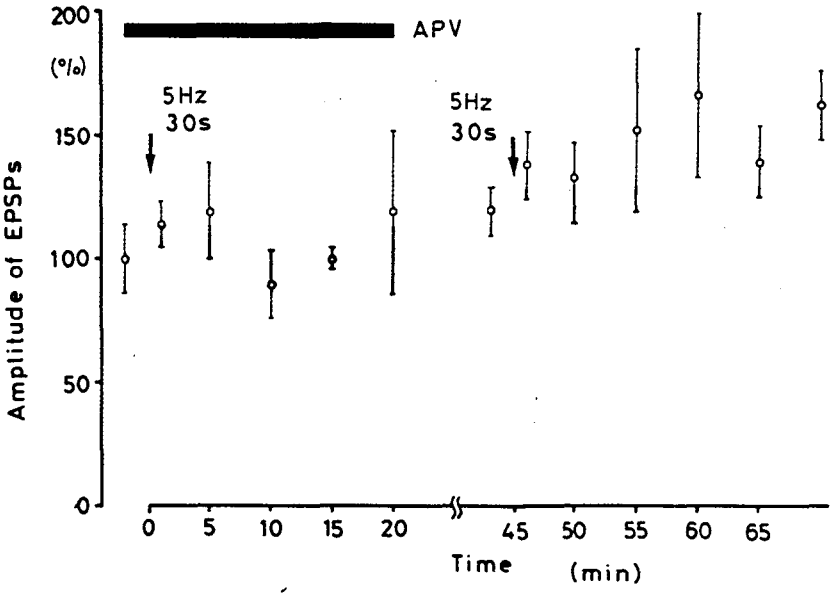

Figure 3. Effects of APV on induction of LTPs. In the ordinate, amplitudes of EPSPs evoked by test shocks were expressed as percent of the control value before starting the tetanus during the application of APV. Time when APV was applied is indicated by shaded, horizontal bar. Open circels and vertical bars represent the means and twice the standard deviations (S.D.).

tetanic stimulation with the same parameters as before was given to the white matter. This time, it induced a significant enhancement of EPSPs 10-30 min after stopping the tetanus (right half of Fig. 3).

DISCUSSION

The present results have demonstrated that tetanic stimulation of the underlying white matter induces potentiation of synaptic transmission in layers II/III of the rat visual cortex. This potentiation seems quite similar in its long duration to that reported in the hippocampus (Bliss and Lomo, 1973), although we did not follow up EPSPs or field potentials longer than one hour after stopping the tetanus. In contrast with the tremendous number of reports on LTPs in the hippocampus, there have been only a few

studies reporting LTPs in the visual cortex (Tsumoto and Suda, 1979; Komatsu et al., 1981; Lee, 1982; Perkins and Teyler, 1988). In these studies, only field potentials evoked by stimulation of the afferents were measured or the current source density analysis was done on the basis of the field potentials. With such an analysis of field potentials, it was not completely clear whether excitatory synaptic transmission is potentiated or inhibitory synaptic activities are depressed. Recently, Artola and Singer (1987) reported that EPSPs recorded from visual cortical cells of the adult rat were actually potentiated following high frequency stimulation of the underlying white matter. On the basis of intracellular and extracellular recordings the present results have also established that the tetanic stimulation at frequencies of 5 Hz for 30-60 sec does induce LTPs in the visual cortex of 4-6 week-old rat pups.

The present results have also demonstrated that the induction of LTPs in the visual cortex is blocked by the application of APV. The concentration of APV was carefully adjusted so as to antagonize NMDA receptors selectively. Thus, it seems reasonable to conclude that NMDA receptors play a role in inducing LTPs in the visual cortex, as already reported in the hippocampus (Collingridge et al., 1983). In the hippocampus, NMDA receptors were shown to play a crucial role in LTPs probably through Ca^{2+} entry into neurons (see Fagg et al., 1986 for review). So, processes similar to those in the hippocampus may also take place in the visual cortex. For example, an activity-related entry of Ca^{2+} into postsynaptic sites might trigger Ca^{2+} -dependent, biochemical processes underlying long-lasting changes in synaptic function in the developing visual cortex.

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