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The spread of excitation in neocortical columns visualized with infrared-darkfield videomicroscopy

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A combination of darkfield techniques and infrared videomicroscopy was used to measure the intrinsic optical signal (IOS) in slices of adult rat neocortex. The IOS, which reflects changes in light transmittance and scattering, provides a means of studying the spread of neuronal excitation and its modulation with high sensitivity and spatial resolution. The column-like IOS elicited by orthodromic stimulation is in accordance with models of neocortical circuitry. Blockade of synaptic transmission by the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-2-amino-5-phosphovaleric acid (D-APV) reduced the IOS. The $GABA_A$ agonist muscimol and the neuroactive steroid 5α-tetrahydrodeoxy-corticosterone (5α-THDOC) decreased the spread of excitation, whereas the $GABA_A$ antagonist bicuculline increased it. The present data suggest that the spatial spread of excitation in different neocortical layers is delimited by GABAergic inhibition mediated by the activation of $GABA_A$ receptors.

Key Words: Somatosensory cortex; Optical imaging; Intrinsic optical signal; Neocortical column; Infrared videomicroscopy; Synaptic transmission; Glutamate; GABA; Neurosteroid; Neuromodulator

Introduction

In contrast to the enormous progress in specifying synaptic potentials and ionic currents underlying neuronal excitation, our knowledge of the functional circuitry of mammalian neocortical neurones and the dynamics of the spread of neuronal activity is still rather limited. This deficit is mainly due to the lack of appropriate methods for analysing the activity of neuronal assemblies with sufficiently high sensitivity and spatial resolution. Intrinsic optical signals have previously been used to map neuronal excitation *in vivo*[1] and *in vitro*.[2,3] The nature of the IOS *in vitro* is still obscure, although the involvement of excitatory amino acids has been demonstrated.[4] Changes in light scattering properties due to volume changes in the periaxonal space,[5] or changes in the extracellular space due to dendritic swelling, have been postulated.[4]

The role of GABAergic inhibition in the spread of excitation in neocortical structures has been studied previously using single unit and field potential recording.[6–8] In the present study we combined darkfield illumination, a well-established method for imaging scattered light, with infrared videomicroscopy to visualize the IOS and to use it as an indicator of neuronal excitation in brain slices.[9] As imaging of the IOS in infrared is unhampered by any phototoxicity problems, we were able to study the actions of $GABA_A$ agonists and antagonists on the IOS for several hours in this *in vitro* preparation.

Materials and Methods

The methods of obtaining coronal brain slices from the adult rat neocortex are described elsewhere.[10,11] In brief, slices were cut at 400 μm from the somatosensory cortex of male rats (Sprague–Dawley, 100–150 g) with a vibratome. The slices were stored until needed in oxygenated standard Krebs-Ringer solution at room temperature.

The slice chamber, with a coverslip bottom (Luigs and Neumann, Germany), was mounted on the stage of an inverted microscope (Zeiss Axiovert) and inspected from below.[12–14] The slices were illuminated with a condenser modified for infrared-darkfield microscopy. The darkfield images were projected onto the target of a video camera (Newvicon) and five times analog contrast enhancement was applied with the camera control unit (C 2400 Hamamatsu). To visualize the intrinsic optical signal the images were processed in the following way with a real time image processing system (DVS 1000, Hamamatsu): a darkfield image was averaged (16 frames), stored in the computer memory and subsequently subtracted

from the incoming image during brief tetanic stimulation of the slice. The resulting difference image was smoothed with a digital 3×3 filter and the rolling average mode of the system, the contrast was digitally enhanced (64×) and the image was displayed in false colours.[14]

The slices were kept submerged at 30°C. Electrical stimulation was applied with concentric bipolar electrodes to the white matter using 50 Hz trains of 2 s with pulses of 0.2 ms and 1.5–3.5 V amplitude. This stimulation induced a column-like pattern of increased brightness in the neocortex slice. Field potentials were elicited using single 0.2 ms pulses of 4-8 V and recorded by means of conventional glass pipettes (filled with 1 M NaCl), placed in different areas of the IOS. The substances were added to the perfusion medium (flow rate 4 ml min^{-1}). 5α-tetrahydrodeoxy-corticosterone (5α-THDOC) was dissolved in dimethyl sulphoxide (DMSO). Aliquots of this stock solution were added to the perfusion medium. The resulting DMSO concentration of 0.01% had no effect on the IOS as tested in control experiments ($n = 8$ slices). The IOS was measured at 5 or 10 min intervals. Measurements were taken at the peak of the optical signal, 3 s after the offset of stimulation. The spread of neuronal excitation was quantified by measuring the area of the image for which pixel intensities were more than half of the maximum signal. For this purpose the pixel intensity was measured in the centre of the IOS and the threshold for the area measurement was set to half of the maximum intensity value. The area covered by the IOS before substance application was set to 100% and the subsequent measurements were expressed as a percentage of this control value. This parameter quantifies the spread of neuronal excitation and represents an even more sensitive indicator for pharmacological effects than changes in light transmittance. To establish a signal to noise ratio, we also measured relative changes in the light transmittance (dT/T, $n = 10$ slices). dT/T reached 3.3 ± 0.3% at the peak of the optical signal whereas the noise was typically 0.1%. The signal to noise ratio (SNR) was on average 35.

Results

Brief tetanic stimulation (50 Hz trains of 2 s with pulses of 0.2 ms duration and 1.5–3.5 V amplitude) of the white matter generally evoked a column-like pattern of the IOS in the adjacent neocortical areas (Fig. 1). The optical signal reached its maximum amplitude 3 s after the end of stimulation and decreased to baseline level in 30–60 s. The column-like area of increased brightness often exhibited a clearly discernable waist in the region of lamina IV. To prove a correlation between the IOS and the electrical activity of neurones, field potentials were recorded in the region where the IOS was observed previously. The amplitudes of the field potentials and the intensity of the IOS closely matched their spatial distribution. The maximum amplitude of the field potential was recorded in lamina II/III in the centre of the column of increased brightness. The amplitude of the field potential decreased towards the border of the IOS (Fig. 1).

To prove that the IOS is driven by synaptic transmission, mediated via the activation of excitatory amino acids, we added the L-glutamate antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) plus D-2-amino-5-phosphovaleric acid (D-APV; 100 μM) simultaneously to the perfusion medium. This application reduced the IOS to 13 ± 8% ($n = 5$, Wilcoxon signed rank test $p < 0.03$). This marked depression of the IOS was partially (> 50%) reversible after 45 min of washing (Fig. 2).

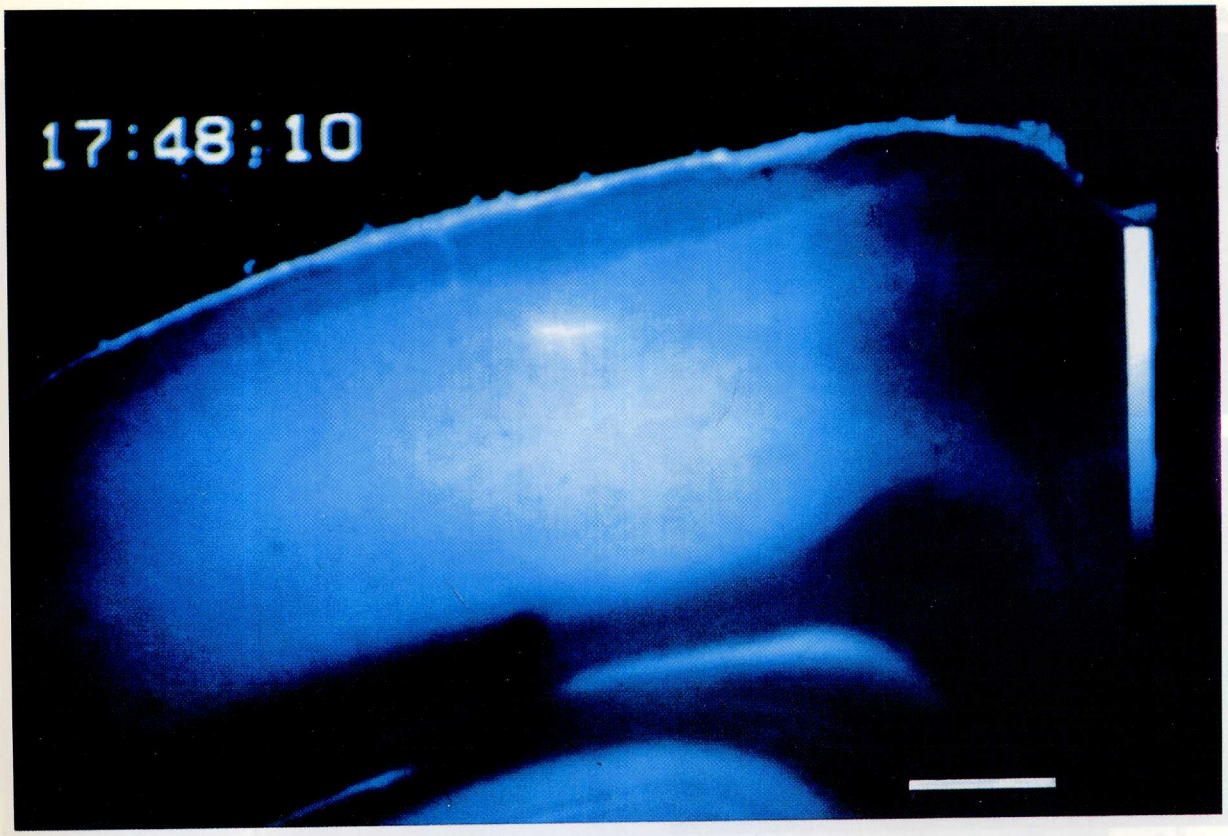
Since there is evidence that inhibitory GABAergic interneurones in lamina IV of the neocortex may delimit the spatial spread of synaptic excitation, we tested the effects of $GABA_A$ agonists and the $GABA_A$ antagonist bicuculline on the IOS. The waist-like narrowing of the IOS in lamina IV was clearly discernable in most recordings (> 80%). The substances were applied for 15 min to the bath in 10 μM concentrations (Fig. 3A). The $GABA_A$ antagonist bicuculline reduced the narrowing and markedly increased the area covered by the IOS to 202 ± 30% of the control ($n = 7$, $p < 0.01$) whereas the $GABA_A$ agonist muscimol (10 μM) reduced the spread of excitation to 74 ± 15% ($n = 7$, $p < 0.03$). Both effects were reversible within 45 min (Fig. 3B). Similarly to muscimol, the neuroactive steroid 5α-THDOC, which enhances $GABA_A$ receptor-mediated chloride currents,[15] also reduced the spread of excitation to 69 ± 5% ($n = 15$, $p < 0.001$). The reduction induced by the steroid was not reversible within the observation period.

Discussion

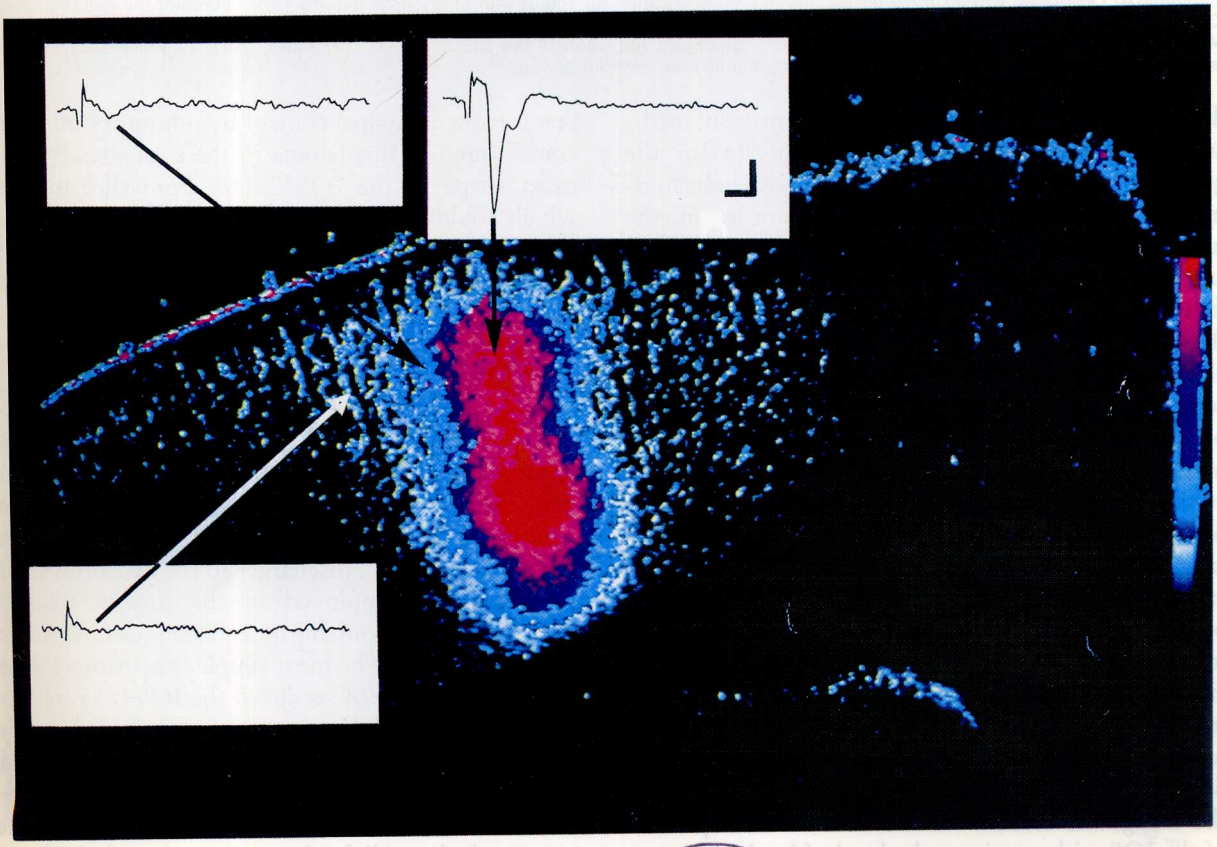
The disappearance of the IOS after the blockade of synaptic transmission by CNQX and D-APV

FIG. 1. The correlation between IOS and electrical activity is demonstrated by simultaneous recording of the optical signal and the field potential. (**A**) Darkfield image of the neocortical slice before image processing. The location of the recording electrode, placed here at the side of the IOS, is indicated by an arrow. Bar = 0.5 mm. (**B**) Tetanic stimulation (3 V, 50 Hz for 2 s) of white matter induces an area of increased brightness, here colour-coded in red. The recording electrode is placed in layer II/III of the neocortex where the maximum of the IOS is observed. The field potential is elicited with single pulses of 5.5 V. The different positions of the recording electrode are indicated by arrows. The field potential with the highest amplitude was recorded in the centre of the IOS. Bars = 5 ms, 1 mV.

(A)



(B)



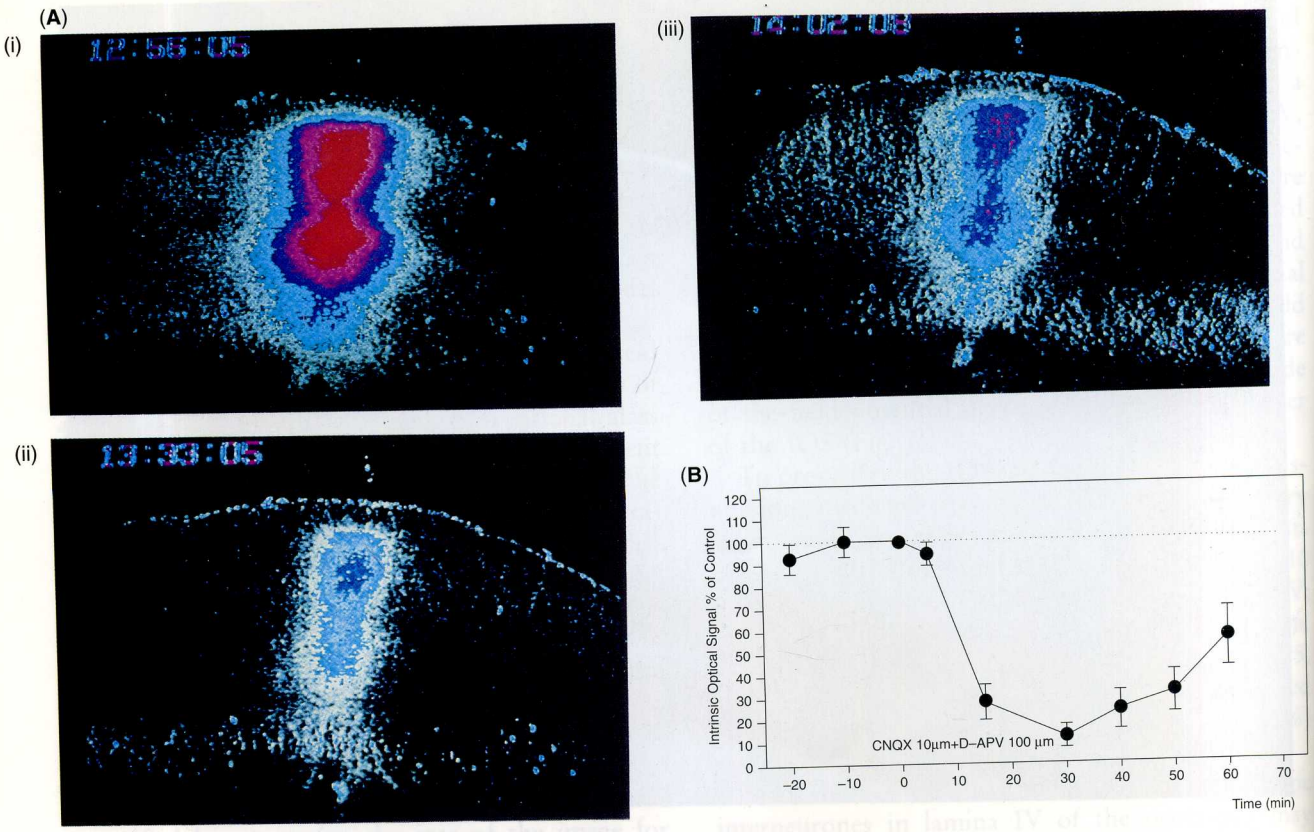


FIG. 2. The L-glutamate antagonists CNQX and D-APV reduced the IOS. Both antagonists were added simultaneously to the perfusion medium. (**A**) Control (i), IOS 30 min after beginning bath application (ii) and 30 min of washout (iii). (**B**) Time course of the reduction of the IOS by CNQX (10 µM) plus D-APV (100 µM). The IOS at t = 0 is taken as 100%, points and bars represent mean ± s.e.m. of measurements obtained in five slices. CNQX plus D-APV induces a significant reduction of the IOS to 13 ± 8% of control ($p < 0.03$, Wilcoxon signed-rank test). This reduction was partially reversible during the 30 min washout period.

demonstrates that intact synaptic transmission, mediated via EAA receptors, is a prerequisite for the generation of the IOS in the neocortex. These findings are in agreement with observations in the hippocampus.[3] The close correlation between the size and intensity of the IOS and the amplitude of the field potential generated in these sites indicates that the IOS can be taken as a measure of postsynaptic electrical neuronal activity. Similar results were reported in a study performed in neocortical slices of 14-day-old rats.[16] Darkfield illumination has been used for the recording of action potentials in cell culture systems, using single photodiodes.[17] In our preparation the time resolution for the IOS is in the range of seconds but this method can reliably monitor excitability changes for several hours without the use of voltage-sensitive dyes, or the need to restrict illumination periods to seconds because of phototoxicity.[18] The importance of GABAergic synaptic inhibition for delimiting the spatial spread of neuronal excitation reflected by the IOS is highlighted by the actions of the $GABA_A$ agonists and the antagonist bicuculline. The column-like pattern of the IOS with a waist at the level of lamina IV may result from a massive control by inhibitory interneurones found in this lamina in the neocortex.[19,20] The exact shape of the IOS differs from slice to slice, which could be attributed to slight variations of the plane of cutting during slice preparation. However this variability was not a factor in the subsequent drug effects as only relative changes of the IOS were considered and all datapoints shown in Figure 3B are averages of at least seven different slices. The $GABA_A$ agonist muscimol and the GABA agonistic neuroactive steroid 5α-THDOC decreased the spread of excitation, whereas the $GABA_A$ antagonist bicuculline increased it. Various studies using single neurone or field potential recording revealed that bicuculline can induce epileptiform discharges in the neocortex at the concentrations employed in the present study,[6–8] probably by antagonizing an endogenous GABAergic inhibitory tonus. The most simple explanation for the finding that THDOC reduced the IOS is an enhancing action on GABAergic synaptic transmission. This is suggested by studies which showed that this steroid increased $GABA_A$ receptor-mediated synaptic potentials in rat neocortical neurones[21] and chloride currents in hypothalamic neurones in culture.[15]

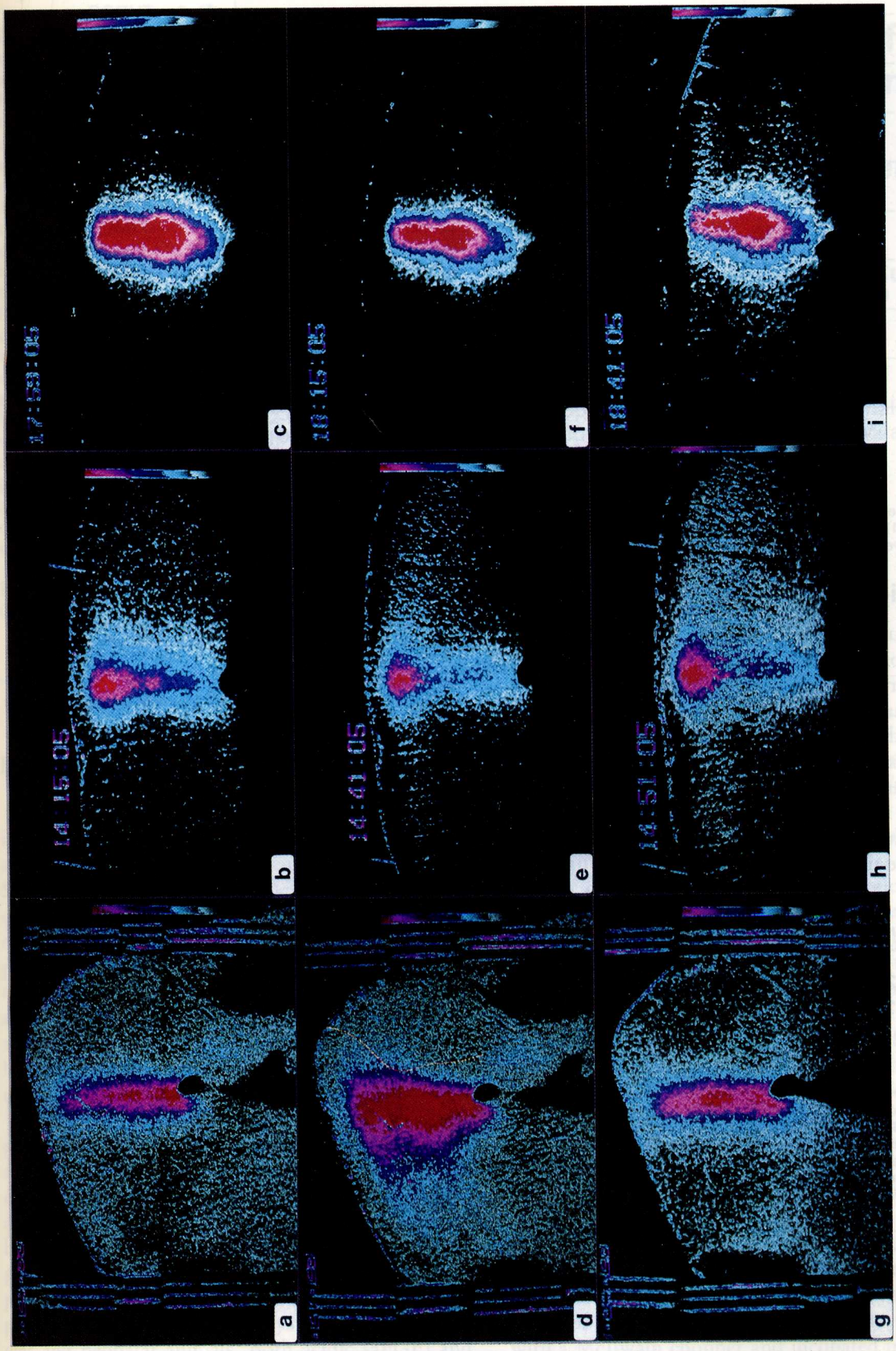


FIG. 3 Effects of bicuculline, muscimol and 5α-THDOC on the spread of excitation. The three substances were added to the perfusion medium to elicit a 10 μM concentration for 15 min. (**A**) control of bicuculline (a), muscimol (b), 5α-THDOC (c), after addition of the substances (d, e, f) and after 10–60 min of washout (g, h,i).

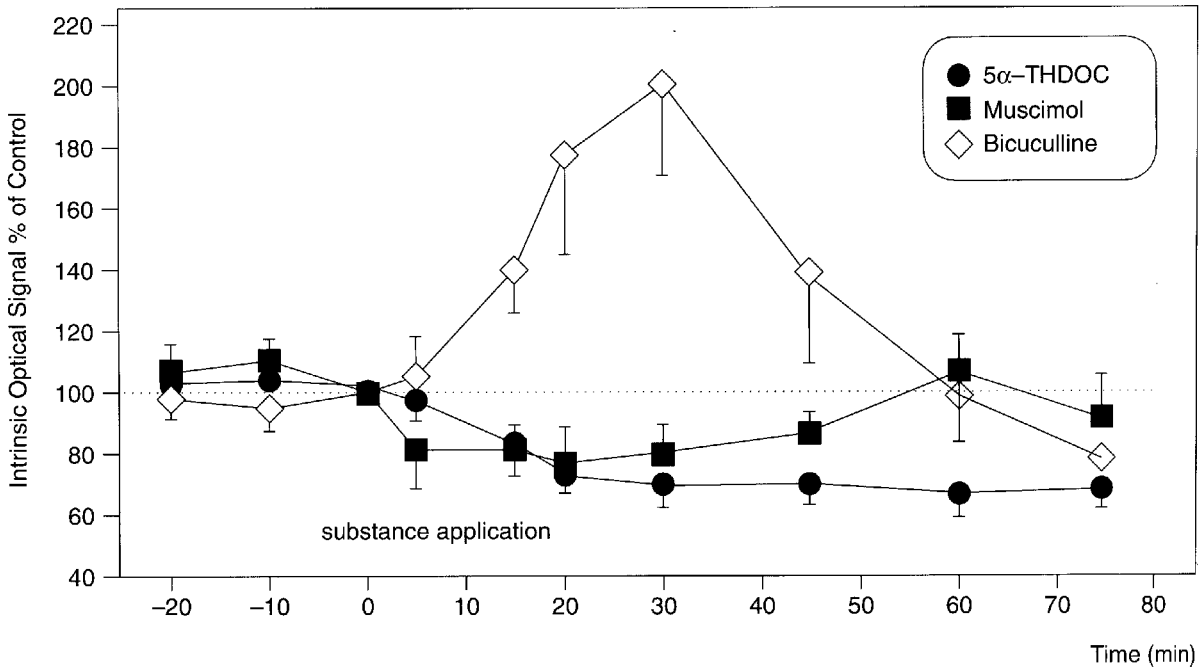


FIG 3 (**B**) Time course of the IOS. Symbols represent the mean ± s.e.m. of values measured in different slices. Bicuculline increased the IOS ($n = 7$, $p < 0.01$), whereas muscimol decreased the signal ($n = 7$, $p < 0.03$) reversibly. 5α-THDOC also decreased the IOS ($n = 15$, $p < 0.001$) but with no recovery during the washout period (see text).

Conclusions

The column-like IOS, elicited in the neocortex of the rat by orthodromic stimulation, depends on intact synaptic transmission via the activation of EAA receptors and is delimited by GABAergic inhibition, mediated through the activation of $GABA_A$ receptors. The experiments reported here demonstrate that infrared-darkfield microscopy can be used in complex systems to image the spatial spread of neuronal excitation with high sensitivity and spatial resolution without the use of voltage-sensitive dyes.

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References

1. Bonhoeffer T and Grinvald A. *Nature* **353**, 429–431 (1991).
2. Grinvald A, Manker A and Segal M. *J Physiol* **333**, 269–291 (1981).
3. MacVicar BA and Hochman D. *J Neurosci* **11**, 1458–1469 (1991).
4. Andrew RD and MacVicar BA. *Neuroscience* **62**, 371–383 (1994).
5. Cohen LB. *Physiol Rev* **53**, 373–418 (1973).
6. Chervin RD, Pierce PA and Connors BW. *J Neurophysiol* **60**, 1695–1713 (1988).
7. Chagnac-Amitai Y and Connors BW. *J Neurophysiol* **61**, 747–758 (1989).
8. Tasker JG and Dudek FE. *Neurochem Res* **16**, 251–262 (1991).
9. Dodt H-U and Zieglgänsberger W. *Brain Res* **537**, 333–336 (1990).
10. Dodt H-U, Pawelzik H and Zieglgänsberger W. *Brain Res* **545**, 307–311 (1991).
11. Howe JR, Sutor B and Zieglgänsberger W. *J Physiol* **384**, 539–569 (1987).
12. Dodt H-U. In: Kettenmann H and Grantyn R, eds. *Practical Electrophysiological Methods*. New-York: Wiley-Liss, 1992: 6–10.
13. Dodt H-U, Hager G and Zieglgänsberger W. *J Neurosci Methods* **50**, 165–171 (1993).
14. Dodt H-U and Zieglgänsberger W. *Trends Neurosci* **17**, 453–458 (1994).
15. Rupprecht R, Reul JMHM, Trapp T *et al. Neuron* **11**, 523–530 (1993).
16. Holthoff K, Dodt H-U and Witte OW. *Neurosci Lett* **180**, 227–230 (1994).
17. Stepnoski RA, LaPorta A, Raccuia-Behling F *et al. Proc Natl Acad Sci USA* **88**, 9382–9386 (1991).
18. Albowitz B, Kuhnt U and Ehrenreich L. *Exp Brain Res* **81**, 241–256 (1990).
19. Swindale NV. *Trends Neurosci* **13**, 487–492 (1990).
20. Szentagothai F. *Proc R Soc Lond B* **201**, 219–248 (1978).
21. Teschemacher A, Zeise ML, Holsboer F *et al. J Neuroendocrinol* **7**, 233–240 (1995).

General Summary
The spread of neuronal excitation in brainslices was investigated with a new imaging method, infrared-darkfield videomicroscopy. Neuronal excitation spreads in the neocortex in columns and is mainly mediated by the neurotransmitter glutamate. The width of these columns could be modulated by substances which act on the inhibitory system of the neocortex. Phenomena resembling epilepsy were studied, suggesting that the method presented could be used for testing anti-epileptic drugs.

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Long-term survival of cortical neurones from adult guinea-pig maintained in low-density cultures

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IN vitro survival of neurones isolated from adult mammalian brain is normally scarce and the postnatal age limit for obtaining viable cultures of cortical, hippocampal and diencephalic neurones is commonly two weeks. Here we describe a novel procedure for the establishment and long-term maintenance of cortical neurones of the adult mammalian brain in low-density cultures. Neurones isolated from the piriform cortex of 30- to 90-day-old guinea-pigs were initially grown in a chemically defined medium enriched with basic fibroblast growth factor (bFGF); later, a small quantity of foetal bovine serum (FBS) was added to facilitate cell differentiation. Under these conditions cells could be maintained in culture for at least 3 weeks, when indirect immunocytochemistry and whole-cell patch-clamp recordings were performed. Cells exhibiting neuronal morphology expressed the neuronal marker microtubule associated *protein-2* (MAP2) and generated action potentials. Moreover, about 70% of the MAP2-immunoreactive cells were simultaneously labelled with anti-γ-aminobutyric acid (GABA) antibody. Cells expressing neuronal antigens were never labelled by antibody raised against the glial marker glial fibrillary acidic protein (GFAP). These results indicate that long-term survival of *adult* neurones can be achieved under definite culture conditions.

Key Words: Neuronal cultures; bFGF; Piriform cortex; Cultures from adult mammalian brain

Introduction

Cultures of central nervous system neurones isolated either from embryos or from postnatal animals are a popular tool in neurobiology research. Since several neuronal properties are known to be differentially expressed during development, the use of mature cultured neurones should be, in principle, preferred to embryonic or neonatal cultures. Unfortunately, *in vitro* survival of neurones isolated from *adult* animals is extremely scarce and the postnatal age limit for obtaining viable cultures of cortical, hippocampal and diencephalic neurones is commonly two weeks.

Although serum is often added to culture media to promote proliferation, survival and differentiation of a large variety of neuronal and non-neuronal cells, many recent studies demonstrate that survival and differentiation of neural cells can also be influenced by specific growth factors. Among these, fibroblast growth factors (FGFs) have been shown to have pleiotropic activity on neurones isolated from the peripheral and central nervous system of both embryonic and neonatal animals. Both acidic (aFGF) and basic FGF (bFGF) support the survival and enhance the outgrowth of neurites of rat neurones from various brain regions.[1–4] Notably, bFGF is a survival factor for cultured CNS neurones from the spinal cord,[5] striatum,[6] hippocampus[7,8] and cortex.[6,9] Furthermore, bFGF promotes the survival and development of dopaminergic and GABAergic neurones in cultures[1,10] and has a protective effect against N-methyl-D-aspartate (NMDA) receptor-mediated toxicity in striatal neurones,[11] glutamate toxicity in hippocampal neurones[8] and hypoglycaemic damage of human cortical neurones[12] in culture. By exploiting the trophic action of bFGF, we aimed to define proper growth conditions enabling the establishment and long-term maintenance of primary low-density cultures from the cortex of adult mammals. Preliminary results have been published in abstract form.[13]

Materials and Methods

Guinea-pigs 30-to 90-days old were anaesthetized with ketamine (150–200 mg kg^{-1}) before extraction of the brain under hypothermic conditions. Coronal slices of the piriform cortex were cut at 500 μm with a McIlwain chopper and incubated in a stirring flask in oxygenated (95% O_2/5% CO_2) artificial