Immature Neocortical Neurons Exist as Extensive Syncitial Networks Linked by Dendrodendritic Electrical Connections

ALEJANDRO PEINADO
Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461
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Peinado, Alejandro. Immature neocortical neurons exist as extensive syncitial networks linked by dendrodendritic electrical connections. J Neurophysiol 85: 620–629, 2001. The properties of immature cortex that may enable it to exhibit large-scale wavelike activity during a brief critical developmental period were investigated by imaging neuronal calcium signals in neonatal cortical slices under conditions of artificially enhanced excitability, conditions that produce a more frequent and robust version of the naturally occurring waves. Using pharmacological manipulation to probe the underlying mechanisms, I show that waves can propagate effectively when excitatory synaptic transmission is blocked. In contrast, propagation is very sensitive to reductions in gap junctional communication. In the barrel field cortex wave propagation is affected by the underlying cytoarchitecture in a way that is consistent with a role for dendrodendritic gap junctions. The ability of cortex to sustain wave activity ends around postnatal day 12, precisely when a major reduction in neuronal gap junctions takes place in cortex. These results suggest that in immature cortex gap junctions link neurons into extensive networks that may allow electrical activity to spread over long distances.

INTRODUCTION

Spontaneous coordinated electrical activity exhibiting different spatio-temporal patterns is emerging as a ubiquitous feature in immature neural networks (O’Donovan 1999). The ability of immature cortex to generate waves of electrical activity has only recently been described (Garaschuk et al. 2000; Peinado 2000). Unlike the waves of electrical activity in retina (Wong 1999), or the biochemically mediated coactivation termed “neuronal domains” in neonatal cortex (Kandler and Katz 1998; Yuste et al. 1992, 1995), the more recently described waves are typically capable of propagating unimpeded over large cortical distances (at least several millimeters). In this respect they resemble another type of wave, the so-called spindle waves, previously described in the lateral geniculate and perigeniculate nuclei of thalamus (Kim et al. 1995; McCormick et al. 1995; Weliky and Katz 1999). In all cases, the neuronal properties and synaptic mechanisms responsible for the various forms of coordinated activity are incompletely understood.

A feature of immature neural networks that may be related to their ability to exhibit large-scale coordinated activity such as waves is the presence of neuronal dendrodendritic gap junctions. The role of these, mostly transient, intercellular channels in neuronal development and circuit formation is not known, in part because it has not been possible to manipulate their function experimentally in vivo. A growing body of evidence, however, is establishing the ubiquitous nature of gap junctional communication between neurons in many regions of the CNS. The recent discovery of a neuron-specific mammalian gap junction protein, connexin36 (Condorelli et al. 1998; Sohl et al. 1998), has provided confirmation at the molecular level of what had previously been shown through electrophysiology, dye coupling, or electron microscopy. Connexin36 mRNA is highly expressed in neurons of many brain areas previously shown to have high levels of neuronal gap junctions. This includes the inferior olive, retina, hippocampus, olfactory bulb, spinal cord, and neocortex, the latter exhibiting highest levels during the early postnatal period (Chang et al. 1999; Condorelli et al. 2000; Sohl et al. 1998; Srinivas et al. 1999). Other connexins have been detected immunocytochemically in developing cortical neurons (Nadarajah and Parvabelas 1999; Nadarajah et al. 1997) and may be involved in neuronal coupling as well (but see Rash et al. 2000).

In developing rat neocortex, neurons are coupled through dendrodendritic gap junctions to multiple other neurons during the first 10–12 days of postnatal development (Peinado et al. 1993; Rorig et al. 1996). This makes gap junctions a likely conduit for propagation of activity. A key unresolved issue, however, is whether gap junction coupling forms extensive networks, such as might be expected to mediate large-scale waves and oscillations, or whether they form restricted groups capable only of local coactivation. The latter possibility finds support in results showing that spread of gap junction tracers following intracellular injection is limited to neurons within a short radius (Peinado et al. 1993). It is also consistent with the restricted spread of biochemical signals through gap junctions, a phenomenon that results in the coactivation of small groups of neurons (Kandler and Katz 1998; Yuste et al. 1992, 1995). Both of these observations, however, could reflect the limited travel potential of the respective nonregenerative signals: the tracer in one case; IP3 or another second messenger in the other. In contrast, monitoring electrical signals, which are regenerative, provides a more sensitive assay for the extent of gap junctional connectivity.

Dendrodendritic gap junctions have always been presumed to mediate electrical coactivation of neighboring neurons. The notion is an intuitive one and dates back to the first demon-
strations of the properties of gap junctions (Bennett 1977; Bennett et al. 1963; Furshpan and Potter 1959). Conventional electrical recording methods, however, are generally inadequate for monitoring activity in extended networks. A more effective approach is to use calcium imaging to monitor electrical activity indirectly, an approach that provides fine anatomical detail while allowing activity to be monitored over large areas of neural tissue. Here I show that under conditions of experimentally enhanced neuronal excitability and electrotonic coupling (partial potassium channel blockade) electrical activity can spread over considerable distances, creating a wavelike activation of the cortical network that can be imaged repeatedly in cortical slices stained with the calcium indicator fura-2 and that can be shown to be dependent on gap junctional communication. These results suggest that, in most regions of developing cortex, gap junctions link neurons into extensive cortical networks, and that this property may be partly responsible for the large-scale activity waves observed under more physiological conditions during early postnatal development.

METHODS

Long-Evans rat pups 0 to 15 days old (day 0 = day of birth) were cryoanesthetized (0–6 days) or anesthetized with pentobarbital so

vated (6.25 mM) [K]o was used because in its presence TEA induces two distinct spatio-temporal patterns of activations (Bennett et al. 1981). As seen

TEA-induced epileptiform events appeared “spontaneously.” In normal (3.5 mM) [K]o, identical events can be induced with any form of local stimulation. This was done for the carbenoxolone experiments, where a bipolar stimulating electrode was used to deliver brief trains of pulses to the cortical slice at a location in layer 2/3 or 4. TEA-containing ACSF was used during slice preparation and staining at all ages, except for the experiments illustrated in Fig. 8, where normal ACSF was used. The inclusion of TEA during cutting and staining was done because, for reasons that are not clear, TEA enhances loading of fura-2 AM in slices older than postnatal day 10 (P10) (unpublished observations). Slices were then placed on a temperature-controlled (30°C) perfusion chamber (rate: 2–3 m/min; volume: 200–400 μl) on the microscope (Zeiss Axioskop FS) stage and viewed with a ×10, ×20, ×40, or ×63 water-immersion objective. A ×0.5 magnification adapter was sometimes placed in front of the camera to obtain a larger (up to 1.5 × 1.5 mm) image area. Optical recordings were done using a cooled charge-coupled device (CCD) digital camera (Princeton Instruments, model 512 EFT) and IPLab Software (Scanalytics). Camera was operated on 4 × 4 pixel binning mode. All fura-2 activity was recorded at a single excitation wavelength using a 380 ± 5 nm band-pass filter. Emission fluorescence was filtered with a 400-nm longpass filter.

For imaging of voltage signals, slices were stained for 5 min with the voltage-sensitive dye di-4-ANEPPS (Molecular Probes, Eugene, OR) (Fluhler et al. 1985) (0.66 mg/ml in ACSF) while in the perfusion chamber. To minimize photobleaching of di-4-ANEPPS and photodynamic damage (Schaffer et al. 1994), for whole cell current-clamp recordings, patch pipettes were pulled from borosilicate glass (3–5 MΩ) and filled with an internal solution containing (in mM) 110 K gluconate, 20 KCl, 10 HEPES, 10 EGTA, 1 CaCl2, and 1 MgCl2, pH 7.2; osmolarity was adjusted to 307 mOsm with sucrose. Signals were amplified with an Axopatch 200A patch clamp amplifier (Axon Instruments), and acquired using Synapse (Synergy Research) and an ITC-16 computer interface (Instrutech). All drugs were dissolved in ACSF from stock solutions and applied through the perfusion. Tetrodotoxin, TEA, picrotoxin, bicuculline, carbenoxolone, L-glutamate, and strychnine were purchased from Sigma-RBI (St. Louis, MO). Glutamate antagonists were purchased from Tocris Cookson (Bristol, UK). Halothane (Halocarbon Laboratories, N. Augusta, SC) was dissolved in ACSF using a halothane vaporizer to bubble a mixture of 2.5% halothane vapor (in 95% O2-5% CO2) into an open ACSF reservoir. The halothane vapor was bubbled continuously during the halothane treatment period. For dye coupling experiments, I used a fluorescent dye, NBD-methylglucamine (Molecular Probes, Eugene, OR), with properties similar to those of the gap junction tracer Neurobiotin. The dye is positively charged at neutral pH and has a molecular weight of 356 daltons. It was dissolved (0.1 mg/ml) in the internal pipette solution, sonicated, and filtered (0.2 μm) before use. The optical filters used to visualize the dye were 470 ± 35 nm band-pass for excitation; 505 nm dichroic; 515 nm longpass for emission.

RESULTS

Calcium imaging was used to visualize neuronal activity in coronal slices of immature rat neocortex stained with the cell-permeant calcium indicator dye fura-2 AM and maintained in conditions of artificially enhanced excitability: ACSF containing 10 mM tetraethylammonium (TEA) and 6.25 mM potassium. The choice of TEA, rather than GABA A blockers or zero [Mg2+] o, as a way to elicit higher levels of excitability in slices was made for three reasons. First, whereas bicuculline or picrotoxin can enhance excitability, these agents can only achieve this effect starting around P9 in neocortical slices. In contrast, as demonstrated here, TEA-induced waves can be elicited as early as P0. The second reason is that, unlike zero [Mg2+] o, the TEA effect does not depend on enhancing a single neurotransmitter-receptor system. The third and most important reason was the expectation that under conditions of reduced potassium conductances, and hence increased input resistance, neuronal interactions mediated by weak gap junctions would be enhanced (Rayport and Kandel 1981).

TEA induces two distinct spatio-temporal patterns of network activation

Analysis of activation patterns in 64 neocortical slices (age P0 to P7) showed that exposure to combined TEA and elevated [K+] o elicits in immature cortical networks two distinct types of “spontaneous” activation, referred to here as “horizontal waves” (H-waves) and “vertical waves” (V-waves). In slices cut in the coronal plane, H-waves are neuronal calcium oscillations that propagate regeneratively along the horizontal length of a slice at a relatively slow speed (Fig. 1A). As seen in a single frame from a sequence, horizontal waves involve simultaneous increases in calcium in hundreds of neurons occupying a region of cortex with a sharply defined leading edge. Horizontal waves constitute the majority of events in the youngest slices. In slices from P0-P3 animals, 191 of 205 events recorded (93%) were H-waves. By using low magnification imaging as well as successive partially overlapping fields, it was possible to demonstrate that H-waves travel...
distances of at least several millimeters. H-waves initiate unpredictably at locations that appear to be distributed randomly throughout the slice. When captured in the early stages, H-waves can be seen to initiate from a small group of cells, within an area <50 μm diam.

In contrast V-waves are calcium transients that initiate simultaneously throughout the horizontal extent of a slice. At low magnification, image sequences demonstrate that this second type of activation initiates in infragranular layers and subsequently propagates to upper layers, giving the appearance of a vertically moving wave (Fig. 1B). The incidence of V-waves increases with age. By P7 they represent 53% (range 26–100%; n = 8 slices) of recorded waves. Despite their very different patterns of spread, both types of activation can often be observed within the same region of cortex and can occur very close in time.

Results described below suggest that H-waves represent activity spreading predominately through dendro-dendritic gap junctions, whereas the later appearing V-waves require glutamatergic synapses. The evidence described below suggests that the long distances traversed by H-waves reflect the fact that gap junctions form extensive syncytia among developing cortical neurons. An exception to this rule is found in the barrel field of somatosensory cortex as described next.

Spread of H-waves through barrel field cortex respects local microarchitecture

In most regions of cortex the horizontal advance of waves occurs at a relatively slow (~1000 μm/s) but constant velocity, suggesting that the mechanism of propagation is a process occurring over short expanses of cortex and is mediated by an anatomical substrate whose dimensions are, on average, constant throughout cortex. Dendrite dimensions are typically uniform among cortical neurons of similar laminar position, and dendro-dendritic gap junctions are a likely candidate for the anatomical substrate mediating propagation. As a test of the role of dendrites in slow horizontal propagation, wave advance was analyzed in layer 4 of the barrel field cortex (n = 6 slices), a region that can be identified in fura-2-stained slices based on differences in staining intensity between barrels and surrounding cortex. In the barrel field, dendrites are mostly confined to individual barrels and do not cross barrel boundaries (Harris and Woolsey 1983; Lubke et al. 2000; Simons and Woolsey 1984; Woolsey et al. 1975). This creates a situation that is unique in neocortex insofar as neurons whose somata are in close proximity have little or no dendritic overlap. As illustrated with the example in Fig. 2, propagation in barrel cortex was always found to be markedly disrupted at boundaries between barrels. Instead of being smooth, propagation is sacadic, with activity spreading normally among neurons in a single barrel but with more difficulty between barrels (Fig. 2A).

Interestingly, spread between adjacent barrels typically occurs through neurons in lower layer 3, where dendritic arbors are not segregated to the same extent as in layer 4. Likewise, spread of activity arriving from a nonbarrel region can end abruptly on encountering a barrel boundary (Fig. 2B). The circuitous but precise path taken by waves as they propagate through the barrel field is consistent with dendrites being involved in wave propagation.

Electrophysiological correlates of waves are depolarization and burst firing

To determine whether depolarization and action potential firing underlie the wave events observed optically in fura-2-stained slices, two different types of experiments were carried out. First, as shown in Fig. 3A, whole cell patch-clamp recordings acquired concurrently with the optical signals (n = 5 experiments) indicate that during calcium oscillations individual neurons are depolarized and fire a burst of action potentials. Electrically, all waves manifest as typical interictal epileptiform events, namely a burst of action potentials riding on a brief paroxysmal depolarizing shift (PDS). Second, as shown in Fig. 3B, simultaneous optical recordings (n = 3 slices) using fura-2 and di-4-ANEPPS to record calcium and voltage signals, respectively, demonstrate that there is generalized cell depolarization occurring simultaneous with the calcium signal. To show that the ability to depolarize and fire action potentials is a necessary aspect of the events recorded with calcium imaging, slices (n = 6) were imaged in the presence of bath-applied sodium channel blocker tetrodotoxin (TTX). TTX reversibly abolishes all TEA-induced wave activity (Fig. 3C).
expression of H-waves.

Slices (Fig. 4) suggests that V-waves, perhaps because of their incidence of H-waves after addition of CNQX in some slices (Fig. 4). As shown in Fig. 4, neither 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) nor CNQX and 2-amino-5-phosphonovaleric acid (APV) together are capable of abolishing TEA-induced waves during the first postnatal week (n = 12 slices). This is in sharp contrast to many previous reports of epileptiform activity induced in mature or immature neocortex by convulsants such as GABA_A antagonists or 0 [Mg^{2+}] (see discussion for exceptions and their possible significance). Time-lapse imaging of activity in which fura-2 fluorescence was sampled once per second for periods up to 30 min show that washing in glutamate antagonists does not abolish wave events induced by TEA (Fig. 4A).

The persistence of TEA-induced waves in CNQX and APV extends to metabotropic glutamate receptor antagonists, as shown in Fig. 4B. The amplitude of calcium transients induced by TEA, however, is noticeably reduced by glutamate antagonists, suggesting that, as might be expected, glutamate release and binding to neuronal receptors during these events contributes to the changes in [Ca^{2+}] recorded with fura-2. Despite this change in signal amplitude, the horizontal propagation of waves, as measured by their speed, is unaffected by glutamate antagonists (mean difference = 38 μm/s; P > 0.5, paired t-test, n = 5; ages: P0–5; Fig. 4C).

A closer look at the effects of glutamate antagonists revealed that, whereas the horizontally propagating waves survive the glutamate receptor blockade, the same is not true of the vertical waves. In fact, these are completely abolished by glutamate antagonists, as shown in Fig. 4D. Thus in slices where a large fraction of waves is of the vertical type before application of CNQX, exposure to this antagonist alone immediately eliminates V-waves without eliminating H-waves (Fig. 4E). As judged by their sensitivity to glutamate receptor antagonists, vertical waves are therefore reminiscent of the interictal events described previously in various seizure models. The increase in the incidence of H-waves after addition of CNQX in some slices (Fig. 4E) suggests that V-waves, perhaps because of their ability to spread rapidly through cortex, can interfere with the expression of H-waves.

Reduced neuronal coupling correlates with the failure of TEA to induce coordinated calcium transients in the presence of glutamate antagonists

The results described above showing an inability of glutamate receptor blockade to eliminate wave events of the horizontal type are consistent with neuronal gap junction involvement in these events. A further test of their involvement is to examine TEA-induced activity when there are fewer gap junctions.

As previously shown using this same preparation, P12 is the time point after which experiments with the gap junction tracer Neurobiotin demonstrate a sharply reduced incidence of neuronal coupling (Peinado et al. 1993; Rorig et al. 1996). Very little coupling is observed by P15 (coupling between inhibitory neurons is an exception to this rule). Unfortunately, the ability to distinguish between horizontal and vertical waves using imaging is lost after P8–9, as the speed of propagation of events in both vertical and horizontal dimensions begins to exceed the ability of the CCD array to keep track of displacements in activity. The increased propagation velocity during development is shown in Fig. 5A, where speed measurements taken from 538 horizontal waves in P0–7 slices are plotted as a function of age. This increase is probably attributable to a general increase in network excitability, as shown by the concomitant increase in the spontaneous incidence of waves during this same period (n = 64 slices; P0–7; Fig. 5A).

Because of this increasing excitability and faster propagation, TEA-induced events recorded on P12, the last time point at which neuronal coupling is high in rat neocortex, appear as rhythmic collective calcium oscillations that are simultaneous in all neurons throughout the field of view. Glutamate antagonists at this age cause some cells to stop oscillating, but large-scale collective activity persists (not shown). After P12, the effect of APV and CNQX on collective calcium oscillations is dramatically different from that observed in younger slices. Experiments done on P15 slices (n = 10) show that application of these two antagonists always results in abolition of activity in the majority of neurons. In neurons that remain active, however, the activity is no longer coordinated among these neurons (Fig. 5B). These results show that glutamatergic syn-
aptic transmission is necessary to coordinate TEA-induced epileptiform activity after P12, but not at earlier ages when gap junctions are abundant.

Horizontal waves are insensitive to blockade of other neurotransmitters

Several experiments were done to address whether synaptic transmission mediated by receptors other than the N-methyl-D-aspartate (NMDA) and \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype glutamate receptors can account for the spread of activity observed in horizontal waves. First, using the voltage-sensitive dye di-4-ANEPPS instead of the calcium indicator fura-2, wave activity was examined in ACSF containing low Ca\(^{2+}\) (0.5 mM) and high Mg\(^{2+}\) (8 mM) to reduce calcium-dependent transmitter release. Under these conditions, spontaneous activation ceases to occur. However, horizontally propagating waves of depolarization reminiscent of those observed with fura-2 are observed following local stimulation, suggesting that the mechanism for wave propagation is not significantly affected by this perturbation (\( n = 5 \) slices).

A more effective test for the involvement of neurotransmitters in the spread of horizontal waves was to examine the effect of glycine and GABA\(_A\) receptor antagonists. Glycine and GABA\(_A\) receptors may mediate excitation in developing neocortical networks (Ben-Ari et al. 1989; Flint et al. 1998; Owens et al. 1996), and taurine, the endogenous ligand for glycine receptors in immature neocortex, is released through a calcium-independent mechanism (Flint et al. 1998), which may not be affected by the low Ca\(^{2+}\)/high Mg\(^{2+}\) manipulation described above. As shown in Fig. 6, neither of these antagonists was able to block propagation of wave activity. In fact, as shown in Fig. 6A, the result of blockade of GABA\(_A\) with bicuculline was contrary to expectations: bicuculline caused an apparent disinhibition instead of reduced excitation (50 \( \mu \)M; \( n = 5 \) slices). Strychnine, an antagonist of glycine receptors (50 \( \mu \)M; \( n = 6 \) slices), had no obvious effect on wave activity (Fig. 6B).

An experimental manipulation that has been used effectively to block a nonsynaptic form of epileptiform activity (induced by...
0–0.2 mM [Ca$^{2+}$$_o$] in hippocampus is to use hypertonic medium (20 mM sucrose added) (Dudek et al. 1990; Roper et al. 1992). If an epileptiform event is spread through the extracellular space, for example by activity-dependent elevations in [K$^{+}$$_o$] or ephaptic interactions, hypertonic medium might be effective in blocking these events by increasing the extracellular volume fraction (Dudek et al. 1986; McBain et al. 1990). Such interactions are unlikely in a submerged immature neocortical slice, where the extracellular volume fraction is high to begin with (Lehmenkuhler et al. 1993) and extracellular potassium is therefore more likely to equilibrate with the superfusing medium. Nevertheless, the effect of hypertonic medium (40 mM sucrose added) was tested on TEA-induced waves. The speed of propagation of horizontal waves was examined before and during superfusion with hypertonic medium in $P_0$–3 slices. Results showed that there is no significant difference in speed between these two conditions (mean difference: 65 $\mu$m/s faster in hypertonic medium; $P < 0.5$; paired t-test; $n = 4$ slices).

**Gap junction blockers abolish horizontal waves**

To test for involvement of gap junctions in H-wave propagation, I used two gap junction blockers, halothane and carbenoxolone. Halothane has been shown previously to effectively abolish dye coupling between postnatal neocortical neurons (Peinado et al. 1993). On the other hand, it has also been shown to enhance GABA$_A$-mediated inhibitory currents (Li and Pearce 2000; Lukatch and MacIver 1997), an effect that, in light of the effects of bicuculline described above, might by itself suppress TEA-induced activity. Wave propagation speed was therefore monitored with and without halothane, in the presence of the GABA$_A$ channel blocker, picrotoxin (100 $\mu$M). In all experiments ($n = 10$ slices) halothane completely abolished epileptiform waves in immature cortical slices (Fig. 7A). Individual neurons, however, continued to express calcium oscillations during blockade of epileptiform activity by halothane.

Experiments done with carbenoxolone gave qualitatively similar results. Although waves were not completely abolished, wave propagation speed was significantly reduced (mean difference: 145 $\mu$m/s, $n = 7$ slices, $P < 0.01$; Fig. 7B). The effect of carbenoxolone was not as rapid as that seen with halothane and continued to increase over the course of 60 min (Fig. 7C), indicating that perhaps carbenoxolone does not penetrate the brain slice as readily as most compounds. As with halothane, random neuronal activity was not eliminated by this gap junction blocker (Fig. 7D).

**Gap junction coupling can mediate correlated activity in some neuron pairs**

To test the ability of gap junctions to allow spread of activity between neurons in this preparation, an additional set of experiments was performed. For these experiments no TEA or elevated [K$^{+}$$_o$] were used. Instead, slices were bathed in normal medium containing a low concentration of extracellular glutamate receptor antagonists eliminate all TEA-induced coordinated activity at $P15$. $A$: measurements (mean ± SE) of wave speed ($n = 538$ waves) and frequency ($n = 64$ slices) taken over the course of the 1st week. $B$: recordings of coordinated activity in $P15$ slices. APV and CNQX abolish TEA-induced coordinated activity. The few cells that remain active exhibit a dramatic change from being completely correlated before to completely uncorrelated during APV/CNQX superfusion. Image at left shows the locations of the 4 cells whose records are shown on the right. These 4 cells were selected a posteriori as examples of cells that remained active in the presence of APV and CNQX.

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glutamate (10 μM) to induce random (temporally uncorrelated) oscillatory activity in large numbers of neurons without generating large-scale wave events. The goal of these experiments was first to find conditions under which pairs of neurons might exhibit correlated activity, and then to test whether gap junctions are responsible for such correlations. At the ages and layers examined (P2–3; layer 2/3), when synaptic connections are exceedingly sparse (Blue and Parnavelas 1983; DeFelipe et al. 1997; Juraska and Fifkova 1979), such correlations might indicate instances of electrotonic coupling, an inference that can be, and was, confirmed through subsequent dye injection into one of the two cells. Although correlations of this sort are hard to find, they do occur (Fig. 8, A and B). Of six pairs exhibiting correlated activity that were examined with dye injections (Fig. 8C), five were shown to be dye coupled, suggesting that even in normal saline some neurons are coupled strongly enough to generate measurable activity correlations.

The reason for the low incidence of correlated pairs observed under these conditions is presently unknown. One reason might be that, whereas imaging is limited to neurons in a thin optical slice, coupling is likely to involve neurons located at different depths within the slice, thus reducing the probability of simultaneously recording from both neurons using this approach. Another consideration is that electrotonic coupling through typical neuronal gap junctions is probably very weak. This makes it unlikely that pairs of neurons will exhibit activity correlations except in cases where junctional conductance between the two cells happens to be high, or under conditions where intrinsic potassium conductances have been reduced.

As a direct demonstration of the ability of neuronal gap junctions to mediate excitation between pairs of cells, these examples obtained in standard saline strengthen the conclusion, derived from TEA experiments, that activity waves traveling through developing cortical tissue are evidence of an underlying extensive neural syncitium.

**DISCUSSION**

The present study shows that in developing neocortex conditions of partial potassium channel blockade induce two types

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**FIG. 7.** TEA-induced wave activity is reduced by gap junction blockers. A: effect of the gap junction blocker halothane on wave speed in P1 slices (n = 5). Picrotoxin (PTX) was used to exclude possible inhibitory effects of halothane through GABA_A currents. Inset: activity in single neurons in the presence of halothane and picrotoxin. B–D: effect of the gap junction blocker carbenoxolone (CBX) on wave speed (B and C; P1–3 slices; n = 7) and on activity induced by 10 μM bath-applied L-glutamate (D) (P3; n = 2). B: time course of carbenoxolone effect in a single slice; each data point represents 1 wave. Group data are shown in C.

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**FIG. 8.** Dye injections confirm the ability of gap junctions to mediate interactions between pairs of neurons in immature neocortex in the absence of TEA. Random activation of neurons was obtained by applying 10 μM L-glutamate to the bath while fura-2 activity was imaged in the region shown in A. Activity of 5 neurons, 2 of which exhibited correlated activation (cells 1 and 2), is shown in B. Cells 3–5 are representative of all other cells in the field of view. Cells 1 and 2 were the only 2 cells with correlated activity out of approximately 50 cells in this field. C: the spread of a fluorescent gap junction tracer, NBD-methylglucamine, from cell 1 to cell 2, but not to other cells, after loading cell 1 with dye from a whole cell patch pipette. Bar, 50 μm.
of neuronal activity waves, one of which (the horizontally propagating type) involves gap junctions. The spatial extent of wave propagation along the horizontal dimension of cortex demonstrates that gap junctions link neurons into extensive syncitia.

Waves and gap junctions in developing cortex

The involvement of neuronal gap junctions in TEA-induced H-waves is supported by experiments in which the gap junction blockers halothane and carbenoxolone were used to reduce this form of intercellular signaling. Conclusions derived using these pharmacological agents, however, need to be justified in light of the known ability of these agents to produce nonspecific effects. Halothane is known to alter nonjunctional membrane conductances and neurotransmitter action in neurons. A suppressive effect on excitatory glutamatergic neurotransmission (Kirson et al. 1998; Narimatsu et al. 1996) is unlikely to be involved in its ability to block waves, since waves are resistant to glutamate antagonists. It could, however, explain why V-waves were also blocked in its presence. However, halothane can also have an enhancing effect on GABA_{A}-mediated inhibition (Li and Pearce 2000; Lukatch and MacIver 1997). The possibility that this effect could abolish waves is consistent with the result shown here that biccuculline has a disinhibitory effect on waves. To exclude an inhibitory effect by halothane, experiments were carried out in the presence of the GABA_{A} channel blocker, picrotoxin. Finally, effects on intrinsic conductances that might alter neuronal excitability were also considered. For effects of this nature to adversely affect waves, halothane would have to suppress excitability. Previous studies addressing this question do not support such an effect (Nishikawa and MacIver 2000), and some studies suggest instead that halothane may enhance intrinsic neuronal excitability (Butterworth et al. 1989; Southan and Wann 1989).

A second gap junction blocker, carbenoxolone (Davidson et al. 1986), was tested and had qualitatively similar effects on waves. Carbenoxolone was selected because previous studies have shown that it can be used to reduce electrotonic coupling without affecting intrinsic neuron properties such as membrane potential, input resistance, and action potential waveform (Draugluhn et al. 1998; Moortgat et al. 2000; Osborne and Williams 1996; Travagl et al. 1995). All carbenoxolone experiments were carried out between P_0 and P_3, when both glutamatergic and GABAergic synapses are poorly developed (Blue and Parnavelas 1983; De Felipe et al. 1997; Juraska and Fikova 1979). The results are therefore unlikely to be affected by potential effects on signaling via these transmitters.

Another potential confounding effect, blockade of astrocyte gap junction coupling, is unlikely since there are very few astrocytes during the first postnatal week in neocortex. Moreover, although the frequency and speed of waves in neocortex increases with age in parallel with the numbers of astrocytes and this might suggest a causal connection, the abrupt cessation of glutamate-independent synchronous activity after P_12 suggests that this network behavior is not related to astrocyte gap junctions, which continue to be present at high levels into adulthood.

In fact, the sudden disappearance of glutamate-independent coordinated activity provides not only evidence against astrocytic gap junction involvement, but further evidence for neuronal gap junction involvement in waves since it coincides precisely with the disappearance of gap junctions from most cortical neurons at P_12. Thus experiments done in P_15 neocortex, where neurobiotin tracer experiments show a very low incidence of neuronal coupling, show that TEA is unable to induce APV- and CNQX-resistant coordinated activity. In contrast, throughout the early postnatal period up to P_12, when coupling incidence is high, coordinated activity that is resistant to APV/CNQX is readily expressed as either slow horizontal waves (early time points) or fast large scale synchronous events (later time points).

Beyond TEA: gap junctions and physiological waves in immature brain

The effects of neuronal coupling examined in this study need not be restricted to experimental situations such as described here, where excitability was enhanced by manipulations purposely chosen to exaggerate physiological conditions. Potassium channels are targets of many neurotransmitter-activated signal transduction pathways in neocortical neurons (McCormick 1992; McCormick et al. 1993), and these may create a milder form (compared with TEA) of enhanced excitability. In a recent study I have shown that, during a brief period in postnatal neocortical development, activation of muscarinic cholinergic receptors can induce waves that are qualitatively similar, although slower and possibly more restricted spatially, to the horizontally propagating waves described here (Peinado 2000). Because waves induced through muscarinic receptor activation are also insensitive to APV and CNQX, they raise the possibility that enhancement of interactions mediated by gap junctions may be a frequent outcome of modulatory transmitter action in immature neocortex.

In the case of waves expressed spontaneously by immature neocortex in standard physiological saline (Garaschuk et al. 2000), the role of gap junctions has not been tested. There is evidence that CNQX does abolish them (Garaschuk et al. 2000), which suggests a role for glutamatergic synapses in some aspect of their expression. However, it is unclear whether glutamate is required for tonic excitation or whether it is involved in the mechanism of wave propagation per se.

The results obtained in the barrel field provide physiological evidence that even at early postnatal ages connectivity in this area of cortex is strongly compartmentalized. Just as there is very strong anatomical segregation of dendritic arbors in mature barrel field, signaling between barrels and surrounding cortex at immature ages also appears much weaker than what is seen within each of these compartments. A consequence of this segregation is that any spontaneous activity expressed by neurons at these ages is less likely to exhibit temporal correlations if neurons are located in different barrels than if they are in the same barrel, independently of any differences in timing caused by differences in thalamic input to these cells. Such a mechanism, if set in motion by an initial anatomical bias in dendritic arbors, possibly caused by molecular cues, could provide a key link in the chain of events responsible for strengthening local synaptic connections inside barrels.

Waves in mature neural networks as indicative of neural syncitia

Three very interesting previous studies of slowly propagating epileptiform activity in neocortex are worth mentioning.
given the similarity of their findings to the slow wave events described here. The first is a study by Wong and Prince (1990) in which ictal activity was induced in mature guinea pig neocortical slices using a 0 [Mg2+]o protocol. Multiple electrodes spaced along the horizontal extent of coronal slices revealed two types of epileptiform spread: fast (generalized) and slow (focal) ictal events. Their fast events were sensitive to APV and are reminiscent of the V-waves described here. Their slow events are very similar, although somewhat slower (0.29 mm/s), to the slow waves induced by TEA in immature rat neocortex. Their sensitivity to gap junction blockers was not tested by Wong and Prince, but more recently de Curtis et al. (1998), using the in vitro isolated guinea pig brain preparation, have shown that gap junction blockers abolish epileptiform bursts induced by brief intracortical application of bicuculline in piriform cortex. It is interesting that, in contrast to mature rat neocortex, mature guinea pig neocortex retains a high level of coupling between neurons, although perhaps not quite as high as that found in immature rat neocortex (Connors et al. 1983, 1984; Gutnick and Prince 1981). Thus the difference between rat and guinea pig in the behavior of epileptiform events in the mature neocortex is possibly related to this difference in neuronal coupling, suggesting that in guinea pig neocortex synaptic coupling may persist into adulthood.

Two other studies (Aram et al. 1991; Avoli et al. 1994) suggest that a phenomenon similar to the slow waves described here in developing neocortex may be possible in mature rat neocortex through the involvement of GABAergic interneurons, which have been shown to remain coupled in maturity (Galarreta and Hestrin 1999; Gibson et al. 1999; Sloper 1972). In these studies, the potassium channel blocker 4-aminopyridine (4-AP) was used to induce epileptiform events in mature neocortex. As in the present study, one variant of epileptiform activity induced by 4-AP in those studies is resistant to excitatory amino acid receptor antagonists. The events, recorded as field potentials by pairs of electrodes placed several millimeters apart along the horizontal extent of neocortex, propagate at speeds ranging from 2.8 to 8 mm/s. Unlike the horizontal waves described here, however, these events cannot be observed in the presence of the GABAA antagonist bicuculline, suggesting that they reflect GABAergic currents on cortical neurons resulting from the propagation of activity through a network of coupled interneurons. In the present study I did not observe this type of interneuron activity in P15 slices exposed to TEA and glutamate antagonists. At high magnification it is often possible to identify putative GABAergic interneurons by their nonpyramidal morphology. However, many of these presumed interneurons do not exhibit calcium oscillations under the conditions tested here (unpublished observations), which may account for the inability of the imaging approach to detect the phenomenon described by Aram et al. and Avoli et al.

Another wavelike phenomenon that may be related to neuronal gap junctions is the spindle waves of the lateral geniculate nucleus (LGN) (Kim et al. 1995; McCormick et al. 1995). In this case, coupling between interneurons in the perigeniculate nucleus could enable activity to propagate through this network as a wave. This in turn would result in a wave of rebound excitation in the LGN network, which receives topographically organized inhibitory projections from perigeniculate interneurons. It remains to be demonstrated, however, whether perigeniculate interneurons are in fact coupled, but this is likely.

In summary, the results presented here taken together with inferences derived from other studies, including studies of mature neural circuits, suggest the possibility that gap junction coupling between neurons and the formation of neural syncitium are a necessary condition for the expression of glutamate-independent activity waves in both mature and developing neural circuits throughout the brain.

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